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A Novel Phosphorylation Site, Serine 199, in the C-Terminus of Cardiac Troponin I Regulates Calcium Sensitivity and Susceptibility to Calpain-Induced Proteolysis

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Abstract

Phosphorylation of cardiac troponin I (cTnI) by protein kinase C (PKC) is implicated in cardiac dysfunction. Recently, Serine 199 (Ser199) was identified as a target for PKC phosphorylation and increased Ser199 phosphorylation occurs in end-stage failing compared with non-failing human myocardium. The functional consequences of cTnI-Ser199 phosphorylation in the heart are unknown. Therefore, we investigated the impact of phosphorylation of cTnI-Ser199 on myofilament function in human cardiac tissue and the susceptibility of cTnI to proteolysis. cTnI-Ser199 was replaced by aspartic acid (199D) or alanine (199A) to mimic phosphorylation and dephosphorylation, respectively, with recombinant wild-type (Wt) cTn as a negative control. Force development was measured at various [Ca^{2+}] and at sarcomere lengths of 1.8 and 2.2 μm in demembranated cardiomyocytes in which endogenous cTn complex was exchanged with the recombinant human cTn complexes. In idiopathic dilated cardiomyopathy samples, myofilament Ca^{2+}-sensitivity (pCa_{50}) at 2.2 μm was significantly higher in 199D (pCa_{50}=5.79±0.01) compared to 199A (pCa_{50}=5.65±0.01) and Wt (pCa_{50}=5.66±0.02) at ~63% cTn exchange. Myofilament

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Disclosures

None

Ca^{2+}-sensitivity was significantly higher even with only 5.9±2.5% 199D exchange compared to 199A, and saturated at 12.3±2.6% 199D exchange. Ser199 pseudo-phosphorylation decreased cTnI binding to both actin and actin-tropomyosin. Moreover, altered susceptibility of cTnI to proteolysis by calpain I was found when Ser199 was pseudo-phosphorylated. Our data demonstrate that low levels of cTnI-Ser199 pseudo-phosphorylation (~6%) increase myofilament Ca^{2+}-sensitivity in human cardiomyocytes, most likely by decreasing the binding affinity of cTnI for actin-tropomyosin. In addition, cTnI-Ser199 pseudo-phosphorylation or mutation regulates calpain I mediated proteolysis of cTnI.

Keywords

cardiomyocyte; heart failure; protein phosphorylation; proteolysis; cardiac troponin I; myofilament function

1. Introduction

Cardiac troponin I (cTnI) is an essential regulator of contraction and relaxation of the heart. cTnI is the “inhibitor” of the trimeric cardiac troponin (cTn) complex, which together with cardiac troponin C (cTnC, the Ca^{2+}-sensor) and cardiac troponin T (cTnT), controls the position of tropomyosin (Tm) on the thin actin filament in response to Ca^{2+} [1]. In diastole (low intracellular [Ca^{2+}]), cTnI binds actin at multiple sites maintaining Tm at the outer domain of actin, and thereby blocks myosin-binding sites and prevents force development (blocked, B-state). In systole (high intracellular [Ca^{2+}]), Ca^{2+} binds to cTnC and induces a conformational change in the cTn complex. This results in the release of cTnI from actin and a shift of Tm closer to the inner domain of actin (Ca^{2+}-activated, C-state), thereby enabling actin-myosin interactions (myosin-induced, M-state) and force development [2].

The functional properties of the cTn complex are regulated by phosphorylation of cTnI, which therefore has an important role in tuning cardiomyocyte performance. The best characterized cTnI phosphorylation sites are protein kinase A (PKA) sites Serine 23 (Ser23) and Ser24. Phosphorylation of both sites by PKA, which is activated via the beta-adrenergic receptor pathway during stress and exercise, results in a decreased Ca^{2+}-sensitivity of force development and thereby improves relaxation of the heart [3–6]. In addition, PKA-mediated cTnI phosphorylation has been demonstrated to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity[7,8]. Myofilament length-dependent activation is the cellular basis of Frank-Starling’s law of the heart, which describes the ability of the heart to adjust the force of its contraction (stroke volume) to changes in ventricular filling (end-diastolic volume). However, also less favorable effects of cTnI phosphorylation for cardiac performance have been reported. Protein kinase C (PKC)-mediated phosphorylation of cTnI at Ser42/44 and Thr143 has been implicated in myofilament dysfunction and cardiac disease [9,10].

Recently a new PKC phosphorylation site, Ser199, was identified in human myocardium at the C-terminus of cTnI [11]. Increased phosphorylation at Ser199 was demonstrated in human end-stage heart failure compared to non-failing donor myocardium and in a canine model of dyssynchronous pacing-induced heart failure (HF_{dys}) [10]. Ser199 is highly

conserved among all the three TnI isoforms and across a wide range of species including human, dog, mouse, rat, chicken and frog, indicating a high selection pressure on its physiological function [12]. In the canine HF_{dys} model, resynchronization therapy reversed the phosphorylation status of Ser199 (in dog Ser198) [10]. Moreover, a mutation at the Ser199 position on cTnI that has been found in different families with hypertrophic cardiomyopathy has been associated with the occurrence of arrhythmias and sudden cardiac death [13]. Therefore, in this study, we examined the effects of phosphorylation of cTnI-Ser199 on human myofilament function.

Proteolysis is another post-translational modification of cTnI found in both physiological and pathological conditions [14–17]. The selective cleavage of 17 amino acid residues (a.a.) at the C-terminus of cTnI is the primary effect of cTnI proteolysis in ischemia/reperfusion injury and results in cardiac dysfunction [18–20]. While the truncation of the unique N-terminus of cTnI is present in normal human hearts and believed to be a compensatory response in microgravity [17]. The mechanism underlying the selective cleavage of cTnI is not clear. It was indicated that calpain I, a ubiquitous Ca^{2+} (1–20μM) activated protease, is an active agent, especially in ischemia/reperfusion hearts [21–23]. Additionally, regulation of proteolysis by phosphorylation has been reported in both myofilament [24,25] and non-myofilament proteins [26,27]. Indeed, the sensitivity of cTnI to calpain I cleavage is depressed when cTnI is phosphorylated by PKA while promoted by PKC [28]. However, to date, there is no direct evidence to identify the specific phosphorylation sites on cTnI responsible for the cross-talk between its phosphorylation and proteolysis and whether the C-terminus phosphorylation sites influence proteolysis. Since Ser199 is a substrate of PKC-α and the only phosphorylation site within the C-terminal 17 a.a. fragment proteolysed during ischemia/reperfusion injury, we tested whether cTnI-Ser199 phosphorylation regulates cTnI proteolysis.

2. Materials and methods

2.1. Exchange of human cTn complex

Human recombinant cTn complex was prepared as described before [20]. Besides wild-type (Wt) cTnI, two different cTnI forms were made via site-directed mutations of Ser199. Ser199 was replaced by aspartic acid (D; 199D) to mimic phosphorylation or alanine (A; 199A) to mimic dephosphorylation (creating a non-phosphorylatable site). Exchange of these cTn complexes in human cardiomyocytes was done as described previously [5]. Briefly, single cardiomyocytes were mechanically isolated with a glass tissue homogenizer, and permeabilized by Triton X-100 (0.5%; v/v) for 5 minutes. They were subsequently incubated overnight at 4°C in exchange solution (10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT (pH 6.9)) containing recombinant human cTn complex at concentrations ranging between 0.0625 to 1.0 mg/mL with the addition of 4 mM $CaCl_2$, 4 mM DTT, 5 μl/mL protease inhibitor cocktail (Sigma, P8340) and 10 μl/mL phosphatase inhibitor cocktail 2 and 3 (Sigma, P5726 and P0044) (pH 6.9). The next day, the cardiomyocytes were washed twice in rigor solution and finally in relaxing solution (5.95 mM Na_2ATP, 6.04 mM $MgCl_2$, 2 mM EGTA, 139.6 mM KCl, 10 mM Imidazole, pH 7.0). This method results in a homogenous distribution of recombinant cTn complex within the

exchanged cardiomyocyte [20]. Troponin exchange did not significantly affect maximal force development compared to control cardiomyocytes incubated overnight in exchange solution without cTn.

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 years) or from non-failing donor myocardium obtained during heart transplantation surgery. The tissue was perfused with cold cardioplegic solution, transported to the laboratory and rapidly frozen and stored in small (~1g vials) in liquid nitrogen. Samples were obtained after informed consent and with approval of the Human Research Ethics Committee of The University of Sydney (#2012/2814). 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 cTnI phosphorylation) in a previous study [29].

2.2. Determination of the degree of cTn exchange

To determine the degree of cTn exchange and to assess the 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% (w/v) SDS and 2% (w/v) DTT. Protein concentration measured with RCDC Protein Assay Kit II (BioRad) ranged between 2 to 4 mg/mL.

Immunoblotting was used to determine the degree of exchange of endogenous cTn by recombinant cTn complex. Recombinant cTnT was labeled with a Myc-tag to allow discrimination between endogenous and recombinant cTn complex. 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 hour at 75 V. A specific monoclonal antibody against cTnT (Clone JLT-12, Sigma; dilution 1:1250) 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 to cTnT-Myc and that cTnT loading was within the linear range [5].

2.3. Myofilament protein phosphorylation

Phosphorylation levels of sarcomeric proteins were determined before and after cTn exchange using ProQ-Diamond phospho-stained 1D-gels, as described previously [5]. The phosphorylation signals (cardiac myosin-binding protein-C, myosin light chain 2 and desmin) 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 [30]. The ratio of the intensities of ProQ-Diamond and SYPRO Ruby stained ovalbumin band was used to correct for inter-gel differences.

The distribution of endogenous phosphorylated species of cTnI was analyzed using Phos-tag™ acrylamide gels (FMS Laboratory; Hiroshima University, Japan) as described before [29].

2.4. Isometric force measurements

Force measurements in cardiomyocytes exchanged with recombinant cTn were performed as described previously [20]. Isometric force was measured at 15°C and myofilament length-dependent activation (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_{pas}), maximal force at pCa 4.5 (F_{max}: total force minus F_{pas}), Ca^{2+}-sensitivity of force development (pCa_{50}), steepness of the sigmoidal force-pCa relation (nHill) and the rate of force redevelopment (k_{tr}) at maximal and at submaximal [Ca^{2+}]. Rate of force redevelopment was determined as described previously [5].

2.5. F-actin cosedimentation assay

Rabbit skeletal actin and rabbit skeletal (alpha/alpha) Tm was prepared according to the published methods [31,32]. Recombinant human cTnI (Wt, 199D and 199A) was incubated at different concentrations (0–8 μM) with 5μM actin at room temperature for 30 minutes in binding buffer (pH 7.0) contains 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA and 1 mM DTT. Another cosedimentation assay, actin and cTnI and Tm were mixed in a molar ratio of actin:cTnI:Tm = 7:1:1. A high ionic strength (250 mM NaCl) was used to solubilize cTnI [33] and control groups containing only cTnI without actin were included in every experiment. After centrifugation, both pellet and supernatant were collected and resolved by SDS-PAGE followed by Coomassie staining. For quantification protein gels were scanned for densitometry and quantified by using software ImageJ.

2.6. Degradation of recombinant cTn with calpain I

Recombinant human whole cTn complexes containing cTnI Wt, 199D or 199A, were incubated with calpain I, a Ca^{2+}-dependent protease, at 37°C for 30 min, in sodium borate buffer (pH 7.4) containing 1.25 mM $CaCl_2$, 3 mM $MgCl_2$ and 50 mM NaCl. The protein to enzyme ratio is 1 μg: 0.025 or 0.05 units. The reaction was stopped by adding Laemmli sample buffer and heated 5 min at 95°C. After digestion, cTnI content in the sample was analyzed by western blotting with cTnI monoclonal antibody (McAb 8I-7, 1:5000, International Point of Care Inc.). Protein bands were quantified by using ImageJ software.

2.7. Data analysis

Data analysis was performed as previously described using the Hill equation to fit force-Ca relations: $F(Ca^{2+})/F_0=[Ca^{2+}]^{nHill}/(Ca_{50}{}^{nHill}+[Ca^{2+}]^{nHill})$, Where F is steady-state force, F_0 the steady-state force at saturating [Ca^{2+}], nHill the steepness of the relationship and Ca_{50} (or pCa_{50}) 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 to control (Wt for Table 2). When two-way 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). One-way ANOVA followed by post hoc Newman–Keuls tests was used to compare between different cTnI variants in F-actin cosedimentation assays and cTnI proteolysis experiments. Values are given as means ± S.E.M. of n myocytes.

3. Results

3.1. Quantification of cTn exchange in human cardiomyocytes

Fig. 1A depicts two representative immunoblots loaded with samples of cardiomyocytes incubated overnight with various concentrations (left: all 1 mg/mL cTn, right: 0, 0.0625, 0.125 and 1.0 mg/mL cTn) of recombinant cTn complex containing Wt, 199D or 199A. Myc-tagged cTnT migrates more slowly through the gel in comparison to endogenous cTnT, resulting in 2 cTnT bands. The percentage of cTn exchange was calculated from the ratio of myc-tagged cTnT and the total amount of cTnT and reached an average of 63.4±0.8% with the 3 recombinant complexes at a concentration of 1 mg/mL in 3 human IDCM samples (Fig. 1B). No significant differences in percentage of cTn exchange were found with the different recombinant complexes, indicating that exogenous cTn complexes incorporated similarly in the myofilaments.

3.2. Sarcomeric protein phosphorylation before and after cTn exchange

Fig. 1C shows a gradient gel stained with ProQ-Diamond and Sypro Ruby. Cardiomyocytes were collected before and after cTn exchange containing Wt, 199D or 199A. No significant changes were found in the level of phosphorylation of myofilament proteins upon exchange with the cTn complexes (Table 1). This indicates that endogenous myofilament protein phosphorylation was not altered during overnight exchange with recombinant cTn. Since endogenous cTnT and cTnI phosphorylation level was low, no significant reduction in cTnT and cTnI phosphorylation was detected upon exchange with unphosphorylated whole troponin complex via ProQ-diamond staining. The level of cTnT and cTnI phosphorylation did not differ between cells exchanged with the different cTn complexes (Table 1).

3.3. Pseudo-phosphorylation of Ser199 increases myofilament Ca^{2+}-sensitivity

To determine the effects of Ser199 phosphorylation on myofilament force development at optimal overlap of the myofilaments, force measurements were performed at 2.2 μm sarcomere length in failing cardiomyocytes (3 IDCM hearts) exchanged with 199D. Data were compared to cells exchanged with unphosphorylated Wt cTnI, and to 199A, where alanine mimics dephosphorylation at Ser199. Compared to unphosphorylated Ser199 (Wt, pCa_{50} = 5.66±0.02; 199A, pCa_{50} = 5.65±0.01), myofilament Ca^{2+}-sensitivity was significantly higher after exchange with pseudo-phosphorylated Ser199 (199D, pCa_{50}=5.79±0.01) evident from the leftward shift of the force-pCa curve for 199D compared to Wt and 199A (Fig. 2A–B).

Since cTnI phosphorylation at Ser23/24 has been demonstrated to affect the length-dependent increase in myofilament Ca^{2+}-sensitivity [7,8], it was studied whether Ser199

phosphorylation affects length-dependent activation. Therefore, force was measured at a sarcomere length of 1.8 and 2.2 μm after cTn exchange (1 mg/mL: average exchange of 64.6±5.6%) in IDCM cardiomyocytes (2 IDCM hearts, 8 cells per group). A length-dependent increase in myofilament Ca^{2+}-sensitivity was found that was similar for all cTn complexes (Wt, 199D and 199A: ΔpCa_{50} 0.036±0.009, 0.047±0.010 and 0.039±0.003, respectively). Therefore, pseudo-phosphorylation of Ser199 does not seem to affect sarcomere length dependence of force development.

3.4. Saturation of the effect of Ser199 phosphorylation on myofilament Ca^{2+}-sensitivity

It was reported recently that phosphorylation of Ser199 on cTnI is higher (respectively, 136% and 127% greater) in human left ventricles from failing ischemic and dilated cardiomyopathy hearts compared to donor hearts [10]. Furthermore, an almost 5-fold increase of phosphorylation at Ser199 was demonstrated in a canine model of dyssynchronous pacing-induced heart failure (HF_{dys}) [10]. However, the level at which Ser199 phosphorylation alters myofilament function is unknown. Therefore, in addition to 199D exchange at 1 mg/mL, 199D exchange was done in IDCM cardiomyocytes at lower cTn concentrations (0.0625, 0.125, 0.25 and 0.5 mg/mL; 4–7 cardiomyocytes per concentration)(Fig. 2C). Interestingly, a relatively low exchange with 199D (5.9±2.5% cTn exchange at 0.0625 mg/mL complex) significantly increased myofilament Ca^{2+}-sensitivity (pCa_{50}=5.75±0.01) compared to control cardiomyocytes incubated overnight in exchange solution without cTn (pCa_{50}=5.68±0.02). Exchange experiments with lower concentrations of cTn containing 199A (0.0625, 0.125, 1 mg/mL) induced no significant differences compared to control cardiomyocytes. This demonstrates that the increase in Ca^{2+}-sensitivity after exchange with 199D is induced by pseudo-phosphorylation at Ser199 rather than the exchange method itself. Fig. 2C demonstrates the effect of 199D exchange on Ca^{2+}-sensitivity at different levels of cTn exchange. The effect of pseudo-phosphorylated Ser199 was calculated as the difference in pCa_{50} between 199D and 199A exchange. The maximal increase in pCa_{50} was reached with ~12% pseudo-phosphorylated Ser199, as a similar increase in Ca^{2+}-sensitivity was found with 12.3±2.6% and 74.7±0.6% 199D. Overall our data indicate that the cTnI-Ser199 phosphorylation-mediated increase in pCa_{50} is already evident at very low phosphorylation levels.

Although it is unknown whether Ser199 phosphorylation levels can be increased in the human healthy heart (for example during exercise- or stress-induced overload of the heart), we also studied the effect of pseudo-phosphorylation of Ser199 in donor cardiomyocytes, since it has been previously demonstrated that the “background” phosphorylation of myofilament proteins other than cTnI can affect the functional impact of cTnI phosphorylation [34]. To test this, exchange experiments were performed in non-failing donor cardiomyocytes with 199D and 199A at 0.0625 and 0.125 mg/mL in donor cardiomyocytes (4–5 cardiomyocytes per concentration). Donor control cardiomyocytes incubated overnight in exchange solution without cTn displayed a calcium sensitivity of pCa_{50} = 5.57±0.01. 199D exchange increased pCa_{50} by 0.14±0.02 units compared to 199A at 13.7±0.5% of cTn exchange. A similar increase in pCa_{50} was found at 37.1±1.8% exchange of 199D (0.15±0.02 units). The average values of the other parameters in donor cardiomyocytes were the following: F_{max}: 18.5±0.8 kN/m^2; F_{pas}: 2.4±0.2 kN/m^2; nHill:

2.7±0.1. Although donor cardiomyocytes demonstrated a lower calcium sensitivity and passive force compared to failing cardiomyocytes, a similar increase in pCa_{50} was found in donor compared to IDCM upon 199D exchange. Therefore, the functional influence of Ser199 may operate independently from the background environment.

3.5. Effects of pseudo-phosphorylated Ser199 on F_{max}, F_{pas} and nHill

Besides Ca^{2+}-sensitivity, other parameters of myofilament function (F_{max}, F_{pas} and nHill)) were determined at various sarcomere lengths (2 IDCM hearts; 8 cells per cTn complex) after exchange with the recombinant cTn complexes (1 mg/mL: average exchange of 64.6±5.6%). F_{max}, F_{pas} and nHill (Table 2) were not significantly different after exchange with cTn complex containing 199D compared to Wt or 199A. F_{max} and F_{pas} were higher at a sarcomere length of 2.2 μm compared to 1.8 μm after exchange with all cTn complexes (Table 2).

3.6. The rate of tension redevelopment after pseudo-phosphorylation of Ser199

The rate of tension redevelopment after exchange with pseudo-phosphorylated Ser199 did not differ from unphosphorylated cTnI (Wt or 199A) measured over a pCa-range from 5.6 to 4.5. Figure 3 depicts the k_{tr}-values plotted against the relative force. In line with a previous study [35], k_{tr} was not affected by sarcomere length (Table 2).

3.7. Effects of pseudo-phosphorylated cTnI-Ser199 on actin binding

The increase in myofilament Ca^{2+}-sensitivity in 199D may be explained by reduced binding of pseudo-phosphorylated Ser199 to actin compared to unphosphorylated Wt cTnI. In order to test whether cTnI-Ser199 phosphorylation affects cTnI binding to actin or actin-Tm, F-actin cosedimentation assays were performed using recombinant cTnI. Rabbit skeletal F-actin (5 μM) was incubated with different concentrations (0–8 μM) of the cTnI variants (Wt, 199D, 199A) and pelleted by centrifugation. At all cTnI concentrations, less cTnI-199D was detected in the actin pellets compared to cTnI-Wt, while less cTnI-199A was detected in the actin pellets compared to cTnI-Wt only at the highest cTnI concentration (Fig. 4A–B). When Tm was added to the actin-cTnI mixture at a physiological ratio of actin:cTnI:Tm = 7:1:1, significantly less cTnI-199D was co-pelleted with F-actin relative to cTnI-Wt, whereas cTnI-199A was similar to cTnI-Wt (Fig. 4C). These results indicate that pseudo-phosphorylation of cTnI-Ser199 decreases cTnI binding to both actin and actin-Tm, whereas the cTnI-199A mutant has minimal effect on F-actin binding only noticeable in saturating condition without Tm, but not affecting F-actin binding when Tm is included. One limitation of the F-actin binding assays with cTnI alone is that only one saturating concentration of cTnI (8 uM cTnI and 5 uM actin) was studied, which was not sufficient to demonstrate a possible plateau of saturating curves in figure 4A–B.

3.8. Alterations on Ser199 affect the sensitivity of cTnI to proteolysis

In order to test whether cTnI-Ser199 phosphorylation regulates cTnI susceptibility to proteolysis, calpain I was employed to partially proteolyze recombinant cTn containing the cTnI variants (Wt, 199D, 199A) *in vitro*. The proteolysis effect of calpain I on cTnI is demonstrated in figure 5A. With increasing concentration of calpain I in the reaction system,

the remaining amount of intact cTnI decreased while more degradation bands showed up. In addition, when the calpain-specific inhibitor calpastatin was added, cTnI proteolysis was completely inhibited.

It is worth noting that, when calpain I concentration is lower than 0.05 unit/μg cTn, only one degradation band was observed, which is named “major degradation band” in figure 5 and 6. Therefore, in order to simplify and optimize the quantification process, 0.025 or 0.05 unit calpain I /μg cTn was used in subsequent experiments to study the effect of calpain I on 199D and 199A. At both concentrations, both 199D and 199A significantly altered cTnI proteolysis compared to Wt (Fig. 5B–C).

Three different cTnI antibodies to different epitopes across cTnI were used to identify the major degradation product of cTnI by calpain I: against its N-terminus (McAb 4C2; epitope $cTnI_{23-29}$), against the inhibitory (middle) region (McAb 8I-7; epitope $cTnI_{137-148}$) and against the C-terminus (McAb P45-10; epitope $cTnI_{195-209}$)(Fig. 6). As shown in figure 6A for Wt, the major degradation band was visible with antibodies against the inhibitory region and C-terminus of cTnI, but not by the antibody directly against the N-terminus, indicating that the major proteolysis product of cTnI with calpain I treatment is N-, rather than C-terminus truncated. This degradation pattern was observed in all three cTnI variants (Fig. 6B).

4. Discussion

Our study shows that pseudo-phosphorylation of cTnI at Ser199 in human cardiomyocytes increases Ca^{2+}-sensitivity of force development, but does not affect maximal and passive isometric forces, steepness of the force-Ca^{2+} relation and the rate of force redevelopment. Also, the length-dependent increase in myofilament Ca^{2+}-sensitivity was unaltered. The most likely mechanism of 199D-mediated increase in myofilament Ca^{2+}-sensitivity is reduced cTnI binding to both actin and actin-Tm. Moreover, amino acid changes at the Ser199 site altered susceptibility of cTnI to proteolysis by calpain I.

The observed increase in myofilament Ca^{2+}-sensitivity upon exchange with pseudo-phosphorylated Ser199 on cTnI may improve contractile performance of the heart during systole, while it may rather limit relaxation during diastole. Interestingly, our results indicate that the maximal Ca^{2+}-sensitizing effect is already reached at relatively low phosphorylation levels (~12%), suggesting that modest phosphorylation at this site has physiological relevance. Recently, a higher phosphorylation at Ser199 was demonstrated in human end-stage heart failure compared to non-failing donors [10]. Moreover, in a dog study, the phosphorylation level raised from 3% in healthy dogs to 14% in dogs with dyssynchronous pacing-induced heart failure (HF_{dys}) [10]. This suggests that Ser199 phosphorylation might have a more prominent role in heart failure compared to the healthy heart. Moreover, Ser199 was identified as target of PKCα [11]. It has been demonstrated that PKCα expression [36] and PKC activity [36] is up-regulated in the failing heart. In addition, PKC-mediated phosphorylation of cTnI at Ser42/44 and Thr143 has been demonstrated to impair sarcomere function [9,37–39]. Therefore, increased PKC activity in the failing heart might be responsible for the increased Ser199 phosphorylation levels in human end-stage heart failure

compared to non-failing donors and in the canine model of HF_{dys} [10]. Our data demonstrates that, although phosphorylation levels might be relatively low at Ser199 in human [10], myofilament Ca^{2+}-sensitivity may already be affected. In addition, the increased Ser199 phosphorylation in a canine model of HF_{dys} was reversed toward control levels by cardiac resynchronization therapy, suggesting this can be targeted therapeutically [10].

The present study relies on the assumption that aspartic acid incorporation at Ser199 mimics phosphorylation. As we discussed before [5], previous studies have indicated that phosphorylation and pseudo-phosphorylation by aspartic acid structurally and functionally behave the same at cTnI-Ser23/24. In addition, it has also been demonstrated that aspartic acid incorporation at cTnI-Thr143 [8,40] functionally (maximal force and Ca^{2+}-sensitivity) mimics phosphorylation. We used site-specific pseudo-phosphorylation at Ser199 to study the effects of phosphorylation of this site on myofilament function and cTnI proteolysis, since there are no site-specific kinases or phosphatases known for this novel phosphorylation site. Therefore, at this moment, site-specific effects of Ser199 phosphorylation can only be studied by pseudo-phosphorylation of this site.

Ser199 lies within the mobile C-terminal domain of cTnI. A previous study showed an increase in myofilament Ca^{2+}-sensitivity upon exchange with partially C-terminal truncated human cTnI ($cTnI_{1\text{-}192}$) [20]. Truncation of the cTnI C-terminus is induced by selective proteolysis in human ischemic myocardial disease [16]. The C-terminal domain (residues 193 to 210) has been reported to stabilize Tm on actin filaments during the C-state of cross-bridge interaction [41]. The increased myofilament Ca^{2+}-sensitivity upon exchange with C-terminal truncated cTnI may be explained by destabilization of Tm on the thin filament. It is tempting to speculate that phosphorylation of Ser199 on cTnI is sufficient to destabilize Tm position on the thin filament during Ca^{2+}-activation, which would result in increased exposure of myosin-binding sites on actin at submaximal [Ca^{2+}]. This may explain the increased Ca^{2+}-sensitivity upon Ser199 phosphorylation without changes in force at low (F_{pas}) and saturating [Ca^{2+}] (F_{max}).

There is evidence that the C-terminal mobile region (C-MR) of cTnI including Ser199 is disordered at high [Ca^{2+}] and senses and interacts with actin upon low [Ca^{2+}]; the coupling of folding to binding of C-MR during its interaction with actin plays a crucial role in the kinetics of thin filament regulation [42,43,44]. Therefore, we tested whether Ser199 phosphorylation affects cTnI affinity for actin-binding. Our F-actin cosedimentation assay showed that pseudo-phosphorylated cTnI in the absence and presence of Tm has significantly less affinity for actin as compared to Wt, indicating that phosphorylation of Ser199 may lower the ability of cTnI C-MR to bind actin. The recent structural data suggested [45–47] that the end of cTnI C-MR interacts with actin-Tm to stabilize Tm on actin during relaxation, which further demonstrated the importance of the C-terminal end of cTnI for muscle relaxation.

Surprisingly, myofilament Ca^{2+}-sensitivity already increased at 6% Ser199 phosphorylation and the maximal increase in myofilament Ca^{2+}-sensitivity was reached at a Ser199 phosphorylation level of ~12%. Previously it has been observed that systolic and diastolic

heart function was compromised in transgenic mice with only 9–17% truncated $cTnI_{1\text{-}193}$ of total cTnI expressed in the heart [48]. Both studies demonstrate that post-translational modifications (phosphorylation, truncation) at relatively low levels at the C-terminus of cTnI may have significant impact on cardiac function. Interestingly, for comparison, similar experiments using pseudo-phosphorylated cTnI-Ser23/24 showed that the maximal effect on myofilament Ca^{2+}-sensitivity was reached at ~55% [5]. Although in our study phosphorylation of Ser199 did not significantly change cooperativity (nHill) of myofilament force development, some form of communication along the thin filament needs to be present in order to explain why partial Ser199 phosphorylation has a large impact on myofilament function. As described above, the C-terminal domain of cTnI stabilizes Tm in the C-state [41]. Destabilization of Tm by Ser199 phosphorylation would be expected to be propagated beyond a single contractile unit caused by the semi-rigid nature of Tm [49]. Ser199 phosphorylation may be responsible for a transition from the preserved B-state to an 'enhanced C-state' upon Ca^{2+}-activation of the myofilament. Low levels of Ser199 phosphorylation may already induce destabilization of the 'semi-rigid' Tm along the myofilament and thereby increase Ca^{2+}-sensitivity without affecting myofilament cooperativity.

Since cTnI proteolysis is present in both physiological and pathological conditions [14–17] and was found to be regulated by cTnI phosphorylation [28], we studied whether Ser199 pseudo-phosphorylation altered the susceptibility of cTnI to proteolysis. Our study used recombinant cTn complex and the protease calpain I for several reasons. Calpain I is an ubiquitous Ca^{2+}-activated protease that may be involved in ischemia-related cTnI proteolysis [21–23]. cTnI alone can present different proteolysis pattern than with cTnT-cTnC in the cTn complex [25]. However, the subsite specificity of calpain I is determined more by conformation than by the primary amino acid sequences [50] thus we studied proteolysis in the cTn complex. Our proteolysis assay results showed that both cTnI-199D and 199A altered cTnI proteolysis by calpain I. It is notable that mutations around this site do not cause the same effects. In the study of Barta et al, familial hypertrophic cardiomyopathy related mutations on cTnI C-terminus (G203S and K206Q) did not affect calpain-I-mediated cTnI degradation [25]. Therefore, our results indicate that the strongly conserved site Ser199 [12] plays a critical role in regulating cTnI susceptibility to proteolysis and potential changes at this site, including phosphorylation, significantly affects how calpain I acts on cTnI. However, due to the limitation of our *in vitro* assays, it is hard to conclude how cTnI proteolysis was regulated by Ser199. With low concentration of calpain I (0.025–0.05 units enzyme/μg protein), only one degradation band can be detected in our system, and thus the ratio of the degradation band to total cTnI in the same lane (Fig 5) can present the percentage of cTnI degradation; the ratio was increased in 199D and 199A groups relative to Wt group. Since it is not feasible in our assays to elucidate whether and how 199D affects the subsequent steps of cTnI proteolysis after the first cleavage by calpain, it remains unclear to which direction (increase or decrease) Ser199 substitutions affect cTnI and/or cTnI fragments proteolysis.

By using three cTnI antibodies against different parts of cTnI, we were able to identify the major cTnI proteolytic product after calpain I treatment as N-, rather than C-terminus

truncated cTnI. The finding that the primary cleavage of cTnI performed by calpain I is near N-terminus is of interest for several reasons. The N-terminal extension is a unique feature to cTnI as compared to the other two isoforms of TnI and it contains two most important phosphorylation sites of cTnI, Ser23 and Ser24. In addition, N-terminal truncation of cTnI is present in the normal hearts of multiple species including human heart [17]. It has been found that this modification enhances diastolic function [51,52], can rescue function in a model of restrictive cardiomyopathy [52] and is a compensatory response in microgravity [17]. Therefore, proteolytic removal of cTnI N-terminal extension is a potentially important post-translational modification. Our results suggest that calpain I could be involved in the selective proteolysis of cTnI N-terminus and this process is regulated by phosphorylation of cTnI Ser199.

cTnI-S199A behaves similar to Wt in myofilament functional assays but alters cTnI proteolysis mediated by calpain I. This discrepancy is not unexpected because the mechanism by which Ser199 affects actin binding and myofilament Ca^{2+}-sensitivity is not necessarily the same as that of enzyme-substrate interaction. An alternative explanation may be the difference of complex system biology used in both assays - for muscle function the different cTnI forms were tested in the presence of thin and thick myofilament proteins, while for the cTnI proteolysis studies the experiments were conducted only in troponin complex. These studies are limited in that we cannot fully explain the mechanisms by which both 199A and 199D affect calpain-mediated cTnI proteolysis. The C-terminus of cTnI is thought to be an intrinsic disordered peptide that is unstructured during muscle contraction and folds and binds simultaneously to actin to ensure muscle relaxation as proposed in the fly-casting model [44]. Thus these results may be informative for future structural studies.

In conclusion, our studies show that pseudo-phosphorylation at Ser199 of the cTnI C-terminus increases myofilament Ca^{2+}-sensitivity at a relatively low level of phosphorylation, most likely by decreasing the binding affinity of cTnI for actin-Tm. In addition, cTnI-Ser199 pseudo-phosphorylation or mutation regulates calpain I mediated proteolysis of cTnI. Overall, our study indicates that phosphorylation (and possibly other post-translational modifications) at Ser199 may have a significant physiological impact on cardiac disease.

Acknowledgments

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Abbreviations

A	Alanine
cMyBP-C	cardiac myosin-binding protein-C
cTn	cardiac troponin
cTnC	cardiac troponin C
cTnI	cardiac troponin I

cTnT	cardiac troponin T
D	Aspartic acid
F_{max}	maximal force
F_{pas}	passive force
HF_{dys}	dyssynchronous pacing-induced heart failure
IDCM	idiopathic dilated cardiomyopathy
k_{tr}	rate of tension redevelopment
k_{tr-max}	maximal rate of tension redevelopment
MLC2	myosin light chain 2
pCa_{50}	$-\log_{10}$ of the calcium concentration at which 50% of maximal force is reached
nHill	steepness of the force-pCa relation
PKA	protein kinase A
PKC	protein kinase C
Ser	Serine
Tm	tropomyosin
199A	cTnI-Ser199 mutated into alanine (pseudo-dephosphorylated)
199D	cTnI-Ser199 mutated into aspartic acid (pseudo-phosphorylated)

References

1. Kobayashi T, Solaro RJ. Calcium, thin filaments, and the integrative biology of cardiac contractility. Annu Rev Physiol. 2005; 67:39–67. [PubMed: 15709952]
2. Lehman W, Craig R. Tropomyosin and the steric mechanism of muscle regulation. Adv Exp Med Biol. 2008; 644:95–109. [PubMed: 19209816]
3. Solaro RJ, Moir AJ, Perry SV. Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart. Nature. 1976; 262:615–617. [PubMed: 958429]
4. Takimoto E, Soergel DG, Janssen PM, Stull LB, Kass DA, Murphy AM. Frequency- and afterload-dependent cardiac modulation in vivo by troponin I with constitutively active protein kinase A phosphorylation sites. Circ Res. 2004; 94:496–504. [PubMed: 14726477]
5. Wijnker PJM, Foster DB, Tsao AL, Frazier AH, dos Remedios CG, Murphy AM, et al. Impact of site-specific phosphorylation of protein kinase A sites Ser23 and Ser24 of cardiac troponin I in human cardiomyocytes. Am J Physiol Heart Circ Physiol. 2013; 304:H260–H268. [PubMed: 23144315]
6. Zhang R, Zhao J, Mandveno A, Potter JD. Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. Circ Res. 1995; 76:1028–1035. [PubMed: 7758157]
7. Konhilas JP, Irving TC, Wolska BM, Jweied EE, Martin AF, Solaro RJ, et al. Troponin I in the murine myocardium: influence on length-dependent activation and interfilament spacing. J Physiol. 2003; 547:951–961. [PubMed: 12562915]
8. Wijnker PJM, Sequeira V, Foster DB, Li Y, dos Remedios CG, Murphy AM, et al. 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. 2014; 306:H1171–1181. [PubMed: 24585778]

9. Sumandea MP, Burkart EM, Kobayashi T, De Tombe PP, Solaro RJ. Molecular and integrated biology of thin filament protein phosphorylation in heart muscle. Ann N Y Acad Sci. 2004; 1015:39–52. [PubMed: 15201148]
10. Zhang P, Kirk JA, Ji W, dos Remedios CG, Kass DA, Van Eyk JE, et al. Multiple reaction monitoring to identify site-specific troponin I phosphorylated residues in the failing human heart. Circulation. 2012; 126:1828–1837. [PubMed: 22972900]
11. Kooij V, Zhang P, Piersma SR, Sequeira V, Boontje NM, Wijnker PJM, et al. PKCα-specific phosphorylation of the troponin complex in human myocardium: a functional and proteomics analysis. PLoS One. 2013:e74847. doi:10.1371:8. [PubMed: 24116014]
12. Jin JP, Yang FW, Yu ZB, Ruse CI, Bond M, Chen A. The highly conserved COOH terminus of troponin I forms a Ca^{2+}-modulated allosteric domain in the troponin complex. Biochemistry. 2001; 40:2623–2631. [PubMed: 11327886]
13. Mogensen J, Murphy RT, Kubo T, Bahl A, Moon JC, Klausen IC, et al. Frequency and clinical expression of cardiac troponin I mutations in 748 consecutive families with hypertrophic cardiomyopathy. J Am Coll Cardiol. 2004; 44:2315–2325. [PubMed: 15607392]
14. Murphy AM. Heart failure, myocardial stunning, and troponin: a key regulator of the cardiac myofilament. Congest Heart Fail. 2006; 12:32–38. 39–40. [PubMed: 16470090]
15. Feng J, Schaus BJ, Fallavollita JA, Lee TC, Canty JM Jr. Preload induces troponin I degradation independently of myocardial ischemia. Circulation. 2001; 103:2035–2037. [PubMed: 11319190]
16. McDonough JL, Labugger R, Pickett W, Tse MY, MacKenzie S, Pang SC, et al. Cardiac troponin I is modified in the myocardium of bypass patients. Circulation. 2001; 103:58–64. [PubMed: 11136686]
17. Yu ZB, Zhang LF, Jin JP. A proteolytic NH_2-terminal truncation of cardiac troponin I that is up-regulated in simulated microgravity. J Biol Chem. 2001; 276:15753–15760. [PubMed: 11278823]
18. Murphy AM, Kogler H, Marban E. A mouse model of myocardial stunning. Mol Med Today. 2000; 6:330–331. [PubMed: 10904251]
19. Foster DB, Noguchi T, Van Buren P, Murphy AM, Van Eyk JE. C-terminal truncation of cardiac troponin I causes divergent effects on ATPase and force: implications for the pathophysiology of myocardial stunning. Circ Res. 2003; 93:917–924. [PubMed: 14551240]
20. Narolska NA, Piroddi N, Belus A, Boontje NM, Scellini B, Deppermann S, et al. Impaired diastolic function after exchange of endogenous troponin I with C-terminal truncated troponin I in human cardiac muscle. Circ Res. 2006; 99:1012–1020. [PubMed: 17023673]
21. Gao WD, Liu Y, Mellgren R, Marban E. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis? Circ Res. 1996; 78:455–465. [PubMed: 8593704]
22. Urthaler F, Wolkowicz PE, Digerness SB, Harris KD, Walker AA. MDL-28170, a membrane-permeant calpain inhibitor, attenuates stunning and PKC epsilon proteolysis in reperfused ferret hearts. Cardiovasc Res. 1997; 35:60–67. [PubMed: 9302348]
23. Van Eyk JE, Murphy AM. The role of troponin abnormalities as a cause for stunned myocardium. Coron Artery Dis. 2001; 12:343–347. [PubMed: 11491198]
24. McConnell BK, Popovic Z, Mal N, Lee K, Bautista J, Forudi F, et al. Disruption of protein kinase A interaction with A-kinase-anchoring proteins in the heart in vivo: effects on cardiac contractility, protein kinase A phosphorylation, and troponin I proteolysis. J Biol Chem. 2009; 284:1583–1592. [PubMed: 18945669]
25. Barta J, Tóth A, Jaquet K, Redlich A, Edes I, Papp Z. Calpain-1-dependent degradation of troponin I mutants found in familial hypertrophic cardiomyopathy. Mol Cell Biochem. 2003; 251:83–88. [PubMed: 14575308]
26. Litersky JM, Johnson GV. Phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain. J Biol Chem. 1992; 267:1563–1568. [PubMed: 1730702]
27. Elvira M, Díez JA, Wang KK, Villalobo A. Phosphorylation of connexin-32 by protein kinase C prevents its proteolysis by mu-calpain and m-calpain. J Biol Chem. 1993; 268:14294–14300. [PubMed: 8390988]

28. Di Lisa F, De Tullio R, Salamino F, Barbato R, Melloni E, Siliprandi N, et al. Specific degradation of troponin T and I by mu-calpain and its modulation by substrate phosphorylation. Biochem J. 1995; 308:57–61. [PubMed: 7755588]
29. Hamdani N, Borbely A, Veenstra SP, Kooij V, Vrydag W, Zaremba R, et al. More severe cellular phenotype in human idiopathic dilated cardiomyopathy compared to ischemic heart disease. J Muscle Res Cell Motil. 2010; 31:289–301. [PubMed: 21132354]
30. Zaremba R, Merkus D, Hamdani N, Lamers JM, Paulus WJ, dos Remedios CG, et al. Quantitative analysis of myofilament protein phosphorylation in small cardiac biopsies. Proteom Clin Appl. 2007; 1:1285–90.
31. Pardee JD, Aspudich J, Dixie W, Frederiksen LWC. Purification of muscle actin. Methods Enzymol. 1982; 18:164–181. [PubMed: 7121269]
32. Smillie LB, Dixie W, Frederiksen LWC. Preparation and identification of α- and β-tropomyosins. Methods Enzymol. 1982; 21:234–241. [PubMed: 6289041]
33. Hitchcock SE. Regulation of muscle contraction: binding of troponin and its components to actin and tropomyosin. Eur J Biochem. 1975; 52:255–263. [PubMed: 126151]
34. Kooij V, Saes M, Jaquet K, Zaremba R, Foster DB, Murphy AM, et al. Effect of troponin I Ser23/24 phosphorylation on Ca^{2+}-sensitivity in human myocardium depends on the phosphorylation background. J Mol Cell Cardiol. 2010; 48:954–996. [PubMed: 20079747]
35. Edes IF, Czuriga D, Csanyi G, Chlopicki S, Recchia FA, Borbely A, et al. Rate of tension redevelopment is not modulated by sarcomere length in permeabilized human, murine, and porcine cardiomyocytes. Am J Physiol Regul Integr Comp Physiol. 2007; 293:R20–R29. [PubMed: 17110532]
36. Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, et al. Increased protein kinase C activity and expression of Ca^{2+}-sensitive isoforms in the failing human heart. Circulation. 1999; 99:384–391. [PubMed: 9918525]
37. Belin RJ, Sumandea MP, Allen EJ, Schoenfelt K, Wang H, Solaro RJ, et al. Augmented protein kinase C-alpha-induced myofilament protein phosphorylation contributes to myofilament dysfunction in experimental congestive heart failure. Circ Res. 2007; 101:195–204. [PubMed: 17556659]
38. Kooij V, Boontje N, Zaremba R, Jaquet K, dos Remedios CG, Stienen GJM, et al. Protein kinase C alpha and epsilon phosphorylation of troponin and myosin binding protein C reduce Ca^{2+} sensitivity in human myocardium. Basic Res Cardiol. 2010; 105:289–300. [PubMed: 19655190]
39. Wijnker PJM, Sequeira V, Witjas-Paalberends ER, Foster DB, dos Remedios CG, Murphy AM, et al. Phosphorylation of protein kinase C sites Ser42/44 decreases Ca^{2+}-sensitivity and blunts enhanced length-dependent activation in response to protein kinase A in human cardiomyocytes. Arch Biochem Biophys. 2014; 554:11–21. [PubMed: 24814372]
40. Wang H, Grant JE, Doede CM, Sadayappan S, Robbins J, Walker JW. PKC-betaII sensitizes cardiac myofilaments to Ca^{2+} by phosphorylating troponin I on threonine-144. J Mol Cell Cardiol. 2006; 41:823–833. [PubMed: 17010989]
41. Galinska A, Hatch V, Craig R, Murphy AM, Van Eyk JE, Wang CL, et al. The C terminus of cardiac troponin I stabilizes the Ca^{2+}-activated state of tropomyosin on actin filaments. Circ Res. 2010; 106:705–711. [PubMed: 20035081]
42. Zhou Z, Li KL, Rieck D, Ouyang Y, Chandra M, Dong WJ. Structural dynamics of C-domain of cardiac troponin I protein in reconstituted thin filament. J Biol Chem. 2012; 287:7661–7674. [PubMed: 22207765]
43. Blumenschein TMA, Stone DB, Fletterick RJ, Mendelson RA, Sykes BD. Dynamics of the C-Terminal region of TnI in the troponin complex in solution. Biophysical J. 2006; 90:2436–2444.
44. Hoffman RMB, Blumenschein TMA, Sykes BD. An interplay between protein disorder and structure confers the Ca^{2+} regulation of striated muscle. J Mol Biol. 2006; 361:625–633. [PubMed: 16876196]
45. Galińska-Rakoczy A, Engel P, Xu C, Jung H, Craig R, Tobacman LS, et al. Structural basis for the regulation of muscle contraction by troponin and tropomyosin. J Mol Biol. 2008; 379:929–935. [PubMed: 18514658]

46. Pirani A, Vinogradova MV, Curmi PM, King WA, Fletterick RJ, Craig R, et al. An atomic model of the thin filament in the relaxed and Ca^{2+}-activated states. J Mol Biol. 2006; 357:707–717. [PubMed: 16469331]

47. Yang S, Barbu-Tudoran L, Orzechowski M, Craig R, Trinick J, White H, et al. Three-dimensional organization of troponin on cardiac muscle thin filaments in the relaxed state. Biophys J. 2014; 106:855–864. [PubMed: 24559988]

48. Murphy AM, Kogler H, Georgakopoulos D, McDonough JL, Kass DA, Van Eyk JE, et al. Transgenic mouse model of stunned myocardium. Science. 2000; 287:488–491. [PubMed: 10642551]

49. Li XE, Holmes KC, Lehman W, Jung H, Fischer S. The shape and flexibility of tropomyosin coiled coils: implications for actin filament assembly and regulation. J Mol Biol. 2010; 395:327–339. [PubMed: 19883661]

50. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol Rev. 2003; 83:731–801. [PubMed: 12843408]

51. Barbato JC, Huang QQ, Hossain MM, Bond M, Jin JP. Proteolytic N-terminal truncation of cardiac troponin I enhances ventricular diastolic function. J Biol Chem. 2005; 280:6602–6609. [PubMed: 15611140]

52. Li Y, Charles PY, Nan C, Pinto JR, Wang Y, Liang J, Wu G, Tian J, Feng HZ, Potter JD, Jin JP, Huang X. Correcting diastolic dysfunction by Ca^{2+} desensitizing troponin in a transgenic mouse model of restrictive cardiomyopathy. J Mol Cell Cardiol. 2010; 49:402–411. [PubMed: 20580639]

Highlights

- cTnI-Ser199 pseudo-phosphorylation (199D) increases myofilament Ca^{2+}-sensitivity
- Low levels of 199D (~6%) already increase Ca^{2+}-sensitivity in human cardiomyocytes
- 199D decreases cTnI binding to actin-tropomyosin

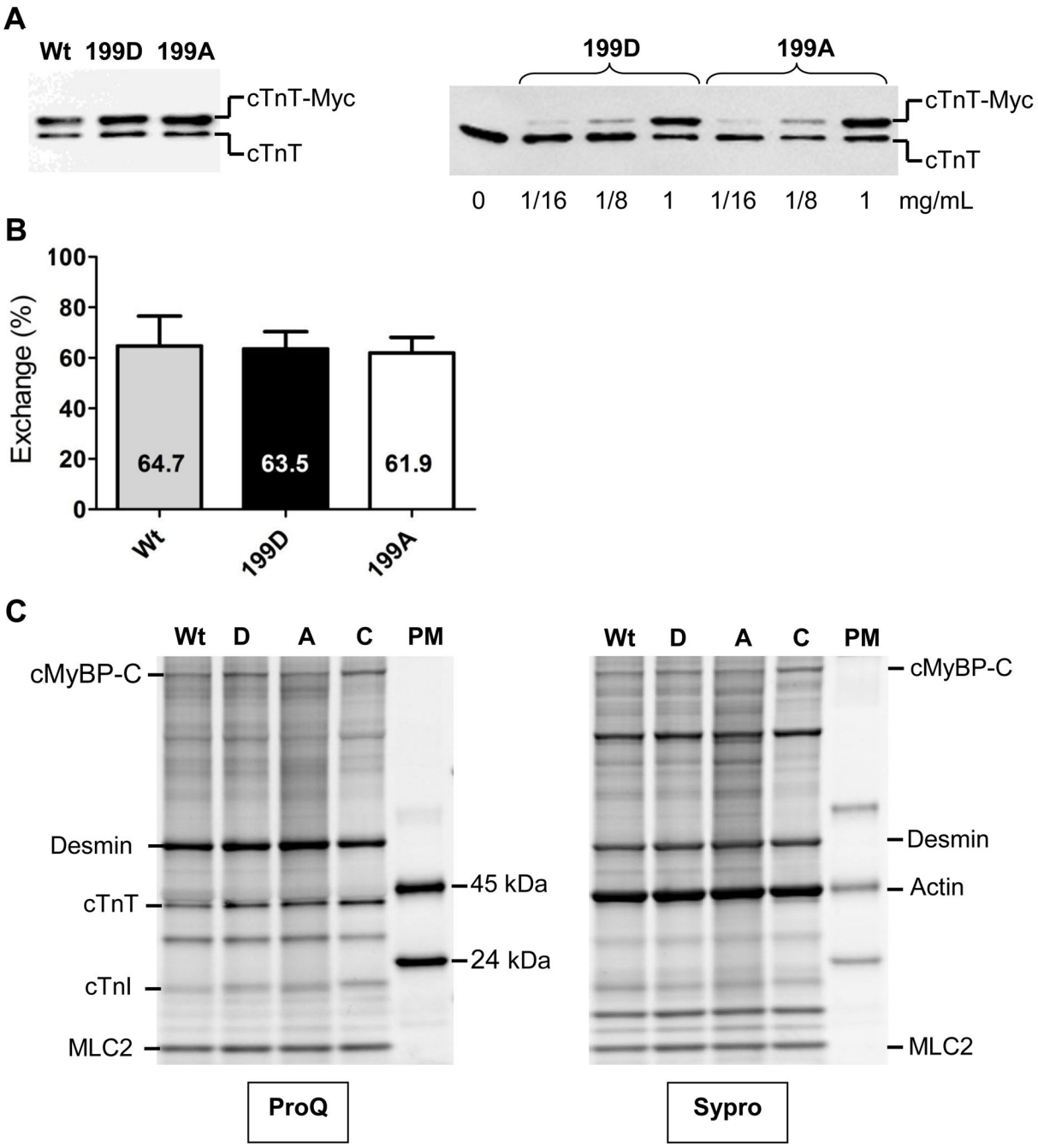

Fig. 1.

Quantification of troponin exchange and myofilament protein phosphorylation. A. Immunoblots stained with an antibody against troponin T (cTnT) that recognizes both endogenous cTnT (lower band) and recombinant myc-tag labeled cTnT (cTnT-myc; upper band). Left: An example is shown of suspensions of cardiomyocytes exchanged with 1 mg/mL recombinant cTn containing Wt, 199D or 199A. Right: An example is shown of a suspension of cardiomyocytes exchanged with 0, 0.0625, 0.125 and 1 mg/mL cTn complex containing 199D or 199A. B. The average percentages of cTn exchange in cardiomyocytes

after overnight incubation in exchange solution with 1 mg/mL cTn complex containing the different complexes (average values represent cTn exchange experiments in cardiomyocytes isolated from 3 IDCM hearts). C. Gradient gel (Criterion Tris-HCl 4–15% gel, BioRad) stained with Sypro Ruby and ProQ-Diamond. Cardiomyocytes were collected before exchange (C, control) and after cTn exchange containing Wt (Wt), 199D (D) or 199A (A). A PeppermintStick Phosphoprotein marker (PM) was added on the gel. Abbreviations: cMyBP-C, cardiac myosin-binding protein-C; 199A, pseudo-dephosphorylated cTnI; 199D, pseudo-phosphorylated cTnI; Wt, wild-type cTnI; MLC2, myosin light chain 2.

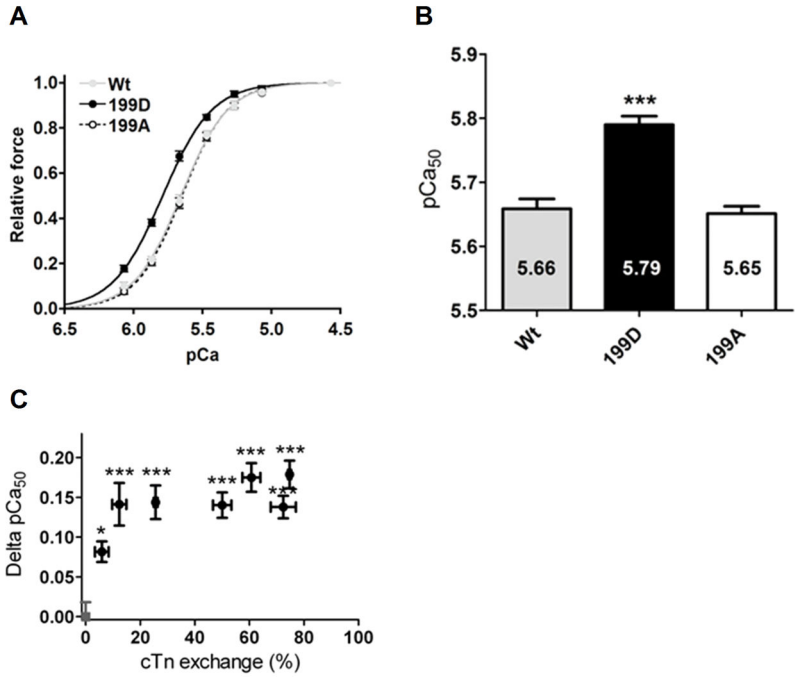

Fig. 2.

Phosphorylation of cTnI at Ser199 increases myofilament Ca^{2+}-sensitivity. A. Myofilament force development measured at a sarcomere length of 2.2 μm at various [Ca^{2+}] in permeabilized IDCM cardiomyocytes in which endogenous cTn was partially exchanged (63.4±0.8%) with 1 mg/mL of the recombinant myc-tag labeled cTn complexes (12–13 cardiomyocytes from 3 IDCM hearts). B. Ca^{2+}-sensitivity derived from the midpoint of the force-pCa relationship (pCa_{50}) was significantly increased after exchange with the pseudo-phosphorylated cTnI (199D) compared to unphosphorylated wild-type cTnI (Wt) or cTnI-199A. C. Relation between phosphorylated Ser199 and myofilament Ca^{2+}-sensitivity in IDCM cardiomyocytes (4–7 cells) exchanged with different concentrations of recombinant 199A and 199D (concentrations ranging between 0.0625 to 1.0 mg/mL). Cardiomyocytes immersed in exchange solution without complex during the overnight exchange served as control (0% cTn exchange). A linear fit through 199A pCa_{50}-values at different levels of exchange was used as a control to exclude that cTn exchange itself influences Ca^{2+}-sensitivity. This made it possible to calculate the increase in pCa_{50} after 199D exchange compared to control cardiomyocytes (199A) exchanged with the same amount of cTn. *P< 0.05, ***P<0.001, control versus 199D in post-test Bonferroni analyses of one-way ANOVA.

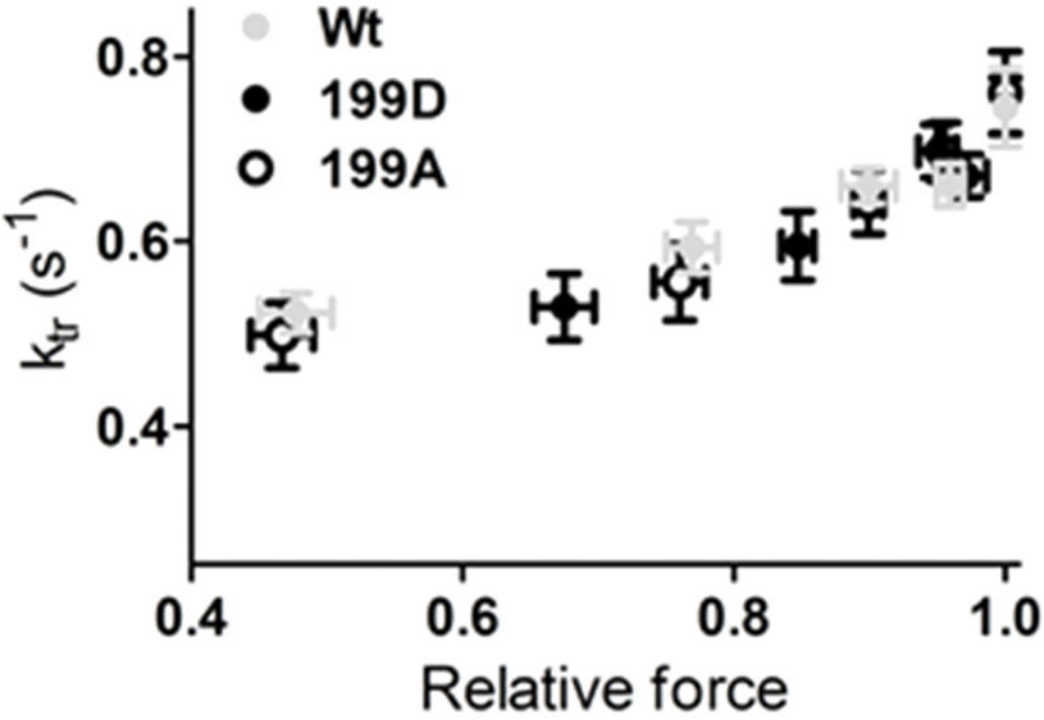

Fig. 3.

Rate of tension redevelopment upon pseudo-phosphorylation of Ser199. The rate of tension redevelopment (k_{tr}) determined from a single exponential curve fit of force redevelopment after a slack-restretch test at maximal and submaximal [Ca^{2+}] was plotted against relative force. No significant differences were found in k_{tr} at maximal and submaximal Ca^{2+}-concentrations between the different cTn complexes.

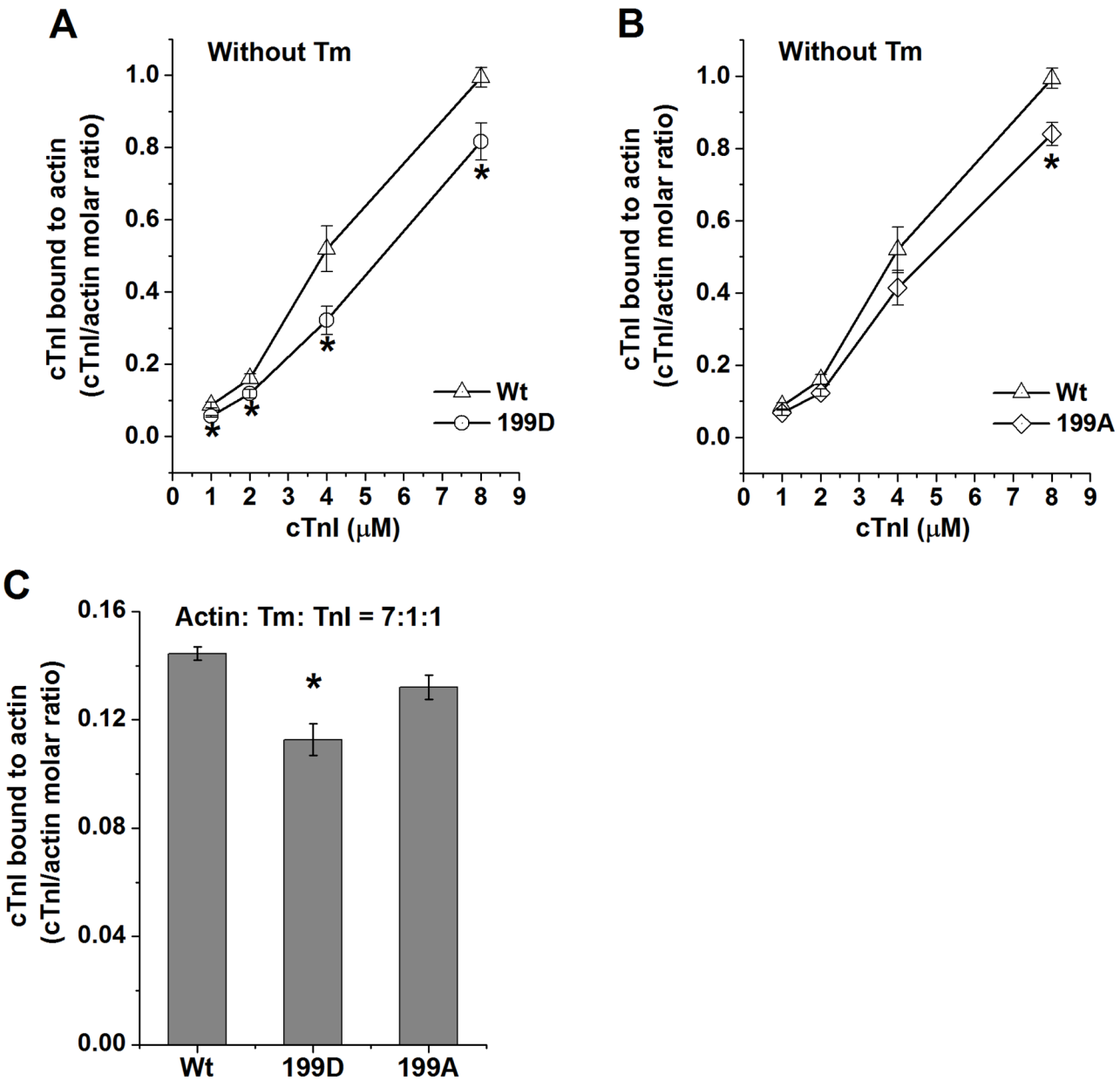

Fig. 4.

F-actin binding of the cTnI variants. A and B. F-actin cosedimentation assays using rabbit skeletal F-actin (5μM) and recombinant human cTnI (from 0 to 8 μM) were resolved by SDS–PAGE followed by Coomassie staining. The molar ratio of cTnI to actin in pellets after centrifugation is shown as a function of cTnI concentration. * $P < 0.05$ compared to Wt. C. Data of cTnI-actin co-sedimentation after incubating cTnI, Tm and actin in a molar ratio of 1:1:7. * $P < 0.05$ compared to Wt. Values are presented as means ± SEM ($n = 5$). Statistical significance was determined by one-way ANOVA followed by post hoc Newman–Keuls tests.

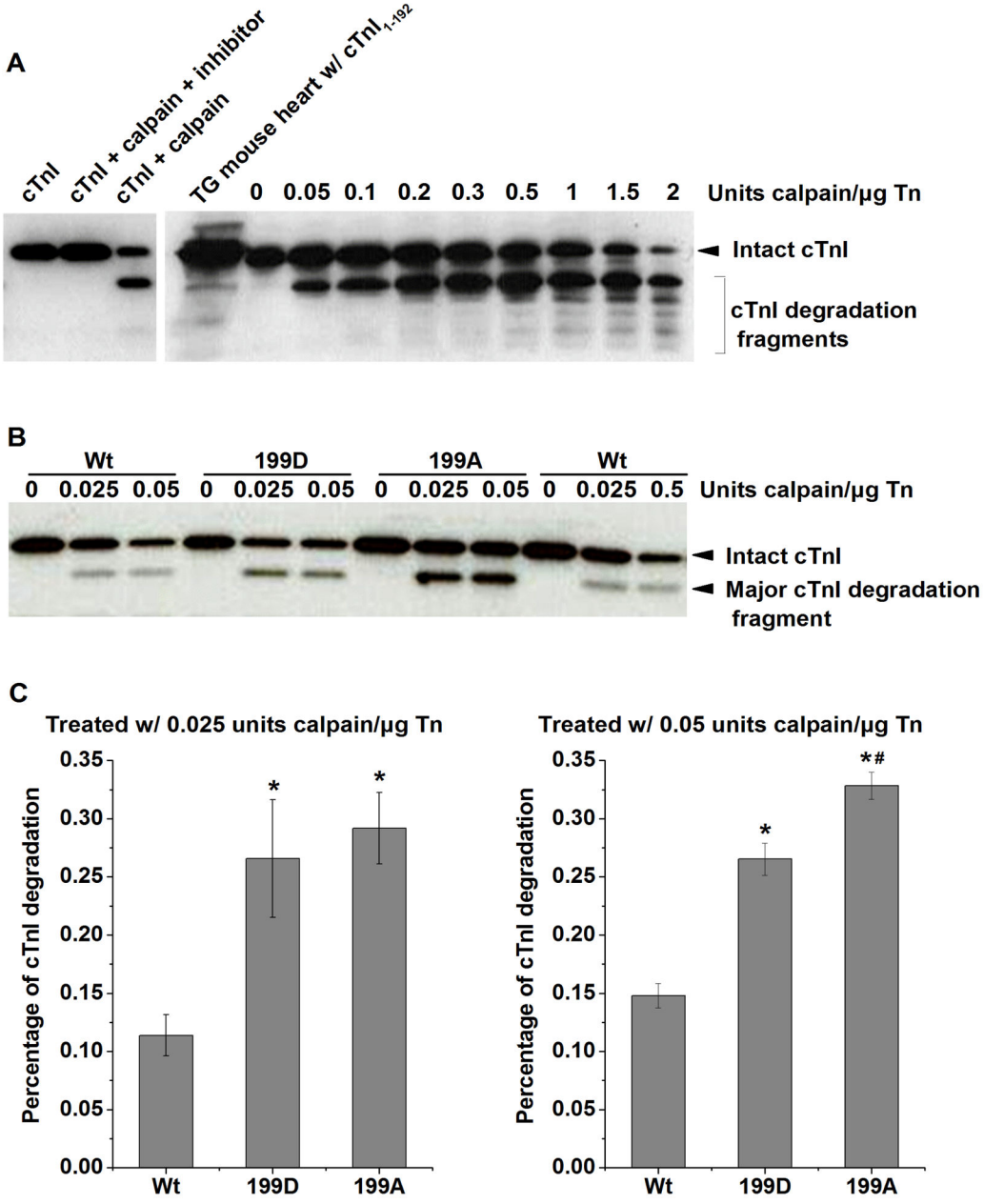

Fig. 5.

Proteolysis of recombinant cTnI by calpain I. A. A representative blotting of cTnI (McAb 8I-7) and its fragments after digestion with different concentrations of calpain I for 10 min with or without the presence of calpain-specific inhibitor calpastatin. B. A representative immunoblot of the different cTnI species (Wt, 199A, 199D) and its fragments (McAb 8I-7) after treatment with 0.025 and 0.05 units of calpain I per μg cTn. C. Quantitative results of cTnI degradation. Percentage of cTnI degradation was calculated within the same lane by using the formula: Rd = Dm/(Dr+Dm). Rd represents percentage of cTnI degradation, Dr represents the density of the remaining intact cTnI after degradation and Dm represents the density of major degradation. Values are presented as means ± SEM (*n* = 5). Statistical

significance was determined by one-way ANOVA followed by post hoc Newman–Keuls tests. * $P < 0.05$ compared to Wt, $^{\#}$ $P<0.05$ as compared with 199D.

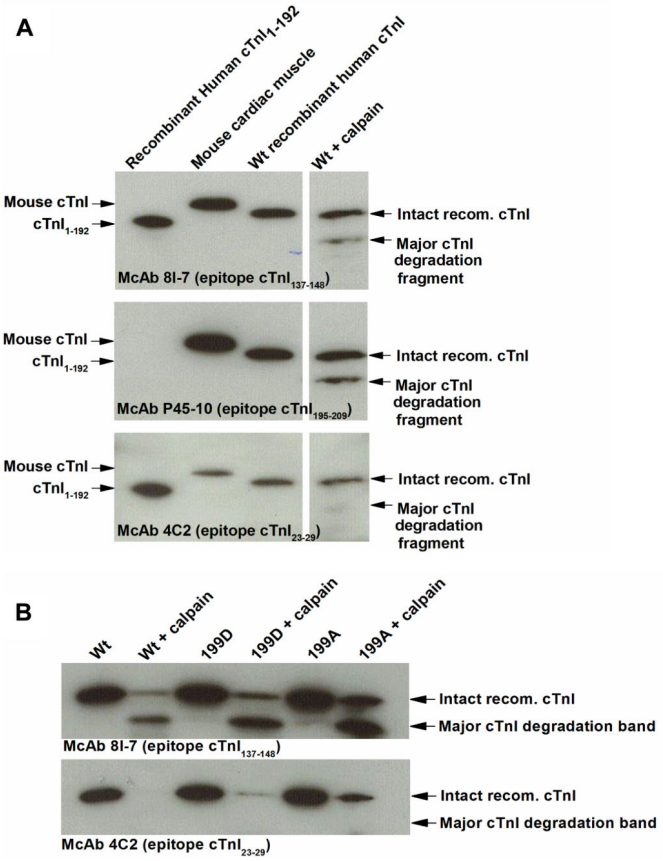

Fig. 6.

Identification of the major degradation product of cTnI after exposure to calpain I. A. Three different cTnI monoclonal antibodies (McAb), 8I-7, P45-10 (1:5000, HyTest Ltd.) and 4C2 (1:1000, HyTest Ltd.) which are directly against the middle part, C-terminus and N-terminus of cTnI, respectively, were used to identify the cTnI degradation products by western blotting. The homogenate of wild-type mouse cardiac muscle and recombinant human $cTnI_{1-192}$ protein were used as control. The wild-type mouse cTnI was recognized by all three McAbs, while recombinant protein $cTnI_{1-192}$ was recognized by McAb 8I-7 and McAb 4C2, but not by McAb P45-10. B. A representative image to show the major degradation bands of all the three cTnI variants (Wt, 199D and 199A) are not recognized by McAb 4C2 which is against cTnI N-terminus. Please to be noted that, a higher concentration of calpain I was used in the assays of this figure than in the quantitative assays demonstrated in figure 5 in order to optimize the density of the major degradation band for identification purpose.

Table 1

Myofilament protein phosphorylation before and after cTn exchange.

	cMyBP-C	Desmin	MLC2	cTnI	cTnT
Before exchange	0.46±0.02	0.81±0.05	0.45±0.06	0.25±0.04	0.77±0.16
Wt	0.43±0.02	0.88±0.13	0.42±0.04	0.17±0.04	0.51±0.07
199D	0.47±0.01	0.85±0.08	0.38±0.03	0.18±0.03	0.51±0.02
199A	0.41±0.02	0.85±0.18	0.35±0.08	0.17±0.04	0.56±0.05

Values are means ± SEM of the ProQ/SYPRO intensity ratio. Shown is myofilament protein phosphorylation determined before and after cTn exchange in 3 IDCM samples via ProQ-Diamond phospho-staining of 1D gels (Fig. 1C). Troponin exchange did not affect myofilament protein phosphorylation (by one-way ANOVA comparing before exchange to the exchanged groups). Abbreviations: cMyBP-C, cardiac myosin-binding protein-C; MLC2, myosin light chain 2; cTnI, cardiac troponin I; cTnT, cardiac troponin T; A, alanine substitution; D, aspartic acid substitution; Wt, wild-type.

Table 2

Force measurements in failing cardiomyocytes after exchange with recombinant troponin at sarcomere length 1.8 and 2.2 μm.

		Wt	199D	199A
F_{max} (kN.m^{-2})	1.8 μm	14.5±0.8	14.0±1.1	13.4±0.9
	2.2 μm	18.0±1.0*	17.3±0.9*	17.1±1.0*
F_{pas} (kN.m^{-2})	1.8 μm	2.2±0.1	2.5±0.2	2.4±0.3
	2.2 μm	3.3±0.3*	3.6±0.5*	3.2±0.2*
nHill	1.8 μm	2.9±0.1	2.8±0.1	2.9±0.2
	2.2 μm	2.7±0.1	2.5±0.1	2.8±0.1
$k_{tr\text{-}max}$ (s^{-1})	1.8 μm	0.77±0.05	0.75±0.05	0.75±0.04
	2.2 μm	0.73±0.06	0.73±0.04	0.77±0.05

IDCM cardiomyocytes exchanged with Wt, 199D and 199A troponin complex (2 samples; 8 myocytes per complex). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 μm at different Ca^{2+}-concentrations. Stretching of cardiomyocytes from 1.8 to 2.2 μm significantly increased maximal (F_{max}) and passive force (F_{pas}) in all groups. There was no significant effect of sarcomere length on nHill (steepness of the force-pCa curves) and $k_{tr\text{-}max}$ (maximal rate of tension redevelopment). Two-way ANOVA repeated measures followed by a Bonferroni post-hoc test revealed no significant differences between the 3 cTn complexes for all 4 parameters (#P<0.05, significant difference compared to Wt). When two-way 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). Wt data has been published before [8]. Values are means ± SEM.