Impact of site-specific phosphorylation of protein kinase A sites Ser\textsuperscript{23} and Ser\textsuperscript{24} of cardiac troponin I in human cardiomyocytes

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During stress and exercise, sympathetic activation of the heart increases heart rate and stroke volume to meet the demands of the body. This is mediated via the stimulation of \(\beta_1\)-adrenergic receptors, which leads to the activation of a downstream kinase, PKA. PKA enhances cardiomyocyte contraction and relaxation by phosphorylation of proteins involved in Ca\textsuperscript{2+} handling and myofilament proteins such as cardiac troponin (cTnI), cardiac myosin-binding protein-C (cMyBP-C), and titin (for reviews, see Refs. 1 and 37).

PKA-mediated phosphorylation of myofilament proteins is thought to exert a positive lusitropic effect, which enables the heart to relax more rapidly when heart rate increases. This positive lusitropic effect may be induced by a decrease in myofilament Ca\textsuperscript{2+} sensitivity (32, 35, 51) and by enhanced cross-bridge cycling kinetics (11, 20, 33). It is well established (mainly from studies in rodents) that phosphorylation of cTnI at the PKA sites Ser\textsuperscript{23} and Ser\textsuperscript{24} leads to a decrease in myofilament Ca\textsuperscript{2+} sensitivity, through a conformational change of the troponin complex. This structural change reduces the affinity of Ca\textsuperscript{2+} binding to cTnC (16, 30). The role of phosphorylation of cTnI at the PKA sites as a regulator of cross-bridge cycling is less clear. Some studies (11, 20, 36) have reported an increase in cross-bridge kinetics via phosphorylation of cTnI. However, others (7, 33) have attributed an increase in cross-bridge kinetics to phosphorylation of cMyBP-C independent of cTnI phosphorylation, whereas several studies (8, 15, 17, 44) did not find an effect of PKA on cross-bridge kinetics at all. In the present study, we aimed to study the effect of site-specific phosphorylation of cTnI on myofilament Ca\textsuperscript{2+} sensitivity and cross-bridge kinetics in human cardiomyocytes since insights into the functional effects of cTnI phosphorylation and the relation between the level of phosphorylation and the functional effects in the human myocardium are lacking.

The effects of PKA-mediated phosphorylation on the contractile function of cardiomyocytes are challenging to study. First, Ca\textsuperscript{2+}-handling and myofilament proteins are simultaneously phosphorylated by PKA. Triton permeabilization of cardiomyocytes eliminates the effects of Ca\textsuperscript{2+}-handling proteins, which makes it possible to study the specific effects on myofilament proteins. Second, incubation of triton-permeabilized cardiomyocytes with exogenous PKA does not reveal the functional effects of site-specific cTnI phosphorylation as PKA phosphorylates multiple myofilament proteins. The collective effects are important for in vivo function, but to understand the effects in both healthy and diseased myocardium, information is required of each of the contributing factors. Finally, theoretically, four different PKA-phosphorylated cTnI forms could coexist: one unphosphorylated, two monophosphorylated (either on Ser\textsuperscript{23} or Ser\textsuperscript{24}), and one bisphosphorylated (18, 34). A previous study (52) in rodents suggested that phosphorylation of cTnI is required for the reduction in myofilament Ca\textsuperscript{2+} sensitivity, but knowledge of the functional consequences of monophosphorylated cTnI also is important since recent stud-
ies (48, 50) in human postmortem control hearts and fresh donor transplant hearts with normal cardiac function revealed that ~40% of cTnI is monophosphorylated at Ser23. Moreover, differences in the level of monophosphorylated cTnI have been reported between donor and end-stage failing hearts (41, 50).

We therefore studied site-specific functional effects of phosphorylation at both PKA sites separately and in combination in human cardiomyocytes. To this end, myofilament force development was measured at various Ca2+ concentrations in triton-permeabilized cardiomyocytes in which the endogenous troponin complex was exchanged with exogenous recombinant whole human troponin complexes. These cTn complexes contained unphosphorylated PKA sites, pseudo-monophosphorylated cTnI, and pseudo-bisphosphorylated cTnI. Ser23 and/or Ser24 were mutated into aspartic acid and into alanine to mimic phosphorylation and dephosphorylation, respectively. This approach allowed us to study the effect of cTnI phosphorylation on active [maximal force (Fmax)] and passive isometric force (Fpas), the force-Ca2+ relation, and the rate of force redevelopment (kτ, a measure of cross-bridge kinetics). In addition, cardiomyocytes from idiopathic dilated cardiomyopathic (IDCM) tissue were used for titration of the effect of pseudo-bisphosphorylated cTnI as these cardiomyocytes have a low level of cTnI bisphosphorylation at baseline (15, 41).

The results of our study suggests that phosphorylation of both PKA sites on cTnI is required to reduce Ca2+ sensitivity in human cardiomyocytes as no change in Ca2+ sensitivity was observed upon exchange with cTn complexes containing pseudo-monophosphorylated cTnI. The maximal reduction in myofilament Ca2+ sensitivity was reached at ~55% bisphosphorylated cTnI. Fmax, Fpas, the steepness of the force-Ca2+ relation [Hill coefficient (nHill)], and cross-bridge kinetics were not significantly altered by pseudo-phosphorylation of cTnI at Ser23 and/or Ser24. The implications of these findings for in vivo cardiac function in health and disease are detailed in the DISCUSSION.

MATERIALS AND METHODS

Expression and purification of recombinant troponin subunits. Four different cTnI forms were made via site-directed mutations of Ser23 and/or Ser24 into aspartic acid (D) to mimic phosphorylation or into alanine (A) to mimic dephosphorylation: pseudo-bisphosphorylated cTnI (cTnI-DD), pseudo-phosphorylated cTnI at only Ser23 (cTnI-DA) or only Ser24 (cTnI-AD), and unphosphorylated cTnI (cTnI-AA). cDNA encoding human cardiac isoforms [cTnC, cTnT, myc tag-labeled cTnT (cTnT-myc), cTnI, cTnI-AA, cTnI-AD, cTnI-DA, and cTnI-DD] were transformed in Escherichia coli Rosetta2 (27) and cultured under carbenicillin/chloramphenicol selection in Overnight Express TB medium (EMD Biosciences). Cultures were harvested by centrifugation, resuspended in PBS, and centrifuged at 10,000 g. Pellets were stored at ~80°C until use.

Troponin subunits were purified using fast protein liquid chromatography (AKTA-FPLC System, Amersham Biosciences) essentially as previously described (27).

Reconstitution of troponin complexes. Fractions containing equal ratios of cTnT, cTnC, and cTnI subunits were pooled and finally dialyzed against 10 mM imidazole, 200 mM KCl, 5 mM MgCl2, 2.5 mM EGTA, 1 mM DTT, and 0.1 mM PMSF (pH 6.9, 2 times, 1 liter each) before the complexes were concentrated 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 cTnI in human cardiomyocytes was done as previously described (5, 27) with minor modifications. Cardiomyocytes were isolated from three donor hearts and from an IDCM heart, which were obtained during heart transplantation surgery. Tissue was collected in cardioplegic solution and stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the local Human Research Ethics Committee of The University of Sydney (no. 7326). This investigation conformed with the principles outlined in the Declaration of Helsinki (1997). The human cardiac samples have been extensively characterized (force characteristics and cTnI phosphorylation) in a previous study (13).

Single cardiomyocytes were mechanically isolated with a glass tissue homogenizer as previously described (27). Cardiomyocytes were subsequently incubated overnight at 4°C in exchange solution containing the appropriate concentration of recombinant human cTn complex (1.0 or 2.0 mg/ml in donor cells or a range between 0.0625 to 2.0 mg/ml in IDCM cardiomyocytes) with the addition of 4 mM CaCl2, 4 mM DTT, 5 µM protease inhibitor cocktail (P8340, Sigma), 10 µl/ml phosphatase inhibitor cocktails 1 and 2 (P2850 and P5726, Sigma), and 50 mM calyculin A (C5552, Sigma) (pH 6.9). The next day, cardiomyocytes were washed twice in rigor solution and finally in relaxing solution (5.95 mM Na2ATP, 6.04 mM MgCl2, 2 mM EGTA, 139.6 mM KCl, and 10 mM imidazole, pH 7.0). It has been previously demonstrated that this method results in a homogenous distribution of recombinant cTn complex within the exchanged cardiomyocyte.

Determination of the degree of troponin exchange. To determine the degree of cTnI exchange and to assess the protein phosphorylation status, half of the suspension of cells was treated with a 2D-clean-up kit (GE Healthcare) as described by manufacturer’s protocol after overnight cTnI exchange. After treatment, 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 concentrations were measured with a RCDC Protein Assay Kit II (Bio-Rad) and ranged between 2 and 4 mg/ml.

Immunoblot analysis was used to determine the degree of exchange of endogenous cTnI by the recombinant cTn complex. Therefore, recombinant cTnI was labeled with a myc tag to allow discrimination between endogenous and recombinant cTn complexes. Proteins were separated on a one-dimensional 15% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond) using the method 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 cTnI by enhanced chemiluminescence (Amersham Biosciences).

Myofilament protein phosphorylation. Phosphorylation levels of sarcomeric proteins were determined before and after cTnI exchange using ProQ diamond-stained one-dimensional gels, as previously described (49). Phosphorylation signals were normalized to the intensities of SYPRO ruby-stained myosin light chain (MLC2) bands to correct for small differences in protein loading. To correct for differences in staining between gels, the Peppermint Stick phosphoprotein marker (Molecular Probes) was used. The ratio of the intensities of the ProQ diamond- and SYPRO ruby-stained ovalbumin band was used to correct for intergel differences.

The distribution of endogenous phosphorylated species of cTnI in the donor hearts and in IDCM cardiomyocytes was analyzed using Phos-tag acrylamide gels (FMS Laboratory, Hiroshima University, Japan) as previously described (13, 24).

Isometric force measurements. Force measurements in cardiomyocytes exchanged with pseudo-phosphorylated cTnI complexes were performed as previously described (27). Isometric force was measured at 15°C and at a sarcomere length of 2.2 µm (40). The following parameters were determined: Fmax at pCa 4.5, Fpas at pCa 9.0, the Ca2+ sensitivity of force development (pCa0), nHill, and kτ at maximal and submaximal Ca2+ concentrations. kτ was determined using a slack-restretch test. Thereafter, after the cell had reached steady-state force in activating solution, it was shortened within 1 ms...
to 70% of its original length and restretched after 30 ms. As a result of this intervention, force first dropped to zero and then quickly redeveloped to the original steady-state level. A single exponential was fitted to force redevelopment to determine \( k_r \).

Data analysis. Data analysis was performed as previously described using the following Hill equation to fit force-Ca\(^{2+} \) relations:
\[
F(Ca^{2+})/F_0 = [Ca^{2+}]^{\text{Hill}}(C_{50}^{\text{Hill}} + [Ca^{2+}]^{\text{Hill}}),
\]
where \( F \) is steady-state force, \( F_0 \) is the steady-state force at saturating Ca\(^{2+} \) without lanes (A and B) and recombinant myc tag-labeled cTnT, and, therefore, two cTnT bands were found. The percentage of cTn exchange was calculated from the ratio of cTnT-myc and the total amount of cTnT. In Fig. 1B, the percentage of exchanged cTn was plotted against the cTn concentration in the exchange solution. The results shown in Fig. 1, C and D, indicate that the affinity of the cTnT antibody was the same for cTnT compared with cTnT-myc and that cTnT loading (ranging from 60 to 240 ng cTn/lane) was within the linear range.

No significant differences in the percentages of cTn exchange were found between cardiomyocytes with the different recombinant cTn complexes. This indicates that the different pseudo-phosphorylated cTn complexes incorporated similarly in the myofilaments. The percentage of exchange with the four pseudo-phosphorylated cTn complexes at a concentration of 1 mg/ml in three donor samples was, on average, 68.7 ± 1.6% (Fig. 1E). At a concentration of 2 mg/ml cTn during overnight incubation, the average percentage of exchange amounted to 86.2 ± 3.5% in cardiomyocytes isolated from three donor samples.

**RESULTS**

Quantification of troponin exchange in human cardiomyocytes. Figure 1A shows a representative immunoblot loaded with samples of cardiomyocytes incubated overnight with increasing concentrations (range: 0–2 mg/ml) of recombinant cTn. cTnT-myc migrated more slowly through the gel compared with endogenous cTnT, and, therefore, two cTnT bands were found. The percentage of exchange was calculated from the ratio of cTnT-myc and endogenous cTnT.

![Figure 1A](http://ajpheart.physiology.org)

**A** Immunoblot stained with an antibody against cardiac troponin (cTnT) that recognizes both endogenous cTnT (bottom band) and recombinant myc tag-labeled cTnT (cTnT-myc; top band). The example shows a suspension of cardiomyocytes exchanged with increasing concentrations of recombinant cTn. **B**: average percentages of cTn exchange obtained during all exchange experiments plotted against the cTn concentrations (range: 0–2 mg/ml) of recombinant cTn. cTnT-myc and, therefore, two cTnT bands were found. The percentage of cTn exchange was calculated from the ratio of cTnT-myc and endogenous cTnT. In Fig. 1, A and B, showed an average of 67.2 ± 4.6% bisphosphorylated cTnI.

**C** Immunoblot of recombinant cTn containing cTnT without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) the Myc tag label. **D**: average cTnT intensities of four blots plotted against the cTn amount (in ng) loaded per lane for cTnT- and cTnT-myc-containing cTn complexes. Linear regression analysis indicated that the slopes and intercepts did not significantly differ. **E**: average percentages of cTn exchange in cardiomyocytes after an overnight incubation in exchange solution with 1 mg/ml cTn containing the different pseudo-phosphorylated cTn complexes (average values represent cTn exchange experiments in cardiomyocytes isolated from 3 donor hearts). No significant differences were found in exchange percentages between the various cTn complexes, cTnT-AA, pseudo-dephosphorylated cTnI; cTnT-AD, pseudo-monophosphorylation of Ser\(^{24} \); cTnT-DA, pseudo-monophosphorylation of Ser\(^{23} \); cTnT-DD, pseudo-bisphosphorylated cTnI.
31.0 ± 4.6% monophosphorylated cTnI, and 1.8 ± 0.5% unphosphorylated cTnI in the three donor hearts used in the exchange experiments (Fig. 2B), whereas unphosphorylated (60.3%) and monophosphorylated (33.1%) cTnI were the most abundant forms in the IDCM sample used (Fig. 2A). Figure 2, C (1 mg/ml) and D (2 mg/ml), shows the percentages of unphosphorylated (endogenous unphosphorylated cTnI plus recombinant cTnI-AA), monophosphorylated (endogenous monophosphorylated cTnI plus recombinant cTnI-AD/DA), and bisphosphorylated (endogenous bisphosphorylated cTnI plus recombinant cTnI-DD) cTnI species after exchange calculated on the basis of the cTn exchange percentage determined with immunoblot analyses (as shown in Fig. 1E). ProQ Diamond-stained gels demonstrated that no significant differences in the level of phosphorylation of other myofilament proteins (cMyBP-C, desmin, and MLC2) were induced upon exchange with the various cTn complexes (Table 1).

Comparison of functional parameters after exchange with cTn with or without the myc tag. To test whether the myc tag, added to cTnT as a tool to quantify cTn exchange, interfered with myofilament function, recombinant cTn complex containing cTnT or cTnT-myc was exchanged in donor cardiomyocytes. No significant differences were found between cells (2 donors, 6–8 cells) exchanged with these complexes (Table 2), demonstrating that the myc tag did not influence any of the functional parameters studied.

Effects of pseudo-phosphorylated cTn on myofilament Ca2+ sensitivity. The effect of site-specific pseudo-phosphorylation of PKA sites Ser23 and Ser24 on pCa50 was measured at various Ca2+ concentrations in permeabilized donor cardiomyocytes (each complex: 3 donor hearts, 13 cells) in which the endogenous cTn complex was partially exchanged with recombinant cTn complexes (1 mg/ml cTn complex; Figs. 1E and 2C). Endogenous cTnI phosphorylation was largely removed by exchange with nonphosphorylated recombinant cTnI complex (Fig. 2C) and enabled us to compare the functional effects of the four different (un)phosphorylated cTnI complexes in donor myocardium. Compared with unphosphorylated cTnI (cTnI-AA), myofilament Ca2+ sensitivity was significantly lower after exchange with pseudo-phosphorylated cTnI at both sites.

Table 1. Overview of myofilament protein phosphorylation before and after exchange

<table>
<thead>
<tr>
<th>Cardiac Myosin-Binding Protein Binding</th>
<th>Desmin</th>
<th>Myosin Light Chain 2</th>
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<tbody>
<tr>
<td>Before exchange</td>
<td>0.60 ± 0.07</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Control exchange</td>
<td>0.58 ± 0.07</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>cTnI-AA</td>
<td>0.56 ± 0.04</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>cTnI-AD</td>
<td>0.54 ± 0.05</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>cTnI-DA</td>
<td>0.58 ± 0.11</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>cTnI-DD</td>
<td>0.55 ± 0.03</td>
<td>0.23 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE of the ProQ/SYPRO intensity ratio. Shown is myofilament protein phosphorylation determined before and after exchange in three donor samples via ProQ diamond staining. Control exchange cardiomyocytes were kept overnight in exchange solution without recombinant cardiac troponin (cTnI) added. No significant effect was found of the control exchange protocol on myofilament protein phosphorylation compared with before exchange (by Student’s t-test). cTn exchange at 1 mg/ml also did not affect myofilament protein phosphorylation (by one-way ANOVA comparing control exchange and the four cTnI exchange groups). A, alanine substitution; D, aspartic acid substitution.
of Ser24 (cTnI-AD) did not lower myofilament Ca\(^{2+}\) pseudo-monophosphorylated cTnI to 88.1% (Fig. 2). Also, D exchange (86.2 cTn-AD (3 donors, 11 cells) to increase the extent of cTnI exchange, experiments were performed using 2 mg/ml cTn-AA and 1 mg/ml cTn-DD were exchanged at 2 mg/ml (11 myocytes/cTnI mutant) in three donor samples. No significant differences were found in the maximal rate of force development (k_{tr-max}; in s\(^{-1}\)) at saturating Ca\(^{2+}\) concentration (pCa 4.5) between cTn-AA and cTn-AD (as analyzed by a Student’s t-test). F_{max}, maximal force (in kN/m\(^2\)); F_{pas}, passive force (in kN/m\(^2\)); n_{Hill}. Hill coefficient (steepness of the force-pCa curve). ***P < 0.0001 (by one-way ANOVA followed by a Bonferroni post hoc test).

Table 2. Force measurements in cardiomyocytes after exchange with recombinant cTnI

<table>
<thead>
<tr>
<th></th>
<th>F_{max}</th>
<th>F_{pas}</th>
<th>pCa_{50}</th>
<th>n_{Hill}</th>
<th>k_{tr-max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/ml cTn</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cTnI-Wt</td>
<td>20.6 ± 1.7</td>
<td>3.3 ± 0.5</td>
<td>5.54 ± 0.02</td>
<td>2.6 ± 0.2</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>cTnI-AA (myc)</td>
<td>24.6 ± 1.5</td>
<td>3.2 ± 0.2</td>
<td>5.51 ± 0.02</td>
<td>3.0 ± 0.1</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>cTnI-AD (myc)</td>
<td>23.3 ± 1.8</td>
<td>3.3 ± 0.3</td>
<td>5.50 ± 0.01</td>
<td>3.2 ± 0.1</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td>cTnI-DD (myc)</td>
<td>22.3 ± 1.4</td>
<td>3.2 ± 0.4</td>
<td>5.48 ± 0.01</td>
<td>3.1 ± 0.1</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>cTnI-DD (myc)</td>
<td>20.9 ± 1.3</td>
<td>3.2 ± 0.4</td>
<td>5.51 ± 0.01</td>
<td>3.3 ± 0.1</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td>cTnI-DD (myc)</td>
<td>24.4 ± 1.0</td>
<td>2.6 ± 0.2</td>
<td>5.48 ± 0.01</td>
<td>3.3 ± 0.2</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td>cTnI-DD (myc)</td>
<td>22.7 ± 1.2</td>
<td>3.2 ± 0.2</td>
<td>5.47 ± 0.02</td>
<td>3.0 ± 0.1</td>
<td>0.52 ± 0.04</td>
</tr>
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</table>

Values are means ± SE. Recombinant wild-type (Wt) cTnI complexes with or without the myc tag were exchanged at 1 mg/ml in two donor samples (6–8 cardiomyocytes/group). No significant differences were found in the force parameters between cTnI complexes with or without the myc tag label. Myc-tagged cTnI mutated at Ser23 and Ser24 into alanine or aspartic acid was exchanged at 1 mg/ml (13 myocytes/cTnI mutant) in three donor samples. The midpoint of the Ca\(^{2+}\) sensitivity of force development (pCa_{50}) was significantly lower in cTnI-DD compared with cTnI-AA, cTnI-AD, or cTnI-DA. Myc-tagged cTnI-AD complexes served as controls. cTnI-DD exchange caused a gradual decrease in Ca\(^{2+}\) sensitivity (slope: 0.0028) between 0% and 51% of cTnI-DD exchange, it can be calculated that 3.6% of cTnI-DD (Fig. 4). The maximal decrease in pCa_{50} was 0.13 ± 0.02 units. This value corresponds well with the difference in pCa_{50} between cTnI-DD and cTnI-AD in donor cells. Assuming a linear decline in Ca\(^{2+}\) sensitivity (slope: 0.0028) between 0% and 51% of cTnI-DD exchange, it can be calculated that 3.6% of cTnI-DD is sufficient to reduce Ca\(^{2+}\) sensitivity by 0.01 pCa units.

Effects of pseudo-phosphorylated cTn on F_{max}, F_{pas} and n_{Hill}. F_{max}, F_{pas}, and n_{Hill} (Table 2) were not significantly different after exchange with cTnI containing the mono- and bisphosphorylated cTnI species.

Effects of pseudo-phosphorylated cTn on k_{tr-max}. Maximal k_{tr} at pCa 4.5 (k_{tr-max}) after exchange with pseudo-unsphosphorylated cTn did not differ from k_{tr-max} after exchange with pseudo-monophosphorylated or pseudo-bisphosphorylated cTn (Table 2). However, because k_{tr-max} showed a tendency to decrease in cTnI-AD, additional experiments were done at even higher exchange percentages (cTn concentration: 2 mg/ml) where cTnI-AD was compared with cTnI-DD (Table 2). In agreement with the results at 1 mg/ml exchange, k_{tr-max} did not significantly differ between cTnI-AD and cTnI-DD. In addition, no significant differences in k_{tr} values measured at submaximal Ca\(^{2+}\) concentrations were found after exchange with cTnI containing mono- and bisphosphorylated cTnI species (data not presented).

(cTnI-DD), as evident from the rightward shift of the force-pCa curve for cTnI-DD compared with cTnI-AA (Fig. 3, A and B). It can also be seen in Fig. 3 that the curves after cTnI-DA, cTnI-AD, and cTnI-AA exchange were indistinguishable. This indicates that pseudo-monophosphorylation of the PKA sites did not influence Ca\(^{2+}\) sensitivity (Fig. 3, A and B, and Table 2) and suggests that bisphosphorylation is required to cause a change in Ca\(^{2+}\) sensitivity. The difference in pCa_{50} in donor cardiomyocytes upon exchange with cTnI-DD compared with the averaged pCa values of cells exchanged with cTnI-AA, cTnI-AD, or cTnI-DA was 0.11 ± 0.01 pCa units. In a previous study from our group (27), it has been shown that PKA treatment of human donor cardiomyocytes after exchange with wild-type unphosphorylated cTnI decreased Ca\(^{2+}\) sensitivity to a similar extent (0.08 pCa units). The similar decrease in myofilament Ca\(^{2+}\) sensitivity observed in the present study of 0.11 pCa units reflects a change in EG_{50} of 0.91 μM between cTnI-DD (pCa_{50}: 5.39 and EG_{50}: 4.07 μM) and non-phosphorylated cTnI-AD (pCa_{50}: 5.50 and EG_{50}: 3.16 μM; Fig. 3B), which is likely to be of functional significance. Note that myofilament Ca\(^{2+}\) sensitivity after exchange with unphosphorylated cTnI-AD and unphosphorylated wild-type cTnI complex were the same (Table 2). This indicates that cTnI-AA exchange mimics the effects of exchange using the unphosphorylated Ser23- and Ser24-containing cTnI complex. In addition, experiments were performed using 2 mg/ml cTnI-AD and cTnI-AD (3 donors, 11 cells) to increase the extent of cTn exchange (86.2 ± 4%) and thereby increase the level of (pseudo-)monophosphorylated cTnI to 88.1% (Fig. 2D). Also, at higher cTn exchange levels, pseudo-monophosphorylation of Ser24 (cTnI-AD) did not lower myofilament Ca\(^{2+}\) sensitivity compared with unphosphorylated cTnI (cTnI-AA; Table 2). This strengthens the observations at 1 mg/ml cTnI that pseudo-monophosphorylation has no effect on Ca\(^{2+}\) sensitivity.

Saturation of the effect of cTn pseudo-bisphosphorylation on myofilament Ca\(^{2+}\) sensitivity. To test how much PKA-mediated cTnI bisphosphorylation is necessary to maximally reduce myofilament Ca\(^{2+}\) sensitivity, cTnI-DD was exchanged in IDCM cardiomyocytes. This was done at cTn concentrations between 0 and 2 mg/ml to vary the degree of exchange (4–5 cells/concentration). IDCM cardiomyocytes were used in these experiments because the endogenous levels of cTnI Ser23/Ser24 bisphosphorylation in the end-stage failing human myocardium are low compared with donor tissue. The results shown in Fig. 2A demonstrate, in agreement with previous studies (13, 41), that cTnI Ser23/Ser24 bisphosphorylation was low (~7%) in the IDCM cardiomyocytes used. Comparison of the phosphorylation levels of other myofilament proteins in IDCM tissue (cMyBP-C: 0.39 ± 0.03, desmin: 0.23 ± 0.04, and MLC2: 0.21 ± 0.03) with the values in donor tissue (Table 1) also indicates that, in addition, cMyBP-C phosphorylation was lower in IDCM tissue compared with donor tissue. Cardiomyocytes incubated overnight in exchange solution without complex served as controls. cTnI-DD exchange caused a gradual decline in pCa_{50} saturating at 51 ± 5% of cTnI-DD (Fig. 4). The maximal decrease in pCa_{50} was 0.13 ± 0.02 units. This value corresponds well with the difference in pCa_{50} between cTnI-DD and cTnI-AD in donor cells. Assuming a linear decline in Ca\(^{2+}\) sensitivity (slope: 0.0028) between 0% and 51% of cTnI-DD exchange, it can be calculated that 3.6% of cTnI-DD is sufficient to reduce Ca\(^{2+}\) sensitivity by 0.01 pCa units.
Ser²³ and/or Ser²⁴ in human cardiomyocytes. Our study suggests that (mono)phosphorylation of either Ser²³ or Ser²⁴ does not alter myofilament Ca²⁺ sensitivity and that both serines need to be phosphorylated to reduce the Ca²⁺ sensitivity of myofilament force. These results are compatible with a model proposed on the basis of biochemical studies (29) indicating that monophosphorylation and consecutive bisphosphorylation exert a step-wise effect on the affinity of binding between cTnl and cTnT as well as cTnC, whereas bisphosphorylation of cTnl causes a reduction in the affinity of cTnC for Ca²⁺. A more recent study (16) has provided insights on the conformational transitions within the cTnl complex upon bisphosphorylation, but alternative models have been proposed as well (for a recent review, see Ref. 31).

In a previous study by Zhang et al. (52), a chimera of a mouse-bovine cTn complex with alanine mutations was exchanged in skinned porcine cardiac myocytes to mimic dephosphorylation at Ser²³ and/or Ser²⁴. PKA treatment of porcine cells containing alanine-mutated cTn complex did not reduce Ca²⁺ sensitivity, whereas PKA did reduce Ca²⁺ sensitivity in cells exchanged with the wild-type complex. This indicated that Ser²³ and Ser²⁴ were required for the PKA-mediated decrease in Ca²⁺ sensitivity but did not rule out the possibility that the decrease depended on the phosphorylation of other target proteins, such as cMyBP-C and titin. To circumvent this problem, Dohet et al. (10) mutated both Ser²³ and Ser²⁴ of cTnI to aspartic acid or alanine and exchanged human cTn mutants in porcine cardiac muscle fibers. This study showed that bisphosphorylated cTnI complex (cTnI-DD) lowered Ca²⁺ sensitivity compared with the nonphosphorylated cTnI complex (cTnI-AA). Our study extends these previous studies in that human recombinant proteins were exchanged in human tissue (13 cardiomyocytes from 3 donor hearts in all groups).

DISCUSSION

Both Ser²³ and Ser²⁴ need to be phosphorylated to reduce Ca²⁺ sensitivity. The present study is the first to examine the effects of site-specific phosphorylation of cTnl- Ser²³ and/or Ser²⁴ on cTnl. A: myofilament force development measured at various Ca²⁺ concentrations in permeabilized donor cardiomyocytes in which the endogenous cTn complex was partially exchanged (68.7 ± 2%) with 1 mg/ml of recombinant myc tag-labeled cTn complexes (13 cardiomyocytes from 3 donor hearts in all groups). B: compared with unphosphorylated cTnl (cTnl-AA), pCa derived from the midpoint of the force-pCa relationship (pCa₅₀) was significantly reduced after exchange with pseudo-phosphorylated cTnl at both PKA sites (cTnl-DD). Exchange with cTnl-DA or cTnl-AD did not alter Ca²⁺ sensitivity compared with unphosphorylated cTnl. The reduced Ca²⁺ sensitivity upon exchange with cTnl-DD is evident from the rightward shift of the force-pCa curve in cTnl-DD-exchanged cardiomyocytes compared with cells exchanged with cTnl-AA, cTnl-AD, or cTnl-DA. ***p < 0.0001, cTnl-DD vs. all other complexes (by posttest Bonferroni analyses of one-way ANOVA).

Shown). Note that kₑ-max after exchange using cTnl-AA was the same as in the exchange using unphosphorylated wild-type cTnl (Table 2). The effect of pseudo-phosphorylation at the PKA sites on force redevelopment was also analyzed with a double-exponential equation (Fig. 5), as previously described by Caremani et al. (6). A fivefold difference in the rate constants of the fast phase (1.12 ± 0.07 s⁻¹, n = 52) and slow phase (0.22 ± 0.03 s⁻¹, n = 52) were observed. In agreement with the results of the monoexponential fit, no differences in the parameter values were observed between groups. On the basis of these data, it can be concluded that pseudo-phosphorylation at Ser²³ and/or Ser²⁴ did not affect kₑ.

This conclusion relies on the assumption that alanine and aspartic acid mimic dephosphorylation and phosphorylated ser-
Thus, although $n_{Hill}$ itself does not appear to be changed by phosphorylation of the NH$_2$-terminal extension of cTnI, some form of communication along the thin filament needs to be present to explain why partial cTnI phosphorylation maximally reduces the activation state of all participating troponin units.

No effect of exchange of pseudo-phosphorylated cTnI at Ser$^{23}$ and Ser$^{24}$ on maximal and passive force. Our results indicated that pseudo-phosphorylation of Ser$^{23}$ and/or Ser$^{24}$ of cTnI did not affect $F_{\text{max}}$ at saturating Ca$^{2+}$ levels of human cardiomyocytes. This is in line with previous studies (4, 39) showing no effect of PKA incubation on $F_{\text{max}}$ at saturating Ca$^{2+}$ in human skinned cardiomyocytes. In addition, no change was found in $F_{\text{pas}}$ upon exchange with either pseudomonophosphorylated or pseudo-bisphosphorylated cTnI. These results are consistent with a previous study (47) indicating that PKA reduces passive tension in cardiomyocytes via phosphorylation of titin’s cardiac-specific N2B domain.

$k_{tr}$ is not affected by cTnI phosphorylation at Ser$^{23}$ and Ser$^{24}$. A small but statistically insignificant trend for a decrease in $k_{tr-max}$ at 1 mg/ml cTn exchange in cTnI-AD compared with cTnI-AA was found (Table 2). Additional experiments with higher cTn concentrations (2 mg/ml) did not show any difference in $k_{tr-max}$ (Table 2). In accordance with our findings, it has been demonstrated that PKA treatment did not affect cross-bridge cycling in skinned trabeculae (8, 17) and cardiomyocytes (15) from rat hearts and in human myofibrils (44). Other studies (11, 20, 36) in rodents showed that PKA-mediated phosphorylation of cTnI increases cross-bridge kinetics. More recently, it has been shown that PKA-mediated phosphorylation of cMyBP-C increases cross-bridge kinetics in transgenic mice, independent of cTnI phosphorylation (7, 33). The reasons for these differences are unclear, but our data indicate that in the human heart, PKA-mediated phosphorylation of cTnI may induce positive inotropic effects by affecting Ca$^{2+}$ sensitivity, whereas no effect was found on $k_{tr}$, which represents the sum of the apparent rates of cross-bridge attachment and detachment under isometric conditions. However, it cannot be excluded that cross-bridge kinetics are affected by phosphorylation of cTnI in the in vivo situation because Layland and Kentish (21) observed, in line with our findings, in isolated sarcoplasmic reticulum-inhibited cardiac trabeculae no relaxant effect of isoprenaline during isometric contractions, but, on the other hand, an increase in the rate of relaxation during work-loop contractions (which mimic the in vivo situation).

Residual force after the shortening restretch protocol used to determine $k_{tr}$ ranged between 30% and 50%. This residual force is most likely mainly caused by cross-bridge reattachment during restretch (duration: 2 ms). However, the $k_{tr}$ values obtained did not depend on the level of residual force, and the average residual force within the experimental groups after exchange did not differ. Moreover, the $k_{tr}$ values after the exchange protocol were comparable with values reported in previous studies (40, 46) in cardiomyocytes without prior treatment. Therefore, we are confident that the comparison of $k_{tr}$ in the different experimental groups was valid.

Implications of cTnI phosphorylation at Ser$^{23}$ and Ser$^{24}$ in health and disease. In the present study, we provided evidence that bisphosphorylation of PKA sites Ser$^{23}$ and Ser$^{24}$ of human cTnI reduces myofilament Ca$^{2+}$ sensitivity and that the functional range lies between 0% and 55% of bisphosphorylation.
The level of phosphorylation of these two serines is determined by the balance between kinase and phosphatase activity at the myofilaments. PKA phosphorylates Ser23 and Ser24 upon stimulation of the β-adrenergic receptor pathway with different affinities (18). In addition, PKC (34), PKD (14), and PKG (2) are all known to phosphorylate cTnI at Ser23 and Ser24. Although in vitro studies (14, 18, 26, 28, 52) have demonstrated that PKA preferentially phosphorylates Ser24 over Ser23, it has been recently demonstrated using top-down mass spectrometry that cTnI in human cardiac tissue is only monophosphorylated at Ser23 (48, 50). This surprising finding may be explained by preferential dephosphorylation of Ser24 by phosphatases (9, 19, 46). Indeed, it has been demonstrated that protein phosphatase 2A has a preference for Ser24 (18). It cannot be excluded that the level of bisphosphorylation needed to maximally reduce Ca²⁺ sensitivity depends on the phosphorylation status of other myofilament proteins and thus might vary with disease state.

In the donor samples used in this study, the level of endogenous bisphosphorylation amounted to 67.2%, which is above the level of saturation of Ser23/Ser24 bisphosphorylation. Recent studies (48, 50) have reported relatively low levels of bisphosphorylation of cTnI Ser23/Ser24 (~15%) and a relatively large fraction of monophosphorylated cTnI at Ser23 (~40%) in human postmortem control hearts and in fresh transplant donor hearts. This suggests that the healthy human heart has a reserve to decrease myofilament Ca²⁺ sensitivity during β-adrenergic receptor stimulation. In addition, several studies have demonstrated lower cTnI phosphorylation of the PKA sites (3, 23–25, 41, 50) and higher myofilament Ca²⁺ sensitivity in end-stage failing hearts relative to donor hearts (25, 41), which has been ascribed to downregulation and desensitization of the β-adrenergic receptor pathway. Comparison of human cardiac samples from heart failure patients with different disease severity [ranging from New York Heart Association (NYHA) class I to IV] showed increased Ca²⁺ sensitivity only in the end stage (NYHA class IV) of cardiac disease (38), which suggests that the detrimental effects of reduced cTnI phosphorylation may only become evident at the end stage of heart failure. However, recent studies in patients with obstructive familial hypertrophic cardiomyopathy and normal systolic but impaired diastolic function (NYHA class III) showed increased myofilament Ca²⁺ sensitivity (43) and lower cTnI phosphorylation (24, 42) in familial hypertrophic cardiomyopathic myocardium compared with nonfailing myocardium. In addition, a recent study (50) showed that the level of cTnI bisphosphorylation in postmortem hearts with mild hypertrophy was significantly lower (4.1%) compared with control levels (18.4%). Collectively, these studies indicate that cTnI bisphosphorylation and the associated impact on Ca²⁺ sensitivity depends on the stage of heart failure (NYHA class) as well as on etiology.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).


