Effects of Cardiac Troponin I Mutation P83S on Contractile Properties and the Modulation by PKA-Mediated Phosphorylation

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ABSTRACT: cTnIP83S (cTnIP83S in rodents) resides at the I-T arm of cardiac troponin I (cTnI) and was initially identified as a disease-causing mutation of hypertrophic cardiomyopathy (HCM). However, later studies suggested this may not be true. We recently reported that introduction of an HCM-associated mutation in either inhibitory-peptide (cTnIR146G) or cardiac-speciﬁc N-terminus (cTnIR21C) of cTnI blunts the PKA-mediated modulation on myofibril activation/relaxation kinetics by prohibiting formation of intrasubunit contacts between these regions. Here, we tested whether this also occurs for cTnIP83S. cTnIP83S increased both Ca2+ binding afﬁnity to cTn (KCa) and afﬁnity of cTnC for cTnI (KCai), and eliminated the reduction of KCa and KCai observed for phosphorylated-cTnIWT. In isolated myofibrils, cTnIP83S maintained maximal tension (TMAX) and Ca2+ sensitivity of tension (pCa50). For cTnIWT myofibrils, PKA-mediated phosphorylation decreased pCa50 and sped up the slow-phase relaxation (especially for those Ca2+ conditions that heart performs in vivo). Those effects were blunted for cTnIP83S myofibrils. Molecular-dynamics simulations suggested cTnIP83S moderately inhibited an intrasubunit interaction formation between inhibitory-peptide and N-terminus, but this “blunting” effect was weaker than that with cTnIR146G or cTnIR21C. In summary, cTnIP83S has similar effects as other HCM-associated cTnI mutations on troponin and myofibril function even though it is in the I-T arm of cTnI.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM), a familial myocardial disease often correlated with myofilament contractile protein mutations, has been identiﬁed as a major autosomal dominant disease accompanied by a morbidity of 1 per 500 individuals.1,2 To date, over 900 HCM-associated mutations have been identiﬁed in 24 genes,3,4 and a majority of these mutations reside in the thick filament proteins, such as cardiac myosin binding protein C (cMyBP-C), myosin, and titin.5,6 Despite this, several mutations have also been reported in one of the thin ﬁlament regulatory proteins, cardiac troponin I (cTnI), which is the actomyosin ATPase inhibitory subunit of the cardiac troponin (cTn) complex.5 cTnI is the key regulator for muscle contraction, and it regulates the thin and thick ﬁlaments interaction in a Ca2+-dependent manner.6 In diastole, cardiac troponin C (cTnC) presents in its “closed” conformation, and cTnI strongly binds with actin (weak interaction with cTnC), thus inhibiting actin–myosin interaction.6,7 In systole, with the rise of intracellular Ca2+ in cardiomyocytes, Ca2+ binding to the cTnC site II induces an “open” conformation of the regulatory N-domain and increases its interaction with cTnI switch-peptide.6,8 Consequently, this results in a decreased binding of cTnI inhibitory-peptide with actin and increased tropomyosin mobility that the myosin binding sites were exposed on actin, thus allowing myosin interaction with actin and the formation of “cross-bridges”6,8.

During β-adrenergic stimulation, cTnI is phosphorylated by protein kinase A (PKA) at sites Ser-23/Ser-24 (S23/S24), which reside at the cardiac-speciﬁc N-terminus of cTnI.9 We10 and others11–13 have demonstrated that this PKA-mediated phosphorylation weakens cTnC–cTnI (C–I) interaction, decreases the Ca2+ sensitivity (pCa50) of cardiac muscle tension production, increases Ca2+ dissociation rate (koff) from cTnC, elevates cross-bridge cycling kinetics, and accelerates cardiac muscle relaxation. Additionally, we demonstrated that PKA phosphorylation of isolated rat cardiac myofibrils with WT-cTnI accelerates and shortens the initial, slow-phase of relaxation
(especially during submaximal activation that the heart operates in vivo) that is considered to reflect cross-bridge detachment rate and, perhaps, deactivation of thin filament.\textsuperscript{10}

Since the first HCM-associated cTnI mutations were reported by Kimura et al. in 1997,\textsuperscript{11,12} there have been at least 29 mutations in cTnI putatively suggested to be contributory to HCM.\textsuperscript{3,15} Initial studies by Niimura et al. identified cTnIP\textsuperscript{R22S} (cTnIP\textsuperscript{PRSS} in rodents) and cTnIP\textsuperscript{R146G} (cTnIP\textsuperscript{PRSS} in rodents) as a disease-causing mutation of the late-onset hypertrophy, and its pathogenicity is not clear.\textsuperscript{16} Later on, Frazier et al. reported an African American individual (32-year-old, women) with severe HCM (a family history of HCM and sudden cardiac death), and identified that she has both the P82S mutation in cTnI and the R453S mutation in cardiac β-myosin heavy chain (MYH7).\textsuperscript{17} By analyzing DNA from a panel of 100 individuals, Frazier et al. also determined that cTnIP\textsuperscript{R21C} presents in ∼3% of healthy African Americans. Thus, they concluded that cTnIP\textsuperscript{R22S} is likely a non-disease-causing amino acid variant.\textsuperscript{17} Using transgenic mice, Ramirez-Correa et al. reported that cTnIP\textsuperscript{R22S} showed normal cardiac contractility when young, but displayed diastolic dysfunction (for instance, impaired ejection and relaxation time, and prolonged isovolumetric relaxation time) with aging.\textsuperscript{18} Additionally, they found that cTnIP\textsuperscript{R22S} mice blunted the β-adrenergic response and impaired myofilament cooperativity.\textsuperscript{18} Considering this, it is of importance to further investigate whether cTnIP\textsuperscript{R22S} alters myofibril contraction/relaxation kinetics, and whether structural-function changes are similar to those of other HCM-associated cTnI mutations.

Among all HCM cTnI mutations, cTnIP\textsuperscript{R146G} (located in the inhibitory-peptide) and cTnIP\textsuperscript{R21C} (resided at the cardiac-specific N-terminus) have been extensively studied by several groups.\textsuperscript{19–33} Recently, we also reported the blunted β-adrenergic response for both cTnI HCM-associated mutations.\textsuperscript{34,35} Both mutations significantly left-shifted (increased) Ca\textsuperscript{2+} binding affinity to cTnI (K\textsubscript{Ca}) and the affinity of cTnC for cTnI (K\textsubscript{C-C}). PKA-mediated phosphorylation of cTnI induced a similar reduction of K\textsubscript{Ca} for all complexes, but the reduction in K\textsubscript{C-C} normally seen with cTnI WT did not occur for either mutation in cTnI. Both mutations increased Ca\textsuperscript{2+} sensitivity of tension (pCa\textsubscript{50}) while maximal tension (TMAX) was maintained. PKA-mediated phosphorylation of cTnI right-shifted pCa\textsubscript{50} for cTnI WT myofibrils, however not for either mutation.\textsuperscript{34} PKA-mediated phosphorylation also sped up the initial, slow-phase relaxation for cTnI WT myofibrils, especially at those submaximal Ca\textsuperscript{2+} conditions that the heart performs in vivo. Importantly, this effect on relaxation was eliminated for both cTnIR146G and cTnIR21C, as was PKA-mediated reduction in K\textsubscript{C-C}.\textsuperscript{34}

Our results also suggested that the structural basis of how these two mutations blunted the ability of PKA-mediated phosphorylation on cTnC–cTnI interaction, myofilament contraction, and relaxation is prohibiting the formation of an intrasubunit interaction between cTnI inhibitory-peptide and NcTnI that is normally observed for WT-cTnI and NcTnI that is normally observed for WT-cTnI with phosphorylation of cTnI S23/S24; however, this “blunting” effect was much weaker than cTnIP\textsuperscript{R146G} and cTnIP\textsuperscript{R21C}. Thus, cTnIP\textsuperscript{R22S} appears to have effects on cTnI and myofibril function similar to HCM-associated mutations in the N-terminus and inhibitory-peptide regions of cTnI, but the effect on Ca\textsuperscript{2+} sensitivity of myofibril tension and PKA modulation of relaxation may be more moderate, and this may at least partially explain conflicting reports and conclusions in the literature.

### EXPERIMENTAL AND THEORETICAL METHODS

Proteins, cTnC Labeling, cTnI Phosphorylation, and cTn Complex Reconstitution. WT rat cTnC, cTnI, and cTnT were constructed and expressed as described previously.\textsuperscript{3,6} cTnC\textsuperscript{C35S}, cTnI\textsuperscript{S23D/S24D}, cTnI\textsuperscript{P82S}, and cTnI\textsuperscript{P82S/S23D/S24D} were constructed from WT cTnC and cTnI plasmids, respectively, using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). All WT and mutant proteins were expressed with a pET-24 vector (Novagen, Madison, WI) which contains a T7 promoter, a lac operator, and a kanamycin resistant gene. Finally, the expressed constructs were confirmed by DNA sequence analysis.

The cTnC\textsuperscript{C35S} substitution was labeled with a fluorescent probe {N-[2-(idoacetoyethyl)]-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole (IANBD, M\textsubscript{w} = 406.14, Life technologies, Cat No: I-9) at position C84 (cTnC\textsuperscript{C35S} IANBD) in the dark overnight at
4 °C to monitor the Ca2+-cTn (K_Ca) and cTnC-cTnI (K_Ca/I) binding affinities, as described previously. 10,34,37-40 The labeling efficiency (also known as percentage of labeling) was obtained by comparing the IANBD fluorophore versus protein concentration ratio. 10,39 The protein’s concentration was determined using the Bio-Rad protein assay (based on the Bradford method), and the IANBD fluorophore concentration was computed using the maximal absorbance of labeled protein at a wavelength ~481 nm dividing by IANBD’s extinction coefficient (21 000 M−1 cm−1). 10,39 The labeling efficiency of cTnCC35S substitution was ~90%. Whole cTn complexes were formed using rat cTnC (WT or cTnC(C35S)), rat cTnI (WT, S23D/S24D, P83S or P83S/S23D/S24D), and rat cTnT (WT) at a 1:1:1 molar ratio and then dialyzed through a series of buffers with gradually decreased KCl concentration at 4 °C (without stirring) as described previously. 41,42 Here, cTn complexes with cTnC(C35S), cTnIS23D/S24D (or cTnIP83S/S23D/S24D) were only used for Ca2+-cTn (K_Ca) binding measurement.

**Steady-State Fluorescence Measurements.** Steady-state fluorescence measurements were performed by a luminescence spectrometer (PerkinElmer LS50B) at 15 °C as described previously. 10,34,40 Solution applied for this measurement contains (in mM) the following: 150 KCl, 20 MOPS, 2 MgCl2, 2 EGTA, and 1 DTT (pH 7.0). Fluorescence signal was recorded at ~530 nm during the titration of (1) Ca2+ into 2 mL of cTnCl200 (0.6 μM), or (2) cTn variants (WT, S23D/S24D, P83S or P83S/S23D/S24D) into 2 mL of cTnC(C35S) (0.6 μM) in the presence of Ca2+ (100 μM). The concentration of free Ca2+ was computed using Maxchelator. 43 The Ca2+ sensitivity (measured as pCa, the pCa = −log(Ca2+)) value at half-maximal fluorescence signal change was collected by fitting the titration curve with the S-shaped Hill equation as described previously. 44 Here, the reported data are the means ± SE of 4–6 successive titrations.

**Ethical Approval and Tissue Preparation.** All animal procedures were carried out according to the U.S National Institutes of Health Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Washington (UW) Institutional Animal Care and Use Committee (IACUC). Animals were housed in the Department of Comparative Medicine at UW and cared for in accordance with UW IACUC procedures. After initial exposure to isoflurane (3–5% in oxygen), pentobarbital (50 mg/kg) was intraperitoneal injected to male, Sprague–Dawley rats (3 months old, 150–250 g) for anesthesia. When the rat had no reflexive response, its heart was quickly excised and dissected in oxygenated physiological saline solution, which contains (in mM) the following: 100 NaCl, 2.5 KCl, 24 NaHCO3, 1 Na2HPO4, 1 MgSO4, 7H2O, and 1 CaCl2. 45 After this, both ventricles were cut open, and the whole heart was demembranated in skinnning solution containing (in mM) the following: 100 KCl, 9 MgCl2, 4 Na2ATP, 5 K2EGTA, 10 MOPS, 1% Triton X-100, pH 7.0, 50% v/v glycerol, and 1:100 dilution Protease-Inhibitor-Cocktail (PI) (Sigma-Aldrich, Cat. No.: P8340) overnight at 4 °C. 46,47 The heart was then washed three times in the same buffer without Triton X-100 and kept at −20 °C for use for up to 1 week. Myofibrils from the left ventricles (LV) were used for the mechanical measurements described below.

**Solutions.** Solution composition (for mechanical measurements) was determined using an iterative algorithm, which calculates the equilibrium concentration of ligands and ions according to published affinity constants. 48 The relaxing solution has the composition as follows (in mM): 80 MOPS, 1 Mg2+, 5 MgATP, 15 EGTA, 52 Na+, 83 K+, and 15 creatine phosphate (CP), pH 7.0 at 15 °C. The inorganic P, concentration was 0.5 mM (determined by NMR), and the solution ionic strength was 170 mM. 49 All mechanical measurements were carried out at 15 °C. The Ca2+ levels (measured as pCa) of activating solutions were adjusted by adding CaCl2.

**Exchange of Recombinant cTn Complexes into Myofibrils.** Muscle bundles obtained from the rat LV were rinsed twice in Rigor solution, with a composition of (in mM) the following: 100 KCl, 50 Tris, 2 MgCl2, 1 DTT, and 1:100 dilution of PI. Then, the muscle bundles were homogenized for two 30 s pulses at a high speed on ice. cTn complexes containing cTnWT or cTnPPS (∼1 mg/mL) were passively exchanged into isolated rat myofibrils in a buffer containing (in mM) the following: 150 KCl, 5 MgCl2, 20 MOPS, 4 ATP, 2 EGTA, 1 DTT, and 1:100 dilution of PI (pH 7.0) on a slow rocker overnight at 4 °C. 10,34 Following overnight exchange, myofibrils were washed with relaxing buffer containing ∼1 mg/mL bovine serum albumin (BSA) twice for 30 min to remove any nonspecifically bound exogenous cTn. Myofibrils were then equally split: one-half was used to collect the kinetic/mechanism results prior to PKA treatment, and the other half was incubated with 200 μL relaxing solution containing 100 units of PKA and 6 mM DTT for 45 min at 20 °C for studying the effects of PKA-mediated phosphorylation.

**Myofibril Mechanical and Kinetics Measurements.** Myofibril mechanical/kinetics studies were carried out on a home-built setup as described previously. 10,50,51 In brief, single or small bundles of ∼2–4 cardiac myofibrils were attached between two glass microneedles (one is straight and another is bent) forged from borosilicate glass capillary tubes (OD 1.0 mm, ID 0.5 mm, Sutter Instruments, Novato, CA) over the range of the diodes. The relaxing (10–4 M, pCa 9.0) Ca2+ solutions that can be rapidly switched. The initial sarcomere length (SL) of myofibril was set as ∼2.3 μm. Here, the straight microneedle is mounted to one end of the myofibril and was applied to rapidly shorten and restretch the myofibril through a computer interface and a piezocontroller motor (PZT Servo controller, LVPZT amplifier, Physik Instrumente, Irvine CA). The bent microneedle acts as a force transducer (FT) to hold the other end of the myofibril, and it deflected with application of force in a predictable mode. 50 The stiffness of FT applied for this study ranged from 5 to 9 nN μm−1, and the value was measured under a 40X lens by bending the microneedle with a set of force using a galvanometer. The displacement of FT was recorded by a dual-diode system, and correlated to tension development. At the end of each experiment, a calibration curve was performed in which the FT needle was moved in 1 μm steps using micromanipulators (MP-285, Sutter Instruments, Novato, CA) over the range of the diodes. The relaxing (10–9 M, pCa 9.0) and activating (10–4 M, pCa 4.0) Ca2+ solutions were streamed by a homemade double-barreled borosilicate theta glass pipet (capillary glass tubing OD 2.0 mm, ID 1.4 mm, SEP 0.2 mm, modified in house to OD of 0.65 mm, Warner Instruments, Hamden, CT) to the attached myofibrils. The solutions were stepping an increase or decrease over the mounted preparation, and this rapid switch (within ∼10 ms) was controlled by a computerized motor (SF-77B Perfusion...
Activation—relaxation results were measured at 15 °C and then fit as described previously.6,52–54 The kinetics of myofibril activation ($k_{ACT}$) with a rapid increase in Ca$^{2+}$ level was collected from a single exponential rise to a maximum. Once the myofibril was activated, a rapid release—restretch protocol (the myofibril was 20% shorting in optimal length suddenly, and after 25 ms of unloaded shortening period, the myofibril was rapidly stretching back to the original length) was applied to get the rate of tension redevelopment ($k_{T_{rel}}$).52 The rate of slow-phase relaxation ($k_{REL,slow}$) was computed from the slope of a regression line fit to the force trace and then normalized to the entire tension amplitude. The duration of slow-phase relaxation ($t_{1/2,rel}$) was collected from the onset of buffer switching to the shoulder marking the starting of fast-phase relaxation. An apparent change in slope or in “signal-to-noise” ratio was applied to determine at the transition from slow- to fast-phase. The rate for fast-phase relaxation ($k_{REL,fast}$) was fitted by a single exponential decay. In some cases (decay cannot be well-described by a single exponential), a $t_{1/2}$ estimation was made and then converted to a rate $τ = \ln(2)/t_{1/2}$. In this study, myofibrils that contracted over 10% of their optimal length were considered as nonisometric and excluded from the analysis.

**cTnI Phosphorylation Profile.** The cTnI phosphorylation profile was quantified using Western blot by calculating the amount of phosphorylated cTnI relative to the total amount of cTnI.55,56 The phosphorylated cTnI was detected using a rabbit polyclonal IgG to troponin I (H170, from antitoxin rabbit polyclonal IgG (1:500). The total cTnI was quantified using antibodies rabbit polyclonal IgG-HRP (Santa Cruz Biotechnology, sc-2004) as secondary antibody (1:5000). The total cTnI was quantified using antibodies rabbit polyclonal IgG to troponin I (H170, from Santa Cruz Biotechnology, sc-15368) (1:1000) and goat anti-rabbit IgG-HRP (1:5000).

**Computational Modeling.** The initial structure of the cTn complex was built up on the basis of the crystal structure of Takeda et al.55 with the addition of the N-terminus of cTnI from the NMR structure provided by Howarth et al.66 To mimic phosphorylation, a bis-phosphomimics model (cTnIP83S/S23D/S24D) was constructed by mutating S23/S24 of cTnI to aspartic acid (D). Two systems of human cTn were prepared for simulations: a cTnIP83S/S23D/S24D cTnI–C172-cTnT236–285 (cTnIP83S cTn model), and a cTnIP83S/S23D/S24D cTnI–C161-cTnC1–161-cTnI–172–285 (cTnIP83S cTn model). The cTnIP83S/S23D/S24D cTn model and the cTnIP83S/S23D/S24D cTn model. The cTnIP83S/S23D/S24D cTn model were introduced by using the Mutate Residue Module in VMD.57 The models were immersed in a truncated rectangular box with TIP3P water molecules, which extended minimally 14 Å away from any solute atoms.58 Then, K$^+$ and Cl$^-$ ions were added to neutralize the systems, and brought to 150 mM ionic strength. The fully solvated systems contained 112 739 (cTnIP83S cTn model) and 112 740 (cTnIP83S/S23D/S24D cTn model) atoms, respectively. Prior to the MD simulations, we performed three steps of minimizations. Next, for each of the two simulation systems, three independent sets of 150 ns MD simulations were carried out under 300 K and the NPT ensemble using NAMD 2.9.59,60 and the CHARMM27 force field.60 The SHAKE procedure was applied, and the time step was set as 2.0 fs.60 During the sampling process, the coordinates were saved every 10 ps (cTnIP83S cTn model) and 2 ps (cTnIP83S/S23D/S24D cTn model), respectively. The stability between site II Ca$^{2+}$ and its coordinating residues (Asp-65, Asp-67, Ser-69, Thr-71, Asp-73, and Glu-76) of cTnC was monitored by calculating the corresponding distances for each 150 ns simulation as described previously.53,55,39,62 The residue–residue contacts between cTnC and key regions of cTnI (N-terminus, inhibitory-peptide, and switch-peptide regions) were monitored over the course of the entire 450 ns simulations. Contacts between two residues were defined as described previously, with a carbon–carbon distance of ≤ 5.4 Å and a distance between any other noncarbon atoms of ≤ 4.6 Å being a contact.39,62 Contacts between the NcTnC-switch-peptide of cTnI and cTnC-inhibitory-peptide of cTnI were monitored. The intrasubunit contact between N-terminus and inhibitory-peptide of cTnI were also recorded. For each contact pair, the portion of the simulation time that these residues contacted with each other was calculated for both simulation systems. Lastly, an I–T arm interhelical angle analysis was performed. The interhelical angles were computed using interh (K. Yap, University of Toronto). We calculated the angle between the two structural helices in cTnI as defined by cTnI residues 45–82 and 91–137.

**Statistics.** Comparison between groups of data was carried out using the one-way analysis of variant (ANOVA) test as appropriate. All experimental data are expressed as mean ± SE, and $n$ represents the number of experimental samples in each group; the reported computational (MD simulations) results are expressed as mean ± SD, and $n$ equals 3. Results with $p < 0.05$ were considered statistically significant.

**RESULTS**

**Steady-State Fluorescence Measurements of $K_C$ and $K_C$**. The effects of cTnIP83S mutation ± bis-phosphomimic substitutions on cTnI–Ca$^{2+}$ and $K_C$ were determined by steady-state fluorescence measurements using a fluoroprobe IANBD, as previously described.10,34,39 IANBD, a sulfhydryl-reactive and environment-sensitive extrinsic fluorophore, has been widely applied for monitoring the intramolecular interactions of proteins. The labeling of IANBD at C84 of cTnC1SSS reflects conformational and environmental changes of NcTnC that arise from Ca$^{2+}$ binding and/or interaction with cTnI.10,34,38,39 We first measured the conformational changes with Ca$^{2+}$ binding to cTn containing either cTnIP83S or cTnIP83S/S23D/S24D compared with the cTnWT set. As shown in the Ca$^{2+}$ titration curves (Figure 2A), the Ca$^{2+}$ sensitivity of the fluorescence intensity (reported as $pC_{50}$) was left-shifted 0.09 pCa units, from 7.18 ± 0.02 (cTn with cTnIP83S) to 7.27 ± 0.02 (cTn with cTnIP83S/S23D/S24D). Consistent with our previous finding,10,34 introduction of bis-phosphomimic substitutions on cTnIP83S (cTnIP83S/S23D/S24D) also reduced $K_C$, resulting in a 0.27 pCa unit decrease (right-shifted to 6.91 ± 0.03). Interestingly, with introduction of bis-phosphomimic substitutions on cTnIP83S, this effect was completely eliminated ($pC_{50}$ = 7.24 ± 0.03 for cTn with cTnIP83S/S23D/S24D).

cTnC–cTnI (C–I) interaction acts as a “gatekeeper” in transferring Ca$^{2+}$ signal to other myofilament proteins to initiate cardiac muscle contraction. Therefore, we next measured how introduction of cTnIP83S mutation ± bis-phosphomimetic substitutions affects the affinity of cTnC for cTnI ($K_C$). As shown in Figure 2B, the IANBD fluorescence signal increased with the titration of cTnI in solution containing 0.6 μM cTnC1SSS and there was no further increase beyond 0.6 μM cTnI, suggesting strong binding of cTnI to cTnC such that 1:1 binding was achieved. Similar to $K_C$, cTnIP83S also left-
shifted $K_{C-1}$ compared to cTnWT. As we reported previously, bis-phosphomimic substitutions of cTnWT (cTnS23D/S24D) also reduced $K_{C-1}$. However, this effect was completely blunted for the cTnPS3 mutant.

Recombinant Troponin (cTn) Complex Exchange Profiles. The native cTn in isolated myofibrils was passively exchanged with recombinant rat cTn containing either cTnWT or cTnPS3. The extent of exchange (exchange efficiency) for this procedure was periodically determined by exchanging cTn containing a cTnT labeled at the N-terminus with a c-myc tag, to compare the c-myc tag band versus the native cTnT band in gels and with Western blot analysis. We consistently saw >80% endogenous cTn replacement by cTn containing the c-myc tag in myofibrils using this approach, suggesting our exchange protocol is very efficient. After PKA treatment, the cTn phosphor-
ylation extent in exchanged myofibrils, which is consistent with our previous observation.

cTn-P83S Mutation Has No Effects on Myofibril Contraction and Relaxation. The effect of cTnPS3 mutation on tension development and relaxation kinetics (at 15 °C) was determined from isolated rat LV cardiac myofibrils exchanged with cTn containing cTnPS3, and then compared with the WT cTn complex. Myofibrils mounted by two glass microtools were exposed to continually flowing pCa buffer that was rapidly switched, from relaxing buffer (pCa 9.0) to either maximal (pCa 4.0) or submaximal (pCa 5.4, pCa 5.6 and pCa 5.8) [Ca2+]i buffer, and finally back to 9.0. Representative example tension traces for cTnWT and cTnPS3 exchanged myofibrils during the submaximal [Ca2+]i activation-relaxation protocol (at pCa 5.4) are presented in Figure 4. The magnitude of tension and kinetic for rat LV myofibrils exchanged with cTn containing cTnWT or cTnPS3 is summarized in Table 1 and Figures 5 and 6.

Maximal tension ($T_{MAX}$) did not differ for myofibrils exchanged with cTnPS3 (74.5 ± 5.0 mN/mm²) compared with cTnWT myofibrils (74.6 ± 5.8 mN/mm², Figure 5A). Tension was also measured at multiple submaximal Ca²⁺ levels, and the pCa₅₀ was just slightly left-shifted ($p > 0.05$, not statistic significantly, Figure 5B), from 5.32 ± 0.03 (cTnWT myofibrils) to 5.36 ± 0.03 (cTnPS3 myofibrils), demonstrating that cTnPS3 mutation did not have effects on the Ca²⁺ sensitivity of tension. This finding is clearly demonstrated in the example tension traces that were collected at pCa 5.4 (Figure 4), showing that cTnPS3 myofibrils have same/similar tension development compared to the cTnWT myofibrils.

The contractile activation rate ($k_{ACT}$) after rapid-switching of [Ca²⁺] solution from pCa 9.0 to 4.0 (or submaximal Ca²⁺ level) contains the kinetic of Ca²⁺-mediated thin filament activation,
Figure 4. Representative tension trace (at pCa 5.4) for isolated rat cardiac myofibril after exchanging with recombinant cTn complexes containing cTnIWT and cTnIP83S. The inset is a close up of slow-phase of relaxation demonstrating how $k_{REL,slow}$ and $t_{REL,slow}$ are measured.

Table 1. Tension Generation and Relaxation Parameters after Recombinant Rat cTn Containing Either cTnI-WT or cTnI-P83S Exchange into Rat Ventricular Myofibrils at 15°C

<table>
<thead>
<tr>
<th>myofibril batches</th>
<th>tension generation</th>
<th>kACT ($s^{-1}$)</th>
<th>kTR ($s^{-1}$)</th>
<th>pCa = 4.0</th>
<th>tREL,slow (ms)</th>
<th>kREL,slow ($s^{-1}$)</th>
<th>$k_{REL,fast}$ ($s^{-1}$)</th>
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<tr>
<td>WT</td>
<td>74.6 ± 5.8 (16)</td>
<td>3.3 ± 0.3 (16)</td>
<td>5.3 ± 0.5 (16)</td>
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<td>WT+PKA</td>
<td>71.1 ± 6.5 (17)</td>
<td>2.5 ± 0.2* (17)</td>
<td>5.2 ± 0.7 (17)</td>
<td>63 ± 3* (16)</td>
<td>1.6 ± 0.2* (16)</td>
<td>17 ± 2 (16)</td>
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<tr>
<td>P83S</td>
<td>74.5 ± 5.0 (22)</td>
<td>3.7 ± 0.2* (22)</td>
<td>6.1 ± 0.5 (22)</td>
<td>71 ± 2 (22)</td>
<td>1.0 ± 0.1* (22)</td>
<td>18 ± 2 (22)</td>
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<tr>
<td>P83S+PKA</td>
<td>70.3 ± 3.9 (23)</td>
<td>3.5 ± 0.3* (23)</td>
<td>5.9 ± 0.4 (22)</td>
<td>74 ± 3* (22)</td>
<td>1.1 ± 0.2* (22)</td>
<td>19 ± 2 (22)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>32.7 ± 3.6 (15)</td>
<td>1.8 ± 0.2 (15)</td>
<td>3.5 ± 0.4 (15)</td>
<td>67 ± 4 (15)</td>
<td>1.6 ± 0.2 (14)</td>
<td>17 ± 2 (15)</td>
<td></td>
</tr>
<tr>
<td>WT+PKA</td>
<td>21.5 ± 2.7* (16)</td>
<td>1.5 ± 0.2 (15)</td>
<td>3.1 ± 0.3 (15)</td>
<td>56 ± 4* (14)</td>
<td>3.7 ± 0.6* (14)</td>
<td>18 ± 3 (15)</td>
<td></td>
</tr>
<tr>
<td>P83S</td>
<td>35.7 ± 2.9* (16)</td>
<td>2.1 ± 0.2 (16)</td>
<td>4.0 ± 0.3 (16)</td>
<td>67 ± 3* (15)</td>
<td>1.9 ± 0.1* (15)</td>
<td>17 ± 2 (16)</td>
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</tr>
<tr>
<td>P83S+PKA</td>
<td>33.9 ± 2.4* (15)</td>
<td>2.1 ± 0.2 (15)</td>
<td>3.8 ± 0.3 (14)</td>
<td>68 ± 4 (14)</td>
<td>2.2 ± 0.3 (14)</td>
<td>18 ± 1 (14)</td>
<td></td>
</tr>
</tbody>
</table>

Values given are mean ± SE. Number in parentheses is number of myofibrils. *p < 0.05 vs WT, and †p < 0.05 vs WT+PKA.

Figure 5. Tension (A), pCa-tension relationship (B), the kinetics of activation (C; $k_{ACT}$), and the $k_{ACT}/k_{TR}$ ratio (D) for cTnIWT vs cTnIP83S exchanged myofibrils prior to and after PKA treatment. *p < 0.05.
the subsequent myosin cross-bridge binding, as well as tension development. With respect to the cTnIWT exchanged myofibrils (3.3 ± 0.3 s⁻¹), k_ACT did not differ for the cTnIP83S (3.7 ± 0.2 s⁻¹) exchanged myofibrils either pCa 4.0, or pCa 5.4 (Figure 5C). As previously reported for rodent (or canine) cardiac myofibrils, k_ACT was significantly slower at submaximal Ca²⁺ activation than during maximal Ca²⁺ levels for both cTnIWT and cTnIP83S exchanged myofibrils, suggesting the Ca²⁺ sensitivity of cardiac contraction kinetics is also maintained upon introduction of the cTnIP83S mutations. Once the activation was in or near steady-state, we applied a rapid release–restretch protocol on myofibrils to collect the rate of tension redevelopment (k_TR). This k_TR protocol is designed to measure the myosin cross-bridge attachment rate and the subsequent tension generation when Ca²⁺−→Tn binding is near steady-state (for example: thin filament is activated), thus and can help to differentiate the contribution of Ca²⁺−dependent thin filament activation vs cross-bridge cycling to k_ACT. For all measured conditions, k_TR was faster than k_ACT, as we previously reported, again suggesting thin filament activation (for rat cardiac myofibrils) is rate-limiting at 15 °C. Additionally, comparison of the k_ACT/k_TR ratio serves as a good indicator to show whether thin filament activation is more rate-limiting in the cTnIP83S exchanged myofibrils with respect to the cTnIWT exchanged myofibrils. Figure 4D demonstrates that the k_ACT/k_TR ratio did not change upon introduction of cTnIP83S mutation.

The myofibrils were rapidly deactivated by a second switching from activating [Ca²⁺] solution to relaxing solution (pCa 9.0), which induced a biphasic relaxation: an initial linear and slow decay followed by a rapid (fast) exponential tension decay back to the baseline (see the example tension trace in the inset of Figure 4). The slow-phase relaxation rate (k_REL,slow) predominantly reflects the cross-bridge detachment rate, and the duration of slow-phase relaxation (t_REL,slow) represents the time for the troponin to move back to a “blocked” state. For maximal activations, both k_REL,slow (0.9 ± 0.1 s⁻¹ vs 1.0 ± 0.1 s⁻¹, Figure 6C) and t_REL,slow (72 ± 3 ms vs 71 ± 2 ms, Figure 6D) were unchanged for cTnIP83S exchanged myofibrils compared to cTnIWT exchanged myofibrils. In contrast to slow-phase, the much larger, fast-phase of relaxation (k_REL,fast) is influenced by multiple sarcomeric properties as well as uneven relaxation between sarcomeres in series. There was no difference in k_REL,fast for cTnIP83S (18 ± 2 s⁻¹) versus cTnIWT myofibril (17 ± 2 s⁻¹) exchange. Similar to pCa 4.0, at submaximal Ca²⁺ level, there was also no difference in k_REL,slow, t_REL,slow, and k_REL,fast between cTnIWT and cTnIP83S exchanged myofibrils. As we observed previously, k_REL,slow was twice as fast at pCa 5.4 compared to the maximal activation (pCa 4.0) for all myofibrils (Table 1).

cTnI-P83S Mutation Blunts the PKA Effects on Myofibril Contraction and Relaxation. We next studied the effects of PKA-mediated phosphorylation on myofibril contraction and relaxation. Consistent with previous studies, with PKA treatment, T_MAX (71.1 ± 6.5 mN/mm², Figure 5A) was maintained for the cTnIWT exchanged myofibrils, and pCa0 was right-shifted 0.18 pCa units to 5.14 ± 0.03 (Figure 5B), demonstrating the reduced Ca²⁺ sensitivity of tension development. At maximal Ca²⁺ level (pCa 4.0), PKA phosphorylation of cTnIWT exchanged myofibrils also decreased k_ACT, whereas k_TR was maintained, suggesting that PKA phosphorylation affects the thin filament activation prior to cross-bridge binding and tension development. The above result can be clearly observed in the plots of the k_ACT/k_TR ratio from Figure 5D, demonstrating that this ratio is significantly decreased for PKA treated cTnIWT exchanged myofibril compared with the ones prior to PKA treatment. After treating the cTnIP83S exchanged myofibrils with PKA, T_MAX (70.3 ± 3.9

Figure 6. Slow-phase relaxation at submaximal Ca²⁺ level for (A) WT-cTn and (B) cTnIP83S-cTn exchanged rat cardiac myofibrils before (black) and after (red) PKA treatment. The kinetics (C; k_REL,slow) and duration (D; t_REL,slow) of slow-phase relaxation for cTnIWT vs cTnIP83S exchanged myofibrils prior to and after PKA treatment. *p < 0.05.
mN/mm², Figure 5A) was also maintained; however, the Ca²⁺ sensitivity of tension development (pCa50, Figure 5B) was not reduced (blunted) for cTnIP83S. Additionally, maximal k_{ACT} did not change for cTnIP83S exchanged myofibrils following PKA phosphorylation, which can be clearly seen by calculating the k_{ACT}/k_{TR} ratio (Figure 5D), suggesting the slowed thin filament activation at maximal Ca²⁺ condition was also blunted.

A major influence of β-adrenergic stimulation on cardiac function is increasing heart rate, such that the rate of chamber relaxation needs to increase to allow for preserved diastolic filling. Thus, we studied how myofibril relaxation was influenced following PKA treatment of cTnIWT and cTnIP83S exchanged myofibrils. As we observed previously, 10,34 PKA phosphorylation on cTnIWT exchanged myofibrils significantly increased k_{REL,slow} (1.6 ± 0.2 s⁻¹, Figure 6C) and reduced t_{REL,slow} (63 ± 3 ms, Figure 6D) during maximal Ca²⁺ activation, thus speeding up the overall relaxation. Furthermore, the above effect was greater at submaximal Ca²⁺ level (pCa 5.4) where the heart operates. However, after treating the cTnIP83S exchanged myofibrils with PKA, there was no change in either the rate (1.1 ± 0.2 s⁻¹, Figure 6C) or the duration (74 ± 3 ms, Figure 6D) of the slow-phase relaxation, suggesting that the effect of PKA phosphorylation on accelerating relaxation was blunted for cTnIP83S mutation. The above finding can be clearly observed in Figure 6A,B, which is a set of example tension traces of slow-phase relaxation at submaximal Ca²⁺ level.

**Molecular-Dynamics Simulations.** Triplicate 150 ns MD simulations (three independent 150 ns simulations) were performed on both systems, the cTnIP83S cTn system and the cTnIP83S/S23D/S24D cTn system. Our first goal was to assess the effect of cTnIP83S mutation on the dynamics of cTn complex. To compare the dynamics of cTnIWT versus cTnIP83S cTn, triplicate MD simulations were performed and average RMSF (±SD) values for all residues were calculated. Figure 7A,B shows the average (±SD) RMSF of the cTnC and cTnI subunits for both cTnIWT and cTnIP83S cTn systems. As we previously saw for cTnIR21C and cTnIR146G mutations,34 the fluctuations in the cTnIP83S mutant were comparable to those in the WT system throughout most of the protein structure. The only pronounced difference was seen in the C-terminal domain of cTnC (cTnC residues 115–130), the loop connecting the two C-terminal cTnC EF-hand motifs (that interacts with the cTnI I–T arm). This is one direct consequence of cTnIP83S, which located in the I–T arm region. Most other regions did not exhibit changes larger than the standard deviations. The largest fluctuations were observed in the NcTnI region, underscoring its highly flexible nature. With fluctuations this large, it is appropriate to refer to a range of conformations for NcTnI rather than an actual single structural conformation. The helical bundle known as the I–T arm (cTn residues 45–82 and 91–137) comprised the most stable residues in the cTnI subunits, reflecting their structural rather than regulatory
function. Figure 7A–B also highlights regions of cTn that play an important part in either Ca$^{2+}$ binding (cTnC site I and cTnC site II) or cTnC–cTnI interaction (cTnI inhibitory-peptide and cTnI switch-peptide). In summary, cTnIP83S had little impact on the dynamics of the troponin complex.

Next, we investigated the influence of PKA phosphorylation on cTn containing cTnI P83S. To that end, we performed simulations with simultaneous phosphomimic substitutions S23D and S24D and P83S of cTnI (cTnIP83S/S23D/S24D) in the troponin structure. Figure 7C–D shows the average (±SD) RMSF of the cTnC and cTnI subunits for the cTnIP83S and the cTnIP83S/S23D/S24D systems. The fluctuations in the cTnIP83S/S23D/S24D system were increased with respect to the cTnIP83S system. Interestingly, there were increased dynamics in the entire structure of NcTnC, suggesting NcTnI mobility can have a profound influence on NcTnC dynamics. We also speculate that structural destabilization of cTnI (caused by the introduction of three amino acids substitutions) could be partially responsible for the wide range of RMSF values observed. The largest change of dynamical behavior between the two systems is observed in cTnI residues 20–30 where an increase in local residue fluctuations is observed. The Ser (S) to Asp (D) mutations break stabilizing interactions in the region, suggesting a large increase in the mobility of NcTnI. To better visualize how introduction of the P83S and/or bis-phosphomimic substitutions influences the subunit interactions among the cTn complex, 15 snapshots taken from the entire MD simulations were superimposed (Figure 7E,F). For both structure alignments, cTnC is shown in blue, cTnI in red, and cTnT in yellow. In addition to the greater flexibility exhibited for the NcTnI in the cTnIP83S/S23D/S24D cTn model with respect to the WT model, the introduction of S23D/S24D to the cTnIP83S model also caused increased flexibility in NcTnC.

In addition to the change in entire protein dynamics/structures, we investigated how the cTnIP83S mutation and/or PKA phosphorylation impact Ca$^{2+}$ binding stability in site II. It is very difficult to accurately calculate binding affinities of Ca$^{2+}$, due to the nature of divalent cations. Therefore, we investigated one parameter related to the Ca$^{2+}$ binding in site II in cTnC, the time evolution of distances between the bound Ca$^{2+}$ ion and its coordinating residues. In the crystal structures, Ca$^{2+}$ is coordinated with six atoms in cTnC site II residues: Asp-65 OD2, Asp-67 OD2, Ser-69 OG, Asp-73 OD2, Glu-76 OE1, and Glu-76 OE2. As observed in previous studies, 34,35,39,62 the coordinating behavior of Asp-65, Asp-67, Asp-73, and Glu-76 did not change for WT cTn in the absence or presence of cTnI-S23D/S24D. The most interesting behavior is observed for Ser-69 OG and, perhaps, Thr-71 OG1. Figure 8 shows those distances over the time course of three independent 150 ns simulations for WT, cTnIP83S, and cTnIP83S/S23D/S24D cTn systems. Here, the 1st run result is shown in black, the 2nd run result is in red, and the 3rd run result is in blue.
systems. The coordination behavior for both residues fluctuated over the course of the simulations for all three systems, in contrast to the stability of the other coordinating residues. Thr-71 generally does not coordinate, in agreement with structural data from X-ray crystallography. As demonstrated in Figure 8, the most pronounced difference between the simulations is observed in coordination of Ser-69, which is consistent with our previous observations. The percent-in-contact time of Ser-69 varies among the different systems. With respect to the WT system (10%), the percent-in-contact time is increased to 19% in the cTnI-P83S system, indicating a stronger interaction and a possible stabilization. This may interpret the increased Ca$^{2+}$ binding affinity of cTnI-P83S with respect to the cTnI-WT observed from steady-state fluorescence measurements. Importantly, this contact time was not affected by introduction of the bis-phosphomimic substitutions (17% in the cTnI-P83S/S23D/S24D system), in agreement with the unchanged Ca$^{2+}$ binding affinity ($K_{Ca}$) obtained from the steady-state fluorescence measurements.

Next, we monitored the residue–residue contacts of key regions during the time course of entire 450 ns simulations. We first focused on elucidating the intrasubunit contacts between NcTnI (cTnI residues 1–41) and the cTnI inhibitory-peptide (cTnI residues 138–147). It has been previously shown that this may constitute an important mechanism by which PKA can regulate cTn structure and dynamics. The following interesting question arises: To what degree does the mutation cTnI-P83S impact that mechanism? We hypothesized that, due to its distance from NcTnI and the inhibitory-peptide, cTnI-P83S may not disrupt this mechanism that blunts PKA-mediated modulation of myofibril contraction and relaxation as much as other mutations (cTnI-R21C and cTnI-R146G). To this end, we examined intrasubunit contacts between NcTnI (cTnI residues 1–41) and the cTnI inhibitory-peptide (cTnI residues 138–147). Figure 9 shows the corresponding contact plots for (A) WT, (B) cTnI-S23D/S24D, (C) cTnI-P83S, and (D) cTnI-P83S/S23D/S24D cTn models. The blue end of the spectrum (value 0) reflects no contact within the residue–residue pair, while the red end of the spectrum (value 1) represents 100% contact within the residue–residue pair.
is not surprising since no phosphomimic substitutions or phosphorylations are present. As we expected, introduction of the cTnIP^{P83S} mutation only moderately reduced the NcTnI–cTnI inhibitory-peptide interactions in the bis-phosphomimetic system (cTn[P83S/S23D/S24D] system, panel D). This suggests that interference with PKA regulation may be a mechanism (albeit not a strong one) of action of the cTnIP^{P83S} mutation.

To further examine the region with direct contact between cTnC and cTnI with Ca^{2+} binding, we next studied the contact stability between the switch-peptide of cTnI (cTnI residues 148–164) and the 14 hydrophobic NcTnC residues (Phe-20, Ala-23, Phe-24, Ile-26, Phe-27, Ile-36, Leu-41, Val-44, Leu-48, Leu-57, Met-60, Phe-77, Met-80, and Met-81). Figure 10 displays the different contact maps of residue–residue pairs between those 14 hydrophobic residues of NcTnC and the cTnI switch-peptide for different systems. As we mentioned previously, cTnI residues 148–164 and the switch-peptide of cTnI were present at the beginning of the MD simulations (and we did not remove Ca^{2+} during MD simulations); thus, we did not expect to observe dramatic structural changes in this region. However, a variation in the contacts can be considered as an indicator of the cTnC–cTnI interaction ($K_{cTnC-cTnI}$) stability associated with activation. As compared with the WT cTn complex, there was some change in contact time upon introduction of the P83S mutant (panel A). A more dramatic change was seen upon introduction of the bis-phosphomimetic substitutions to S23/S24 of the WT complex (cTnI-P83S/S23D/S24D), suggesting there was a decreased interaction between NcTnC hydrophobic residues and cTnI switch-peptide upon phosphorylation (panel B). With introduction of the bis-phosphomimetic substitutions to the cTnI-P83S system (panel C), there was still some visible change in fluctuation for the contacts, but the degree (net change) of fluctuation is smaller than that for the WT complex. Together with the information for the intrasubunit contact of cTnI (Figure 9), our MD simulations suggest that phosphorylation of S23/S24 of cTnI results in the formation of an intrasubunit contact between cTnI inhibitory-peptide and NcTnI, which further reduces the stability of the interaction between cTnI switch-peptide and the cTnC hydrophobic-patch, and cTnI^{P83S} moderately abrogates this action. This suggests a potential structure-based mechanism of how cTnI^{P83S} may impair PKA regulation of contraction and relaxation.

### Discussion

CtNI^{P83S} (cTnIP^{P83S} in rodents) has been considered as a potential disease-causing mutation, but others suggest it may be a benign polymorphic variant rather than a disease-causing one. Recently, Ramirez-Correa et al. demonstrated that cTnI^{P83S} resulted in mild diastolic dysfunction in aging mice. However, this mutation itself has not yet been reported to cause HCM in patients. So, it is of importance to study whether cTnI^{P83S} alters myofibril contractile properties, and whether the alterations of structural-function for cTnI^{P83S} are similar to other HCM-associated cTnI mutations. In this study, we characterized the effects of cTnI^{P83S} on structure–function relationship of cTn and on the contractile properties of rat left ventricular myofibrils, to compare with similar measurements made previously with other cTnI mutations that have been associated with HCM. Two HCM-associated mutations, cTnI^{R146G} and cTnI^{R21C} (located in the inhibitory-peptide and cardiac-specific N-terminus of cTnI, respectively), not only altered normal contractile properties, but also blunted the regulatory ability of PKA-mediated phosphorylation on S23/S24 during β-adrenergic stimulation, and we attributed this to the disruption of the formation of an intrasubunit contact between cTnI inhibitory-peptide and NcTnI that is normally seen with phosphorylation. To understand whether these changes in contractile properties occur in all putative HCM cTnI mutations or are restricted to the mutations located in specific regions (such as N-terminus, inhibitory-peptide region), in this work, we studied the cTnI^{P83S} mutation that
is resided in the I–T arm region. The most significant findings for the current study were the following, compared to cTnIWT:

1. cTnIP83S increased both $K_C$ and $K_{C-I}$, but may limit or eliminate the ability of S23/S24 phosphorylation to reduce these properties of cTn. 2. cTnIP83S had no effect on $T_{MAX}$ or the Ca$^{2+}$ sensitivity of myofibril tension, but blunted PKA-phosphorylation-associated decrease in pCa$\_I$.

3. cTnIR146G and cTnIR21C exchanged myofilaments with PKA treatment, suggesting a slowed thin filament activation process. Interestingly, the $k_{ACT}/k_{TR}$ ratio did not differ with PKA phosphorylation for cTnIP83S, which is similar to what we reported for cTnIR146G and cTnIR21C. Also similar was the observation that cTnIP83S blunted the ability of PKA to speed the early, slow-phase of myofilibril relaxation (Figure 6). Using steady-state fluorescence measurements, we found that introduction of the bis-phosphomimic substitutions on cTn resulted in a quite similar reduction of the Ca$^{2+}$ binding affinity to cTn ($K_C$) for all complexes (except cTnIP83S). However, the decrease of C–I interaction ($K_{C-I}$) observed in cTnWT did not occur for all three cTn mutations. Those findings suggest that it may be the alteration of C–I interaction per se that perturbs (blunt) the modulation of PKA-mediated phosphorylation (or S23/S24 of cTnI) on altering the contractile properties for at least some cTnI mutations associated with HCM. It is of importance to point out that, in regard to the steady-state C–I binding measurements (in the presence of Ca$^{2+}$), there are at least two kinds of C–I interactions (the hydrophobic batch of cTnC with switch-peptide of cTnI, and the cTnC with the N-terminus of cTnI, etc). We also measured the C–I binding in the absence of Ca$^{2+}$ (date not present), and the $K_{C-I}$ values were saturated more slowly and were right-shifted (compared to the presence of Ca$^{2+}$) condition for all cTnI variants. In the absence of Ca$^{2+}$, the regulatory domain of cTnC was in the closed conformation, and cTnI may just nonspecifically bind to cTnC, such that the C–I binding without Ca$^{2+}$ is not a good indicator. On the other hand, the presence of Ca$^{2+}$ induces an “open” confirmation of the NcTnC, and allows the hydrophobic-patch of cTnC to interact with the switch-peptide of cTnI. Together, although it is hard to only measure the C–I binding between the hydrophobic-patch of cTnC with the switch-peptide of cTnI, it is the primary C–I binding portion in the presence of Ca$^{2+}$. Additionally, the presence of Ca$^{2+}$ is relevant to the physiology conditions that lead to muscle contraction and tension production.
Interestingly, the blunted or abnormal cardiac response to β-adrenergic stimulation had also been reported for several other cardiomyopathy mutations. For example, Li et al. and Schmidtman et al. found that the cTnC E239Q mutation hindered the effect of PKA-mediated phosphorylation of cTnI on transducing the signal from cTnC to cTnI, and Dong et al. reported that this mutation blunted the enhancement of NcTnC closing rate upon cTnI phosphorylation by PKA. Siegel et al. found that the cTnI R145G mutation suppressed the PKA effects on the maximal force-generating capacity and maximal sliding velocity and maximal acto-S1-ATPase activity. By exchanging the cTn complex into skinned rat trabeculae, Biesiadecki et al. found that cTnI G199D mutation blunted the PKA phosphorylation-induced decrease in the Ca2+ sensitivity of tension development (pCa50). Using an in vitro motility assay, Messer et al. reported that seven HCM-associated mutations in cTnT uncoupled the modulation of Ca2+ sensitivity by PKA phosphorylation of cTnI. To mimic PKA phosphorylation, we built up a bis-phosphomimetic (cTnI S23D/S24D cTnI) model by mutating the S23/S24 of cTnI to aspartic acid (D). Despite the charge and molecular space of real phosphorylation and the phosphorylation mimetics being different, the cTnI S23D/S24D substitution has previously been demonstrated to mimic the effects of PKA phosphorylation on S23/S24 of cTnI (cTnI S23V/S24V) both structurally and functionally.

Following this, several groups (including ours) have used the cTnI S23D/S24D substitution instead of the cTnI P23S/P24S to study the effects of PKA-mediated phosphorylation on cardiac function, as it serves as a useful tool to study the specific effect of PKA-mediated phosphorylation of cTnI, in the absence of cMyBP-C and titin phosphorylation that also occurs during β-adrenergic stimulation. As we reported previously, introduction of the bis-phosphomimetic substitutions at S23/S24 led to the formation of an intrasubunit interaction between the N-terminus and the inhibitory-peptide regions of cTnI for WT cTnI simulations, which has been previously proposed by Solaro et al. based on the spectroscopic and solution biochemistry measurements. Additionally, our simulation results suggested a bending at the N-terminus of cTnI and the formation of a more compact cTnI structure with the introduction of bis-phosphomimetic substitutions (on WT-cTnI), which is in accordance with the biochemical studies by several other laboratories.

Interestingly, this intrasubunit interaction no longer occurs in the presence of either cTnI R146G or cTnI R21C. This result was not surprising since these mutations are located in the interacting regions, either the inhibitory-peptide (cTnI R146G) or N-terminus (cTnI R21C) of cTnI, which may directly inhibit the formation of intrasubunit contacts between these two regions. However, cTnI P82S resides in the I–T arm of cTnI and does not directly interact with either region. Thus, our finding that there is still a moderate amount of intrasubunit interaction (though reduced) makes sense and may explain the lesser influence of cTnI P82S (compared with cTnI R146G or cTnI R21C) in blunting PKA-mediated modulation of function. Our findings may at least partially explain the contradictory reports and conclusions in the literature of cTnI P82S. Taken together, it suggests that cTnI P82S could contribute to a hypertrophic phenotype in the heart, but it would likely require an additional causal genetic mutation or epigenetic conditions.
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