New Insights Into the Functional Significance of the Acidic Region of the Unique N-Terminal Extension of Cardiac Troponin I

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Non-standard Abbreviations and Acronyms

cTn, cardiac troponin

cTnI: cardiac troponin I

cTnT: cardiac troponin T

cTnC, cardiac troponin C

Tm, tropomyosin

Sw, switch peptide

Ip, inhibitory peptide.

Wt, wild type

Abstract

Previous structural studies indicated a special functional role for an acidic region comprised of residues 1-10 in the unique N-terminal peptide of cardiac troponin I (cTnI). Employing LC-MS/MS, we determined the presence of phosphorylation sites at S5/S6 in cTnI from wild type mouse hearts as well as in hearts of mice chronically expressing active protein kinase C-ε (PKCε) and exhibiting severe dilated cardiomyopathy (DCM). To determine the functional significance of these phosphorylations, we cloned and expressed wild-type cTnI, (Wt), and cTnI mutants expressing pseudo-phosphorylation cTnI-(S5D), cTnI(S6D), as well as cTnI(S5A) and cTnI(S6A). We exchanged native Tn of detergent-extracted (skinned) fiber bundles with Tn reconstituted with the mutant cTnIs and measured tension and cross-bridge dynamics. Compared to controls, myofilaments controlled by cTnI with pseudo-phosphorylation (S6D) or Ala substitution (S6A) demonstrated a significant depression in maximum tension, ATPase rate, and k_{tr}, but no change in half-maximally activating Ca^{2+}. In contrast, pseudo-phosphorylation at position 5 (S5D) had no effects, whereas S5A induced a small increase in Ca^{2+}-sensitivity with no change in maximum tension or k_{tr}. We further tested the impact of acidic domain modifications on myofilament function in studies examining the effects of cTnI(A2V), a mutation linked to DCM. This mutation significantly altered the inhibitory activity of cTnI as well cooperativity of activation of myofilament tension, but not when S23/S24 were pseudo-phosphorylated. Our data indicate a new functional and pathological role of amino acid modifications in the N-terminal acidic domain of cTnI that is modified by phosphorylations at cTnI(S23/S24).

Keywords

Cardiac, Ca-activation, phosphorylation, cardiomyopathy, thin filament

1. Introduction

In experiments reported here, we investigated the functional significance of modifications in an acidic region comprised of residues 1-10 in the unique N-terminal peptide of cardiac troponin I (cTnI). cTnI, in conjunction with the Ca-receptor, cTnC, the scaffolding protein, cTnT, and tropomyosin (Tm), functions in a molecular mechanism to switch on and modulate sarcomeric force and shortening [1, 2] In relaxed sarcomeres, two basic domains (an inhibitory peptide, Ip, and a second actin binding region) of cTnI are tethered to actin and together with the N-terminal extension of cTnT, hold Tm in a position to block force generating cross-bridges from reacting with thin filament actins. The actin-binding domains of cTnI flank a switch peptide (SwP) that binds to cTnC upon Ca-binding to its regulatory site. This movement of SwP induces a relocation of the cTnI interaction with actin thereby inducing a release of Tm from its blocking position and the thin filament from inhibition.

Unique domains, especially the cTnI N-terminus consisting of ~30 amino acids are well known to control cardiac function [3-6]. Phosphorylation at S23/S24, which are substrates for several protein kinases, especially protein kinase A (PKA)[7], depresses Ca-responsiveness of myofilament tension [2], the rate of myosin head binding to thin filaments[8], and thin filament sliding velocity in the motility assay [9], but has no effect on maximum tension or open and closed states of the thin filaments [8]. There are also highly conserved serial Ser residues at position 5 and 6 in cTnI, but the significance of these residues has yet to be determined.

Results of recent studies have significantly altered thinking regarding the role of the unique N-terminus in control of cardiac function [3, 10, 11] The N-terminus did not resolve in

the elucidation of the core crystal structure of cTnI [12], but Howarth et al. [10]determined the NMR solution structure of cTnI (aa 1-32) and employed bioinformatic analysis together with data from small angle x-ray scattering experiments to model the N-terminal extension into the core crystal structure of cTn. In the non-phosphorylated state, the model demonstrated a close interaction between the N-terminal extension with cTnC near L29 as previously shown in peptide-array experiments [9]. The NMR solution structure also determined that upon phosphorylation of S23/S24, cTnI undergoes a major conformational transition dependent on a hinge within cTnI-(aa 33-42). Molecular docking of cTnI into the core structure indicated that this structural transition induced close proximity between acidic residues in the N-terminal extension (D3, E4, D7 and E11) with basic residues in the inhibitory peptide and with the switch peptide. The acidic region (aa 1-10) at the N-terminus, which is connected to the region housing S23/S24 by an extended poly-Pro II helix (aa 11-19), thus appeared to have a previously unappreciated functional significance based on an intra-molecular interaction[3, 13]. We [11] tested this idea by engineering a Cys residue into cTnI at positions 5 and 19 and demonstrated cross-linking of these sites to Met residues in the SwP of cTnI.

Following investigation of phosphorylation sites in mouse cTnI employing LC-MS/MS mass spectrometry, we discovered, as reported here, the presence of novel phosphorylation sites at S5/S6 of cTnI. At the same time there has been a report of the existence of these sites in human cTnI with evidence that the relative phosphorylation at these sites decreases with cardiac disorders [14]. To determine the significance of phosphorylation in the unique N-terminus of cTnI at either S5 or S6, we expressed cTnI pseudo-phosphorylated at these sites, and determined their functional effects when exchanged into detergent-extracted (skinned) fiber bundles. To further investigate the significance of the acidic region of the cTnI N-terminus, we also

expressed cTnI(A2V), a mutant linked to DCM, and determined its effects on myofilament function. Our results indicate that phosphorylation and structural modifications depress myofilament tension and that the A2V mutation alters myofilament cooperative activation as well as responsiveness to phosphorylation of cTnI at S23/S24.

2. Methods

2.1 *Animal model of dilated cardiomyopathy*

Experiments using transgenic animals were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Illinois and the National Institute of Health guidelines. PKCε transgenic mice were generated via cardiac-specific expression of constitutively active PKCε(A159E), driven by mouse α-myosin heavy chain promoter on a FVB background as previously described[15].

2.2 *Protein purification and reconstitution into Tn complex and regulated myofilaments*

We expressed and purified recombinant mouse and human cTnI (wild-type and mutant as described in the results) employing pET3d, mouse cTnT containing an N-terminal *myc* tag in pSBET, and human cTnC in pET3d purified as described previously [16, 17]. Tn complex was reconstituted and verified as previously described [16, 18, 19]. Actin was isolated from rabbit fast skeletal muscle acetone powder and tropomyosin was prepared from bovine cardiac ether powder as described previously [19] . Myosin-S1 was made by chymotryptic digestion of rabbit psoas muscle myosin [16].

2.3 *Mass spectrometry*

Sarcomeric fractions were enriched from ventricular lysates of 12-month PKCε and FVB mice according to Scruggs et al.[20]. As a preparatory step to enrich endogenous cTnI prior to mass spectrometry, sarcomeric proteins (1 mg) were separated in solution via OFFGEL

electrophoresis (Agilent Technologies) using a 24 cm, pH 7-10 IPG strip (Biorad) and the following focusing conditions: 8000 V, 64 kVh, 50 µA and 200 mW. To further enrich cTnI, OFFGEL fractions containing cTnI were separated via 12% SDS-PAGE. cTnI protein bands were excised and digested according to Deng et. al.[21]. LC-MS/MS with CID fragmentation was performed on an LTQ instrument (ThermoFisher Scientific) integrated with a Surveyor nano-LC instrument. A PicoFrit C18 column (75 µm x 10 cm, New Objective) containing resin (Biobasic C18, 5 µm particle size, 300-Å pore size, New Objective) was used. ESI conditions for the LTQ were 190 °C capillary temperature, 2.0 kV spray voltage, and a nano-LC source. Reverse-phase chromatography flow rate was 5 µl/min for loading and 220 nl/min for separation (buffer A: 0.1% formic acid, 2% ACN; buffer B: 0.1% formic acid, 80% ACN). Peptides were resolved as follows: time 0 min/2% B, time 100 min/50% B, time 110 min/100% B, time 120 min/100% B; time 121 min/2% B. The LTQ was operated in data-dependent mode; one full MS scan was followed by five MS2 scans. Spectra were searched against the International Protein Index mouse database (Version 3.47) using the Sequest algorithm (Bioworks Version 3.3). Search parameters were set as follows: partial enzymatic digestion (trypsin); two missed cleavages; 2.0 precursor tolerance; 1.0 fragment tolerance; fixed modification of cysteine carbamidomethylation (+57 Da); and variable modifications of methionine oxidation (+16 Da) and serine, threonine, and tyrosine phosphorylation (+80 Da). Identified peptides were filtered according to the following criteria: Xcorr >2.5 (+2) or >3.5 (+3). The reported peptides had a probability better than 95% ($p < 0.05$) in Scaffold. Phosphorylation site(s) were identified in two biological replicates.

2.4 *Exchange of recombinant cTn into skinned left ventricular fiber bundles*

In studies of pseudo-phosphorylation and related mutants of cTnI(S5/S6), left ventricular papillary fiber bundles were freshly dissected from male FVBN mouse hearts, aged 4-5 months, in dissecting solution containing 100 mM BES, 10 mM EGTA, 6.57 mM $MgCl_2$, 10 mM creatine phosphate, 6.22 mM ATP, 2.5 µM pepstatin, 1 µM leupeptin, 50 µM PMSF, 5 mM NaN_3, pH 7.0. Whole papillary fiber bundles were then cut into strips 125-275 µm wide by 125-275 µm deep and incubated in the above solution containing 1% Triton X-100 at 4°C for 2 hours to allow complete skinning. Aluminum T-clips were attached to both ends of the fiber preparations following exchange of endogenous Tn for exogenous Tn. Fibers were exchanged by overnight incubation at 4°C in exchange buffer (200 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 20 mM MOPS, 1 mM DTT, pH 6.5) containing 12-15 µM recombinant mouse cTn as previously described [22]. A similar procedure was carried out in studies of the cTnI(A2V) and related mutants described in the results section with the exception that CD-1 female mouse hearts aged 4-5 months were used. Recombinant cTn complexes containing N-terminal myc-tagged TnT were used in the present study to allow for the quantification of Tn exchange by Western blotting techniques as previously described [22]. Previous studies have demonstrated that myofilament function is not affected by the presence of the myc-tag[17, 23].

2.5 *Measurement of isometric tension, stiffness, ATPase, and* k_{tr}

Isometric tension and ATPase rate of skinned left ventricular fiber bundles were simultaneously measured using experimental apparatus and methodology described in detail previously[24, 25]. After mounting the fibers, sarcomere length was set to 2.2 µm by laser diffraction, and dimensions were measured under a light microscope at 40X power. The fiber bundle was moved sequentially into relaxing solution, then into the pre-activating solution, and finally into the experimental pCa solution where isometric force, stiffness, ATPase, and k_{tr} were

determined. After these parameters were measured, the fiber was returned to the relaxing solution until force decreased to the pre-activation level, followed by placement into the pre-activating solution in preparation for the next activation. Each fiber bundle underwent 8-10 contractions, with the first and last contractions being in maximal activating solution, pCa 4.5. Fibers were discarded if tension decreased by greater than 25% of the initial activation. Steady-state ATPase rate was determined by an enzyme-coupled ultraviolet light absorbance assay previously described, in which ATP consumed by the muscle during contraction was stoichiometrically linked to NAD+ formation. Calibration curves were performed with each experimental contraction as previously described [24, 25]. The rate of force redevelopment, k_{tr}, was measured in each pCa solution by a protocol previously described by Hinken and McDonald [26]. All mechanical experiments were performed at either 15° or 20° C. Following mechanical measurements, each fiber was stored at -80° C for subsequent biochemical analysis.

2.6 *In-vitro PKA phosphorylation*

Purified TnI mutants were incubated with 2.5 mM ATP for 30 min at 27° C in 0.2 M KCl, 5 mM EGTA, 5 mM $MgCl_2$, 20 mM MOPS, 1 mM DTT, pH 6.5 or in with the above solution and 0.025 U PKA (Sigma)/ug TnI. PKA treated and untreated cTnI were dialized overnight in 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM Tris, pH 8.0 prior to formation of Tn complex as described. Phosphorylation was confirmed in samples by 12% SDS-PAGE using ProQ Diamond stain (Invitrogen).

2.7 *ATPase assay with reconstituted myofilaments*

In vitro ATPase assay was performed as described previously [27]. Myofilaments were reconstituted using Tn complex containing PKA treated and untreated TnI (Wt, S6D, S6A), myosin S1, actin, and tropomyosin and were dialized against 70 mM NaCl, 10 mM $MgCl_2$, 40

mM MOPS, pH 7.0. Assay conditions contained 5 µM actin, 0.4 µM tropomyosin, 0.2 µM S1, and 1 mM ATP with various concentrations of Tn complex with a total reaction concentration of 35 mM NaCl, 5 mM $MgCl_2$, 20 mM MOPS, pH 7.0. Reactions were performed in the presence of Ca^{2+} (0.1 mM) or in its absence (2 mM EGTA), incubated at 27° C, and were quenched in 0.2 M perchloric acid at 10° C at various times. Phosphate production was measured following incubation for 20 min at 27° C with a malachite green assay [27, 28] by spectrophotometric absorbance at 655 nm. In studies with the cTnI (A2V) and related mutants, the reaction mixture were the same as described above except that actin, Tm, and myosin S-1 concentrations were 6, 1, and 0.5 µM respectively. ATPase was determined as described above at 25° C.

2.8 *Solutions*

Relaxing solution (pCa 10.0), contained 100 mM BES, 1 mM free Mg^{2+}, 5 mM MgATP, 10 mM EGTA, 48.95 mM potassium propionate, and 1mM DTT. Pre-activating solution contained 100 mM BES, 1 mM free Mg^{2+}, 5 mM MgATP, 0.50 mM EGTA, 9.50 mM HDTA, 49.19 mM potassium propionate, and 1 mM DTT. Maximal activating solution (pCa 4.5), contained 75 mM BES, 1 mM free Mg^{2+}, 5 mM MgATP, 10.00 mM EGTA, 10.01 mM $CaCl_2$, 29.06 mM potassium propionate, and 1 mM DTT. Pyruvate kinase and lactate dehydrogenase were added to all three solutions prior to use at final concentrations of 1 mg/ml and 0.1 mg/ml, respectively. Relaxing solution and maximal activating solutions were then mixed to create experimental pCa solutions ranging from pCa 6.4 to 4.5. All solutions had a final ionic strength of 180 mM and pH 7.0 at 20° C. Solution compositions were calculated by customized software based upon binding constants described previously [29].

2.9 Data and Statistical analysis

Tension-, stiffness, ATPase-, and k_{tr}-pCa relationships were fit with a modified four parameter Hill equation: $P = Po + axb/(cb + xb)$, where P is the parameter of interest (tension, ATPase, k_{tr}); Po is the minimum value, a is the maximum value minus Po ; x is pCa; b is –e (Hill coefficient); and c is the pCa at which 50% of maximum value is reached (pCa50). ATPase-tension, stiffness-tension, and k_{tr} -relative tension (P/Po) relationships were fit with linear curves. All values are presented as mean ± standard error of mean (SEM). Data were analyzed using 2-way ANOVA, a Student-Newman-Keuls post hoc method, with a level of statistical significance set at $P< 0.05$.

3. Results

3.1 *Identification of the cTnI S5/S6 Phosphorylation Site via LC-MS/MS.*

To identify novel sites of cTnI phosphorylation in mouse myocardium that potentially play a role in the maladaptive functional response, sarcomeric sub-proteomes were analyzed via LC-MS/MS. A triply-charged precursor ion (m/z 649.5) corresponding to singly-phosphorylated cTnI peptide (aa 2-20) was identified, and upon fragmentation with CID, the phospho-site was identified as either S5 or S6 (Figure 1). This site represents a novel phosphorylation site on the unique mouse cardiac N-terminus of cTnI. S5/S6 phosphorylation was identified in both wild type and in mice expressing active PKCε.

3.2 *Chemo-mechanical Measurements in Skinned Fiber Bundles Regulated by Control and Pseudo-phosphorylated cTnI.*

In order to determine the functional role of phosphorylation at S5 and S6 of mouse cTnI, we designed recombinant Tn complexes and exchanged them with endogenous Tn from skinned mouse papillary muscle fibers. Serine 5 and 6 of cTnI were pseudo-phosphorylated by

substitution with aspartic acid (S5D and S6D, respectively). Non-phosphorylatable cTnI mutants were generated by substitution of alanine for serine 5 and 6 (S5A and S6A, respectively). Recombinant cTnI proteins were incorporated into Tn complex containing myc-tagged mouse cardiac TnT (myc-cTnT) and mouse cardiac TnC (cTnC). Mutant and wild-type (Wt) Tn complexes were then exchanged into skinned mouse papillary muscle fibers. As illustrated in Figure 2, this method resulted in an exchange efficiency of 50-75% as measured by Western Blot using TnT-specific antibody. Exchange efficiency was determined as the ratio of the slower migrating TnT (myc-cTnT) to total TnT (endogenous + myc-cTnT (Figure 2). There were no significant differences in the exchange efficiency among any of the forms of cTnI.

The functional effects of cTnI mutations were determined by simultaneously measuring tension, ATPase activity, and reestablishment of tension (k_{tr}) over a range of pCa values. The pCa-tension relation revealed a significant decrease in maximum tension in both the cTnI-S6D and -S6A-exchanged fibers compared to Wt-exchanged fibers (Wt: Fmax=20.30 ± 1.60 mN/mm^2; S6A: Fmax=11.34 ± 0.37 mN/mm^2, $P<0.01$; S6D: Fmax=11.90 ± 0.61 mN/mm^2, $P<0.01$; Figure 3A), with no significant effect of either the S5A or S5D mutations. The pCa-tension relation showed increased Ca^{2+} sensitivity in the S5A-exchanged mutant compared to Wt-exchanged fibers (Wt: pCa50=5.76 ± 0.04; S5A: pCa50=5.93 ± 0.04, $P<0.01$), with no significant effect of either the S6A or S6D mutations. There were no effects of any of the mutations on the Hill coefficient of the pCa-tension relationship.

ATPase activity was measured at each pCa value via the rate of decreasing UV absorption at 310 nm within the pCa solution during tension development as described under "Materials and Methods". The resulting pCa-ATPase activity relation (Figure 3B) showed

decreased maximum ATPase activity in S5A-, S6A-, and S6D-exchanged fibers compared to Wt-exchanged fibers (Wt: 183.2 ± 4.7 pmol/s/mm^3; S5A: 152.5 ± 3.3 pmol/s/mm^3, P<0.05; S6A: 136.7 ± 3.4 pmol/s/mm^3, P<0.01; S6D: 135.3 ± 3.1 pmol/s/mm^3, P<0.01). The relationship between ATPase and tension (tension cost) showed no differences among any of the mutant- and Wt-exchanged fibers (Figure 3C), indicating no effect of any of the cTnI modifications on steady-state rate of cross-bridge detachment. The pCa-ATPase activity relation demonstrated a significant decrease in the Hill coefficient in S6A- and S6D-exchanged fibers compared to WT-exchanged fibers (Wt: n_H=3.14 ± 0.35; S6A: n_H=1.22 ± 0.08, P<0.05; S6D: n_H=1.10 ± 0.08, P<0.05). There was no effect of any of the mutations on pCa_{50} of ATPase activity.

As summarized in the data shown in Figure 4, the pCa-stiffness relationship showed a decrease in maximal stiffness in S5A-, S6A-, and S6D-exchanged fibers compared to Wt-exchanged fibers (Wt: 26.12 ± 0.65; S5A: 19.94 ± 0.45 au, P<0.05, S6A: 16.14 ± 0.51, P<0.01; S6D: 17.13 ± 0.54, P<0.01, Figure 4A). The decreased tension and stiffness of S5A-, S6A-, and S6D-exchanged fibers resulted in a normal stiffness to tension ratio, indicating no effect of either mutation on the force generated per cross-bridge (Figure 4B). The pCa-stiffness relation also showed a decreased Hill coefficient with the S6A and S6D mutants compared to Wt (S6A: n_H=1.15 ± 0.11; S6D: n_H=1.08 ± 0.11; Wt: n_H=3.06 ± 0.32).

At steady state for each pCa value, fibers were subjected to a rapid release re-stretch maneuver, resulting in disengagement of attached cross-bridges. The rate of re-establishment of tension (k_{tr}) was quantified at each pCa value with use of a single-exponential equation and plotted versus the percent of maximal tension (P/Po) to generate a linear relationship (Figure 5A). The S6A and S6D mutations showed a decreased slope of the k_{tr}-P/Po relation compared to

Wt (Wt: 16.65 ± 1.44 s^{-1}; S6A: 9.45 ± 0.95 s^{-1}, P<0.01, S6D: 9.03 ± 1.13 s^{-1}, P<0.01), Figure 5B), indicating decreased entry of cross-bridges into force generating states in these mutants.

3.3 *In vitro studies on control of reconstituted myofilaments by cTnI-S5,S6 mutants.*

Data presented so far indicate that modifications at S6 significantly inhibit the actin-cross-bridge reaction. To address the mechanism of this modification, we performed a series of experiments testing whether modifications at S6 affect the ability of cTnI to inhibit the actin-cross-bridge reaction. Data in Figure 6A show that neither the S6A nor S6D mutation influenced the ability of cTnI to inhibit actin-myosin interactions. This fits with our finding that the influence of modifications at S6 alters the Ca-activated state of the myofilaments. To examine this possibility further, we investigated the influence of the modifications at S6 on the ability of cTn to confer activation of the myofilaments in the presence of Ca^{2+}. In this same series we also tested for interactions between cTnI-S6 mutants and S23/S24, which are substrates for PKA [5]. Data shown in Figure 6B demonstrate cTnI-specific phosphorylation, as determined by ProQ analysis. Data illustrated in Figure 6C show the results of ATPase measurements from reconstituted myofilament preparations regulated with increasing concentrations of either control or mutant cTn, with and without PKA-treatment. In Figure 6C, the solid line shows the relation between untreated cTn containing TnI-WT, -S6D, or -S6A and ATPase rate, while the dashed lines show the relation of these cTn complexes with ATPase rate following PKA-treatment. In the presence of Ca^{2+}, cTn complex containing TnI-S6D at 0.5 µM Tn (1:10 molar ratio of Tm:actin) showed decreased ATPase activity compared to TnI-Wt reconstituted myofilaments (0.14 ± 0.02 vs. 0.29 ± 0.02 s^{-1}, P<0.01, Figure 6C). There was no difference between the ATPase activity of preparations controlled by either cTn-TnI-S6A or cTn-TnI-Wt at any concentration tested. In the absence of Ca^{2+}, there was no difference in ATPase activity between

myofilaments reconstituted with Tn containing either mutant or Wt cTnI (grey lines, Figure 6C). Treatment of TnI-Wt with PKA prior to incorporation into Tn complex showed significant inhibition of ATPase activity compared to untreated cTn-TnI-Wt myofilaments ([Tn]=1.0 μM: 0.04 ± 0.01 vs. 0.36 ± 0.04 s^{-1}, P<0.01). Troponin complex containing PKA-phosphorylated TnI-S6D or -S6A also showed PKA-induced inhibition of ATPase activity compared to their respective untreated groups ([Tn]=1.0 μM; S6D: 0.10 ± 0.01 vs. 0.32 ± 0.02 s^{-1}, P<0.01, S6A: 0.15 ± 0.022 vs. 0.44 ± 0.02 s^{-1}, P<0.01). However, PKA-treatment had a reduced effect on inhibiting ATPase activity in Tn containing TnI-S6 mutants compared to PKA-treated TnI-Wt ([Tn]=1.0 μM; S6D: 0.10 ± 0.01 vs. 0.04 ± 0.01 s^{-1}, S6A: 0.15 ± 0.02 vs. 0.04 ± 0.01 s-1, P<0.05).

3.4 *An A2V mutation in the acidic region of cTnI and linked to dilated cardiomyopathy has altered binding to thin filaments and to cTnC.*

In view of data demonstrating the potential of the acidic region of cTnI to influence myofilament function via structural and charge changes, we sought to determine whether other significant modifications in this region might affect myofilament function. We focused on an A2V mutation linked to DCM in order to provide more support for the significance of these cTnI residues. Murphy et al., 2004 reported that the A2V mutation caused a significant impairment in the interaction between cTnI and cTnT using a heterologous cell system. However, virtually nothing is known about whether and how this mutation alters myofilament regulation or the response to β-adrenergic stimulation. We therefore determined the functional effects of the cTnI A2V mutation on myofilament regulation and myofilament response to PKA phosphorylation. To determine the effects of the A2V mutation and PKA pseudo-phosphorylation (S23D/S24D) on cTnI inhibitory activity, we measured actin-Tm-S1 ATPase activity as a function of cTnI

concentration in the absence of Ca^{2+}. Figure 7A shows averaged percent maximum ATPase activity for each form of cTnI. Maximum inhibition occurred near a 1:1 actin:TnI ratio, but the extent of inhibition was significantly less for cTnI-A2V as compared to Wt at all concentrations studied (all concentrations $P<0.05$, Figure 7A). cTnI-S23D/S24D alone demonstrated the same inhibition as Wt, which were also the same as inhibition by cTnI-A2V/S23D/S24D. The lower inhibitory activity of cTnI-A2V was restored to that of Wt by pseudo-phosphorylation at S23/S24.

We measured ATPase activity as a function of TnC:TnI ratio to determine the effects of the A2V mutation and PKA pseudo-phosphorylation on the Ca-TnC as a method to probe the Ca-dependent cTnC-cTnI interactions. Figure 7B shows averaged percent maximum ATPase activities at pCa 4 as a function of varying molar ratios of cTnC:cTnI. Myofilaments controlled by either Wt or cTnI-S23D/S24D showed a similar increase in ATPase rate with increasing molar ratios of cTnC:cTnI. However, compared to these controls, the ability of cTnC to reverse inhibition by cTnI-A2V was significantly depressed (all concentrations $P<0.05$, Figure 7B). Moreover, at all ratios, cTnC reversed inhibition by cTnI-A2V/S23D/S24D to a lower extent than cTnI-A2V (all concentrations $P<0.05$). These results indicate that in this assay the A2V mutation decreases the affinity of the TnI regulatory region for TnC, and PKA pseudo-phosphorylation further decreases the affinity in the presence of the A2V mutation.

3.5 *Myofilaments regulated by cTn-A2V demonstrate altered cooperativity and kinetics of force redevelopment (k_{tr}) dependent on pseudo-phosphorylation of S23/S24.*

To determine the effects of the A2V mutation and PKA pseudo-phosphorylation on myofilament mechanical properties, we exchanged Tn containing cTnI-Wt, A2V, S23D/S24D,

and A2V/S23D/S24D into skinned left ventricular papillary fiber bundles. Data in Figure 8 show tension-pCa relations from each group and demonstrate that Ca^{2+} sensitivity (pCa_{50}) of steady state tension and maximum tension of skinned fiber bundles regulated by cTnI-A2V were the same as the controls (Wt: Fmax=26.7 ± 0.9 mN/mm^2; A2V: Fmax=23.0 ± 1.5 mN/mm^2; Wt: pCa_{50}=5.71 ± 0.02; A2V: pCa_{50}=5.70 ± 0.02, Figure 8A).. However, in fibers regulated by the cTnI-A2V mutation alone, there was a significantly decreased Hill coefficient (n_H), a measure of the cooperativity of tension activation (Wt: n_H=4.18 ± 0.10; A2V: n_H= 3.77 ± 0.10, *P*<0.01). cTnI-S23D/S24D caused a significant desensitization of the tension-pCa relation as previously demonstrated [30] in the presence or absence of the A2V mutation (Wt: pCa_{50}=5.71 ± 0.02; S23D/S24D: pCa_{50}=5.55 ± 0.02, *P*<0.01; A2V/S23D/S24D: pCa50=5.53 ± 0.03, *P*<0.01), with no effect on maximum tension or n_H.

Figure 8B shows results of experiments in which we determined ATPase rate of the skinned fiber bundles. We found that in fibers controlled by TnI-A2V there was no effect on the Ca^{2+} sensitivity of ATPase activity, the Hill coefficient of the ATPase activity-pCa relation, or the maximum ATPase activity. Pseudo-phosphorylation, whether in the absence or presence of the cTnI-A2V mutation, induced a rightward shift of the ATPase activity-pCa relation when compared to Wt (Wt: pCa_{50}=5.70 ± 0.02; S23D/S24D: pCa_{50}=5.55 ± 0.03, *P*<0.01; A2V/S23D/S24D: pCa50=5.54 ± 0.03, *P*<0.01), consistent with previous studies, but did not significantly alter other parameters. We also analyzed the ATPase activity-tension relation, or tension cost (Figure *C). We found that the TnI-A2V mutation alone did not alter the relation compared to Wt (Wt: 5.15 ± 0.21; A2V: 5.25 ± 0.40). In contrast, cTnI-S23D/S24D and cTnI-A2V/S23D/S24D exhibited significantly higher tension costs than their counterparts (Wt: 5.15 ± 0.21; S23D/S24D: 6.18 ± 0.48, *P*<0.01; A2V/S23D/S24D: 6.68 ± 0.47, *P*<0.01). These results

indicate that PKA pseudo-phosphorylation increases the rate of cross-bridge detachment, which has previously been reported [30].

To further investigate rate constants for the cross-bridge reactions, we measured the rate of force redevelopment (k_{tr}) as function of pCa and of relative tension. The measure of k_{tr} reflects the sum of the rate-limiting transition steps ($k_{tr} = f + g$) of myosin cross-bridges to and from the strongly-bound, force-generating state during isometric contraction. We found k_{tr} to be dependent on the pCa as previously reported [31, 32]. As recommended by Patel et al., (2001), we expressed the rate of force redevelopment as a function of relative isometric tension. Figure 9A depicts representative k_{tr} -relative tension relations for each group and shows that the cTnI-A2V mutation significantly decreased the slope (Wt: 8.39 ± 0.50; A2V: 6.82 ± 0.50, $P<0.01$, Figure 9B) and significantly increased the y-intercept of the relationship (Wt: 3.91 ± 0.31; A2V: 5.67 ± 0.58, $P<0.01$) as compared to Wt, whereas both PKA pseudo- phosphorylation mutants were similar to Wt. These results indicate that the A2V mutation increases the kinetics of force redevelopment at sub-maximal, but not maximal Ca^{2+} levels.

4. Discussion

Our results provide the first evidence of a functional significance of conformational changes and novel sites of phosphorylation at the acidic region of the unique N-terminus of cTnI. Our findings also extend understanding of potential control of cardiac function by the N-terminus of cTnI physiological and patho-physiological states. It is apparent from our results that these conformational and charge induced modulations are potentially significant factors in acquired and familial cardiomyopathies. While carrying out the present studies, we became aware of a report by Zhang et al. [14] identifying cTnI-S5/S6 in human myocardium and demonstrating a decrease in phosphorylation of these sites in samples from hearts in failure. However, no functional significance of these sites was assigned in this study. Our data indicate that the depression in phosphorylation of these sites in human heart failure may be compensatory. Our studies involving LC-MS/MS identified the Ser-5/-6 in both wild type and the PKCε over-expressing mouse model, a known model of DCM REF. The development of DCM in these mice has been attributed to decreased Ca-sensitivity, most notably through phosphorylation of cTnI at S43/S45 and T144 [33, 34]. Phosphorylation at these sites depresses myofilament response to Ca^{2+} and maximum tension development [33]. However, to determine whether phosphorylation levels at cTnI-S5/S6 are different in PKCε over-expressing hearts, requires future quantitative analysis.

Our data demonstrate that pseudo-phosphorylation of cTnI-S6 in the acidic domain of the N-terminus induces a depressed maximum tension development of skinned fiber preparations with no apparent change in the stiffness/tension ratio or cross-bridge detachment rate, but a reduction in entry of cross-bridges into force generating states. The particular sensitivity of activation of tension to modification of cTnI-S6 is emphasized by our data demonstrating little or

no effects of cTnI-S5D or cTnI-S5A, but significant effects cTnI-S6D and cTnI-S6A. We think that the mechanism for these effects may involve an intra-molecular interaction, as described above, between the acidic region containing cTnI-S6, and regulatory domains surrounding the Ip of cTnI. Our proposal is that when cTnI-S6 is phosphorylated or conformationally modified, the interaction of the SwP and the acidic region of cTnI impedes release of the SwP/Ip region from actin, and therefore induces a reduction in the number of cross-bridges entering into the force generating cross-bridge cycle. There was no effect of cTnI-S6D on the inhibitory effect of cTnI indicating that reaction with the Ip or a second actin binding region may be important in the inhibition of tension. Other evidence also indicates the functional significance of the acidic region of cTnI. Studies [13] have been carried out a on a mutant cTnI truncated by removal of the acidic region (cTnI(Δ2-11). Compared to controls, hearts of transgenic mice expressing the mutant demonstrated significantly depressed contraction and relaxation kinetics as well as decreased maximal tension in skinned fibers with no influence of the mutation on Ca-sensitivity or phosphorylation at cTnI-S23/S24. Solution NMR studies indicated residues 2-11 do not influence the induction of the open state of cTnC. In contrast to the studies [13]with cTnI(Δ2-11) in which there was no apparent influence of removal of the acidic region on PKA effects on the myofilaments we found that modification at cTnI-S6 suppressed the effect of PKA phosphorylation of cTnI to inhibit activation. Thus, modifications at Ser-6 appear to affect mechanisms by which PKA-dependent phosphorylation of cTnI-S23/S24 suppress response to Ca^{2+}.

Our data indicate a role for cTnI-S6 and cTnI-A2 in modulating the molecular conformation of cTnI and for allosteric interactions with other regions of the cTnI N-terminal peptide. There were significant depressions in tension induced by both Asp and Ala

substitutions at cTnI-S6, which indicates conformational changes as well as charge changes influence the function of the cTnI acidic region. The demonstration of significant effects of the conservative substitution of Val for Ala in the DCM linked mutation also indicates the sensitivity of this region to conformational modulation affecting myofilament response to Ca^{2+} in the form of an altered cooperativity of activation. Moreover the loss of this effect when cTnI-S23/S24 are pseudo-phosphorylated further indicates the allosteric influence of modifications of the acidic region of the cTnI on the phosphorylation motif at S23/S24. These effects are similar to those of demonstrated by another actin-binding protein, calponin [35]. In this case PKC-dependent phosphorylation or Ala substitution at S175 induced the same alteration in affinity of calponin for actin. It is interesting that as with the cTnI-N terminus, calponin is a highly flexible protein. We think in the case of cTnI conformational modulation of the highly flexible N-terminus provides a mechanism of altered direct and allosteric interactions with neighbors, and in the case of the acidic region with intra-molecular regions of cTnI especially the SwP (Warren REF). Overall our data indicate that modifications at the acidic region may influence diverse functional properties of the myofilaments in a manner not previously appreciated.

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Disclosures

None

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Figure Legends

Figure 1. Identification of a novel phosphorylation site in cTnI. cTnI from Wt mice were digested and analyzed via LC-MS/MS on an LTQ mass spectrometer. CID fragmentation spectra from both wild type (A) and PKCε (B) show a triply-charged, singly-phosphorylated precursor ion (m/z 649.5), which localizes the phosphorylation to either Ser-5 or Ser-6 in the (ambiguity denoted by the parentheses). Observed a-, b- and y-ions with a relative abundance above 5% are labeled above each corresponding ion in the spectrum and are illustrated in the peptide sequence (top). Essentially identical results were obtain from heart samples from mice expressing acitive PKCε.

Figure 2. Quantification of cTn exchange in skinned fiber bundles. The exchange of endogenous for exogenous Wt or mutant cTn was determined by Western blot analysis from skinned fiber samples using the cTnT-specific monoclonal antibody CT3. The difference in migration of endogenous cTnT and exogenous myc--tagged cTnT (myc-cTnT) allowed for the determination of the efficiency of exchange. Top: Representative non-exchanged fibers (NE) and an exchanged fiber (Ex). Bottom: Representative individual exchanged fibers and recombinant cTn complex (Control).

Figure 3. Tension- and ATPase-pCa relation in skinned fiber bundles exchanged with Wt or mutant cTn. Tension-pCa relationship (A). ATPase-pCa relationship (B). Tension cost (C). Tension- and ATPase-pCa relationships are presented as mean ± SEM. n = 8-10 fibers, N=3-4 hearts. Tension cost is presented as the linear regression of mean data from A and B.

Figure 4. Stiffness-pCa relation in skinned fiber bundles exchanged with Wt or mutant cTn. Stiffness-pCa relationship (A). Stiffness-tension relationship (B). Stiffness-pCa relationships is presented as mean ± SEM. n = 8-10 fibers, N = 3-4 hearts. Stiffness-tension relationship is presented as the linear regression of mean data from A and Figure 3A.

Figure 5. k_{tr}-relative tension relationship in skinned fiber bundles exchanged with Wt or mutant cTn. Average k_{tr}-relative tension plots from each experimental group (A). Slope of k_{tr}-relative tension between fibers exchanged with Wt and mutant cTn (B). k_{tr}-relative tension are presented as the linear relationship of k_{tr} and relative tension. Slope of k_{tr}-relative tension relationships are presented as mean ± SEM (B). n = 5-10 fibers, N = 3-4 hearts. $^{**}P<0.01$ compared to Wt.

Figure 6. Wt- and mutant-cTn effects on actin-Tm-S1 ATPase activity. All assays contained 7 μM actin, 0.4 μM tropomyosin and 0.2 μM myosin S1, with final buffer conditions of 35 mM NaCl, 5 mM $MgCl_2$, and 20 mM MOPS, pH 7.0. Wt- and mutant cTnI inhibition of ATPase activity (A). Wt- and mutant-cTn effects on ATPase activity in the presence (black lines) and absence (grey lines) of Ca^{2+} (C). ATPase activity was calculated by subtracting the ATPase activity of myosin S1 only at each concentration. Data presented as mean ± SEM. n = 3-4. $^{**}P<0.01$.

Figure 7. Wt- and mutant-cTn effects on actin-Tm-S1 ATPase activity. Wt and mutant cTnI inhibition of ATPase activity (A). All assays contained 6 μM actin, 1 μM tropomyosin and 0.5 μM myosin S1, with final buffer conditions of 10 mM NaCl, 5 mM $MgCl_2$, and 20 mM

MOPS, pH 7.0. Wt- and mutant cTnI inhibition of ATPase activity (A). Effects of increasing ratio of cTnC to Wt- and mutant cTnI- on ATPase activity in the presence of Ca^{2+} (pCa 4.0). Percent maximal ATPase activity was calculated by subtracting the ATPase activity of myosin S1 only and dividing by ATPase activity at zero cTnI (Figure 7A) and at zero cTnC (Figure 7B). Data presented as mean ± SEM. n = 4-6. *P<0.05.

Figure 8. Tension- and ATPase-pCa relation in skinned fiber bundles exchanged with Wt or mutant cTn. Tension-pCa relationship (A). ATPase-pCa relationship (B). Tension cost (C). Tension- and ATPase-pCa relationships are presented as mean ± SEM. n = 11-19 fibers, N=4-8 hearts. Tension cost is presented as the linear regression of mean data from A and B.

Figure 9. k_{tr}-relative tension relationship in skinned fiber bundles exchanged with Wt or mutant cTn. Average k_{tr}-relative tension plots from each experimental group (A). Slope of k_{tr}-relative tension between fibers exchanged with Wt and mutant cTn (B). k_{tr}-relative tension are presented as the linear relationship of k_{tr} and relative tension. Slope of k_{tr}-relative tension relationships are presented as mean ± SEM (B). n = 5-8 fibers, N = 3-5 hearts. *P<0.01 compared to Wt.

Figure 1

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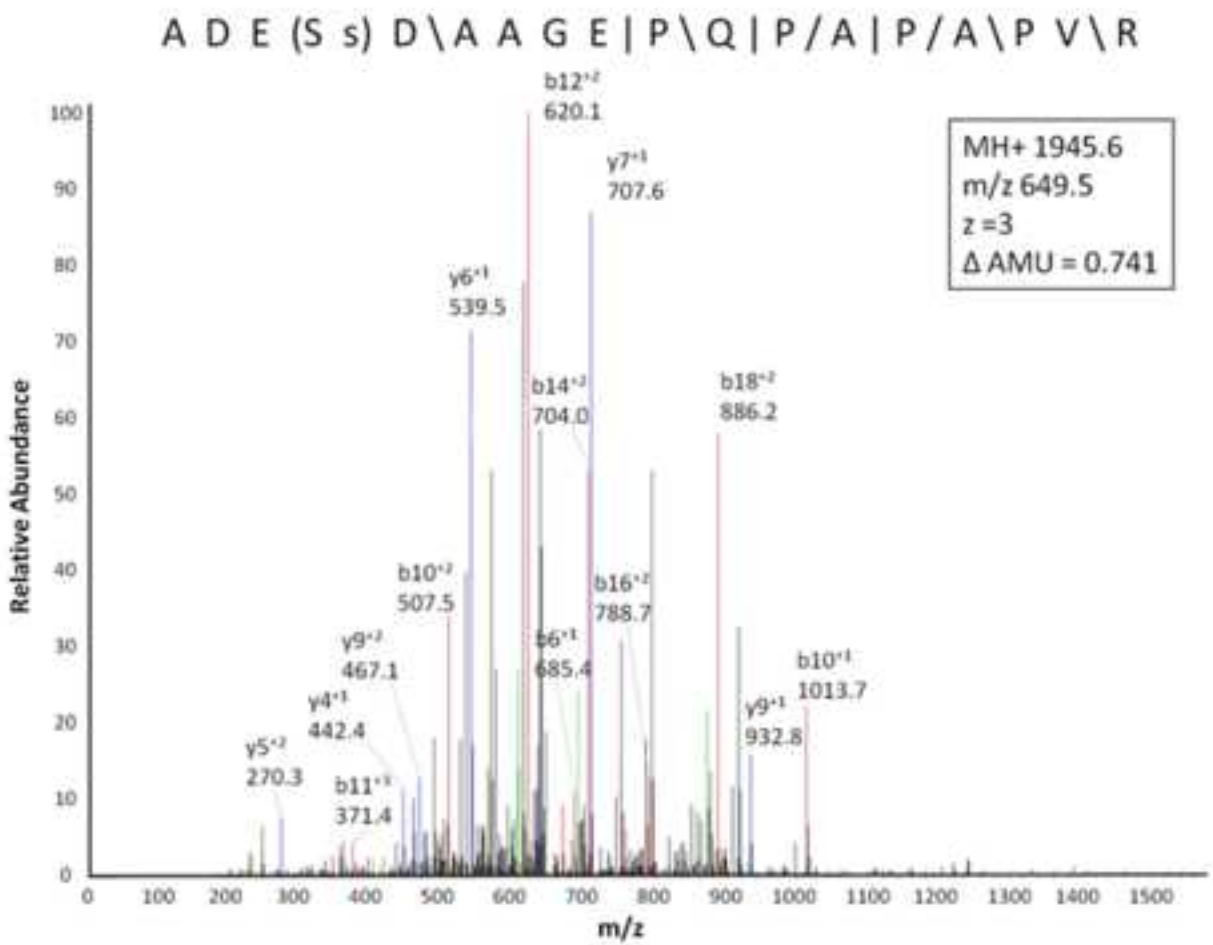

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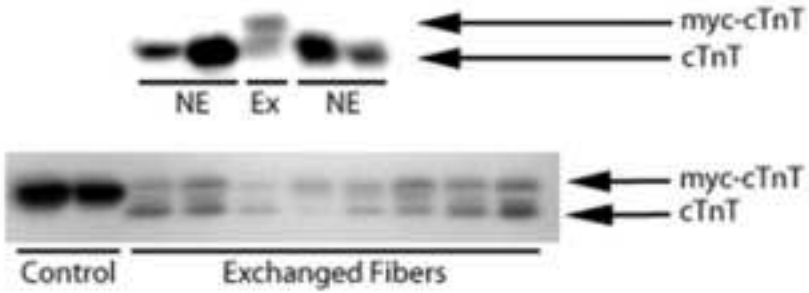

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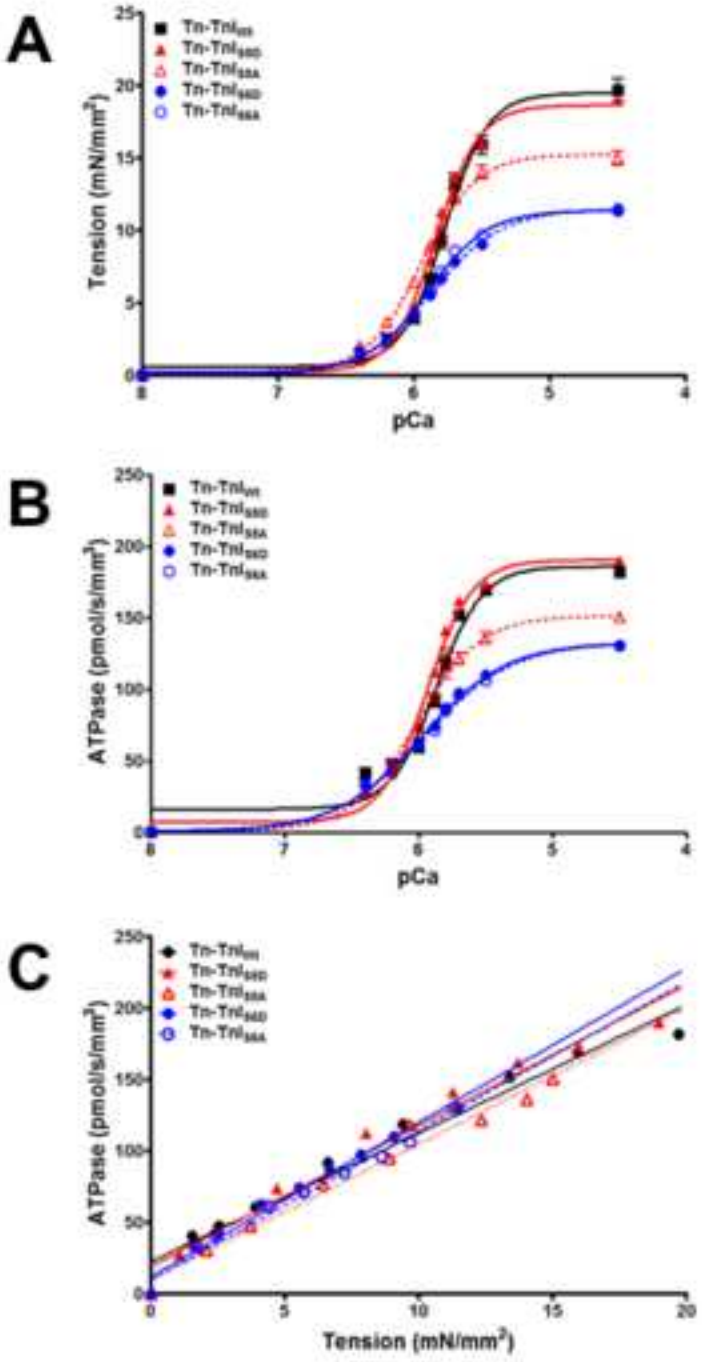

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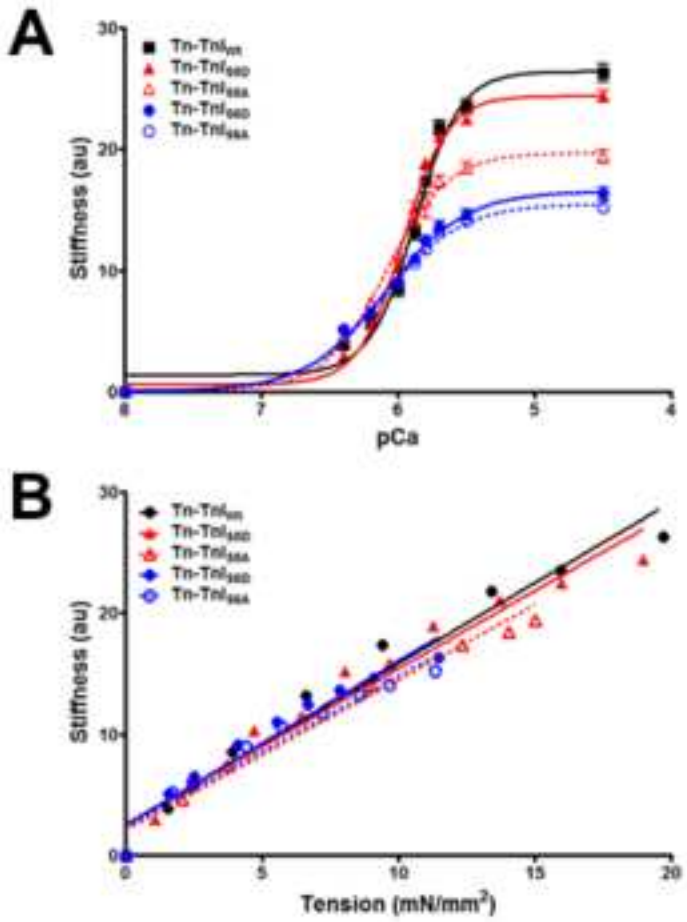

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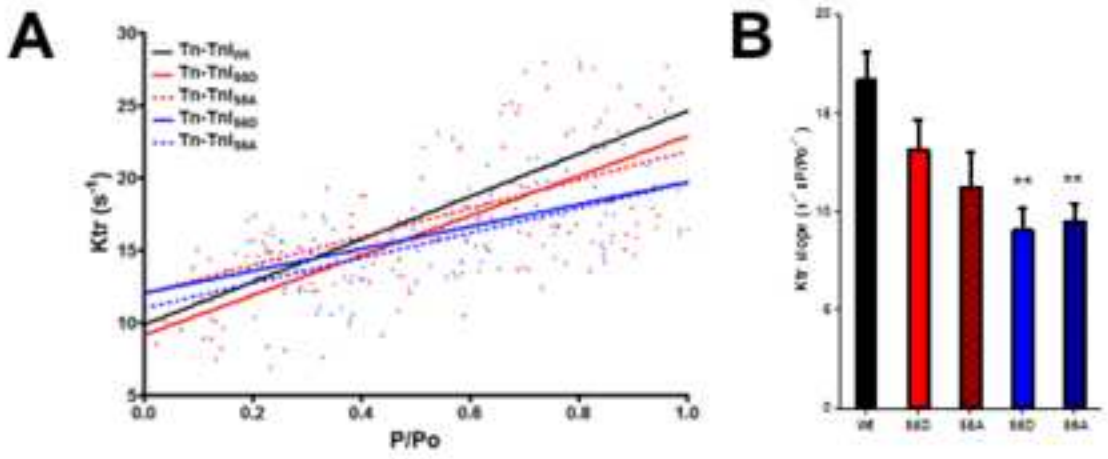

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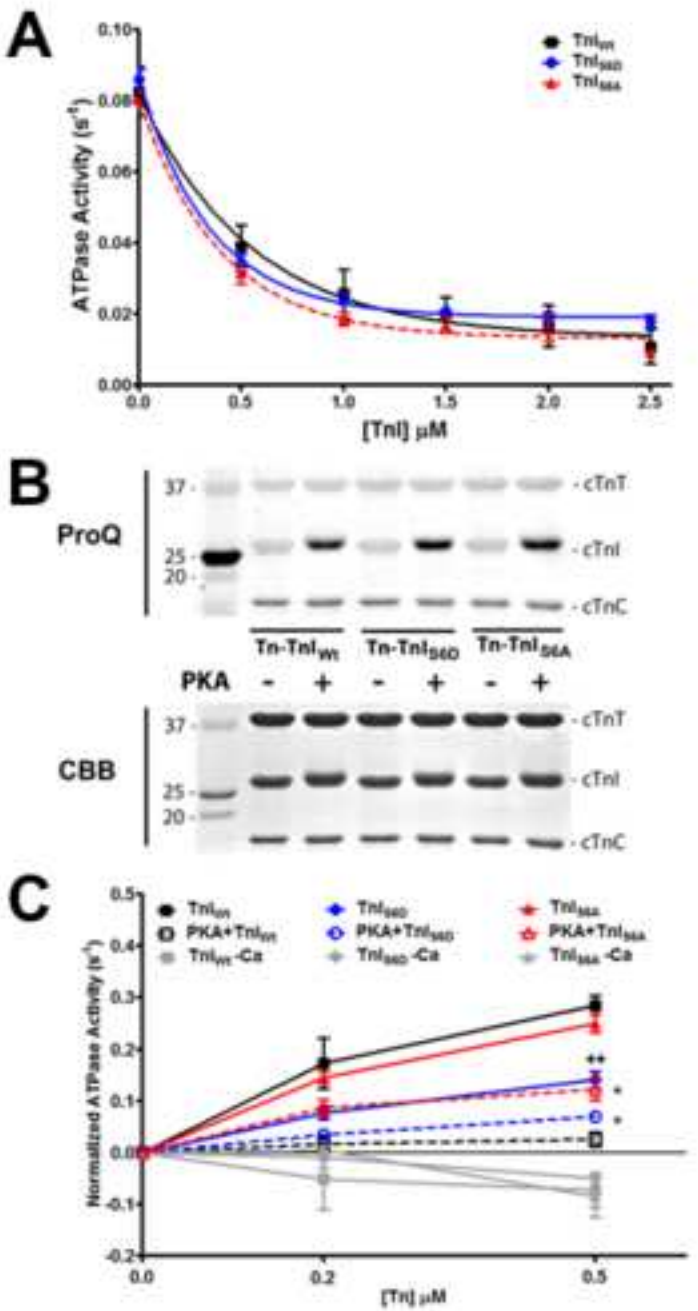

Figure 7

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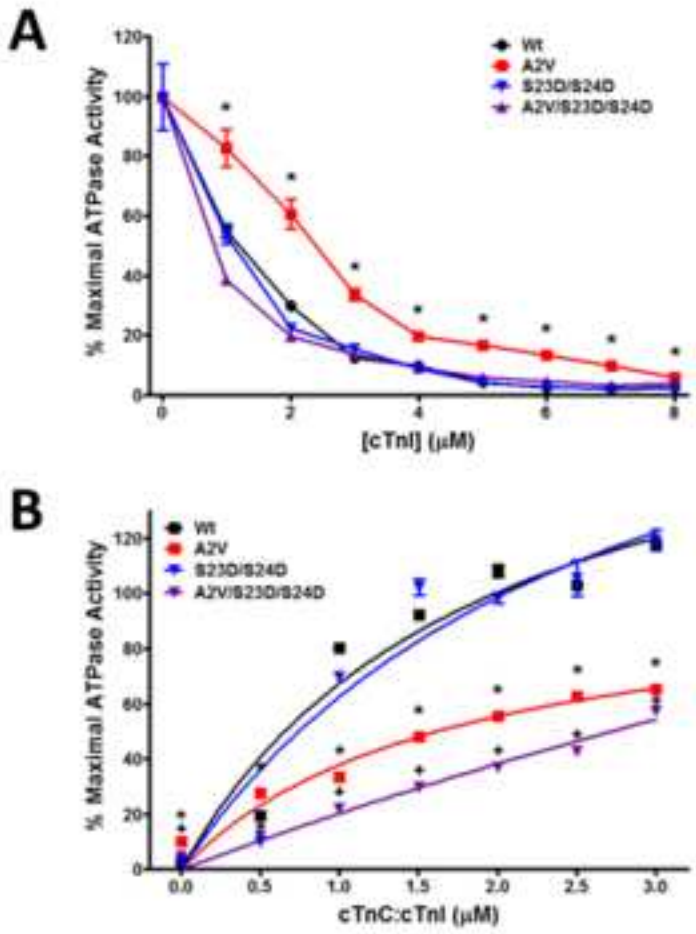

Figure 8

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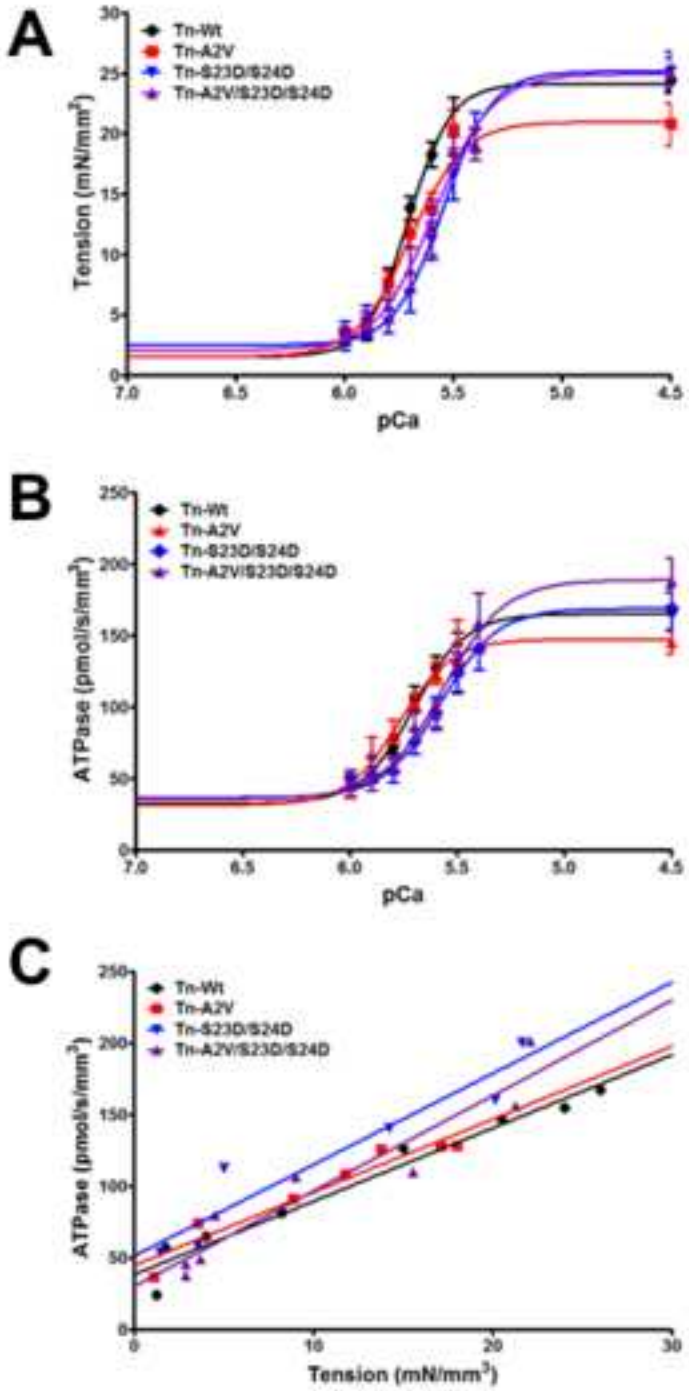

Figure 9

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