Two hypertrophic cardiomyopathy-associated cardiac troponin I (cTnI) mutations, R146G and R21C, are located in different regions of cTnI, the inhibitory peptide and the cardiac-specific N terminus. We recently reported that these regions may interact when Ser-23/Ser-24 are phosphorylated, weakening the interaction of cTnI with cardiac TnC. Little is known about how these mutations influence the affinity of cardiac TnC for cTnI (K_{C-I}) or contractile kinetics during β-adrenergic stimulation. Here, we tested how cTnIR146G or cTnIR21C influences contractile activation and relaxation and their response to protein kinase A (PKA). Both mutations significantly increased Ca^{2+} binding affinity to cTnI (K_Ca) and K_{C-I}. PKA phosphorylation resulted in a similar reduction of K_Ca for all complexes, but K_{C-I} was reduced only with cTnIWT. cTnIWT, cTnIR146G, and cTnIR21C were complexed into cardiac troponin and exchanged into rat ventricular myofibers, and contraction/relaxation kinetics were measured ± PKA phosphorylation. Maximal tension (T_{max}) was maintained for cTnIR146G- and cTnIR21C-exchanged myofibers, and Ca^{2+} sensitivity of tension (pCa_{50}) was increased. PKA phosphorylation decreased pCa_{50} for cTnIWT-exchanged myofibers but not for either mutation. PKA phosphorylation accelerated the early slow phase relaxation for cTnIWT myofibers, especially at Ca^{2+} levels that the heart operates in vivo. Importantly, this effect was blunted for cTnIR146G- and cTnIR21C-exchanged myofibers. Molecular dynamics simulations suggest both mutations inhibit formation of intra-subunit contacts between the N terminus and the inhibitory peptide of cTnI that is normally seen with WT-cTn upon PKA phosphorylation. Together, our results suggest that cTnIR146G and cTnIR21C blunt PKA modulation of activation and relaxation kinetics by prohibiting cardiac-specific N-terminal interaction with the cTnI inhibitory peptide.

Familial hypertrophic cardiomyopathy (HCM) has been identified as a major autosomal dominant disease and is highly correlated with mutations detected in myofilament contractile proteins (1). Although the majority of mutations are found in myosin and cardiac myosin-binding protein C (cMyBP-C), mutations have also been identified in thin filament regulatory proteins such as cardiac troponin I (cTnI), which is a subunit of the cardiac troponin (cTn) complex that has a critical role in the activation and relaxation of cardiac muscle (2). At the beginning of systole, with the rise of intracellular Ca^{2+} in cardiomyocytes, Ca^{2+} binding to cardiac troponin C (cTnC) initiates a chain of events involving dynamic and structural changes in troponin that result in the activation of the thin filament (3). In the absence of Ca^{2+} (diastole), cTnC exists in its “closed” conformation, and cTnI binds actin tightly (and only weakly with cTnC), inhibiting actin-myosin interaction (3, 4). In systole, Ca^{2+} binding to site II of cTnC induces an “open” conformation that increases interaction between the N terminus of cTnC (NcTnC) and the cTnI switch peptide, resulting in decreased binding of the cTnI inhibitory peptide with actin (3, 5). Conseq
quently, this permits increased tropomyosin mobility, myosin interaction with actin to form cross-bridges, resulting in force generation (3).

\[ \beta \text{-Adrenergic stimulation serves as an essential physiological mechanism to meet increases in circulatory demand, acting through positive inotropic-lusitropic effects (6). During } \beta \text{-adrenergic stimulation, } cTnI \text{ is phosphorylated by protein kinase A (PKA) at sites Ser-23 and Ser-24 (Ser(P)-23/Ser(P)-24) that reside in the cardiac-specific N terminus of } cTnI \text{ (NcTnI) (6). We (7, 8) and others (6, 9–11) have demonstrated that phosphorylation of these sites reduces the affinity of } cTnC \text{ for } cTnI (K_{C-I}), \text{ reduces } Ca^{2+} \text{ sensitivity (pCa}_{50} \text{) of tension production, increases cross-bridge cycling kinetics, and accelerates cardiac muscle relaxation. We have also reported that PKA phosphorylation of } cTnI \text{ or bis-phosphomimic substitutions of } cTnI (cTnIS23D/S24D) accelerates and shortens the initial slow phase of cardiac myofibril relaxation, particularly during contraction with physiological (sub-maximal) } Ca^{2+} \text{ conditions, and thus it increases the overall speed of relaxation (7).}

HCM-associated cTnI mutations were first reported by Kimura et al. in 1997 (12), including R145G/R145Q, R162W, G203S, and K206Q. Among them, the cTnIR145G mutation (cTnIR146G in rodent), which is located in the inhibitory peptide of cTnI, has received prominent attention (13–25). Most previous studies investigating this mutation have focused on the \( Ca^{2+} \) sensitivity of tension, and ATPase activity in cardiomyocytes, demembranated cardiac muscle, and transgenic mice. It is well established that cTnIR146G mutation increases \( Ca^{2+} \) sensitivity of myofibrillar ATPase activity and force (18–20), reduces inhibition of actin-tropomyosin-activated myosin ATPase (14, 18, 19), and may have no direct effect on the cross-bridge cycle (20). It has also been reported that the cTnIR145G mutation has a significant effect on energy cost and has been associated with diastolic dysfunction (20). Another mutation, cTnIR21C, is the only identified HCM-associated mutation located at the cardiac-specific N terminus of cTnI (26–29). In transgenic mice, the cTnIR21C mutation has been reported to prevent PKA-mediated phosphorylation \textit{in vivo} (27, 28). It has also been reported that isolated cardiac myocytes from R21C mice older than 12 months of age have significantly delayed \( Ca^{2+} \) transient decay and relaxation (28). However, the mechanism for these effects and how these mutations affect the contraction and relaxation kinetics of cardiac muscle have not been studied.

Previous studies have proposed the formation of an intramolecular interaction between the N terminus and the inhibitory peptide region of cTnI upon PKA phosphorylation of Ser-23/Ser-24 of cTnI (11, 30–32). Recently, our computational modeling results demonstrated that introduction of the S23D/S24D substitutions on cTnI (cTnIS23D/S24D) led to the formation of an intra-subunit interaction between the N terminus and the inhibitory peptide of cTnI (8). We hypothesized that this interaction may be the structural correlate for shortening the duration and increasing the rate of the early phase of relaxation by destabilizing cTnI switch peptide interaction with NcTnC (8). Therefore, we hypothesized that introduction of an HCM mutation located in either the N terminus or the inhibitory peptide of cTnI may disrupt the formation of this intra-subunit interaction and blunt the effects of Ser-23/Ser-24 phosphorylation by PKA during \( \beta \)-adrenergic stimulation. In this work, we tested this hypothesis by studying the two HCM mutations cTnIR146G and cTnIR21C (see Fig. 1 for the location) that are located in the inhibitory peptide and the N terminus of cTnI (respectively) using combined protein biochemistry, myofibril mechanics, and computational (molecular dynamics) simulation studies.
Our studies indicate that both of these cTnI mutants increase Ca\(^{2+}\) binding of cTn (\(K_{Ca}\)) and \(K_{C-I}\) in solution, increase the Ca\(^{2+}\) sensitivity of myofibril tension development, and also prolong the early slow phase of relaxation. Importantly, both mutants blunt the ability of PKA to reduce \(K_{C-I}\) and the Ca\(^{2+}\) sensitivity of tension (\(pCa_{50}\)) and speed relaxation of myofibrils.

Our computational modeling of cTn suggests that introduction of either mutation inhibits the formation of the intra-subunit interaction between the N terminus and the inhibitory peptide of cTnI normally seen for cTn with phosphorylation (or bis-phosphomimic substitutions) of Ser-23/Ser-24. Thus, in addition to being hyper-contractile during systole, hearts with these mutations may have impaired initiation of diastole during \(\beta\)-adrenergic stimulation.

**Experimental Procedures**

**Proteins, cTnC Labeling, cTnI Phosphorylation, and cTn Complex Reconstitution—**WT rat cTnC, cTnI, and cTnT in the pET24 vectors were constructed and expressed as described previously (33). cTnC\(^{C35S}\), cTnIR146G, cTnIR21C, and cTnIR21C/S23D/S24D were constructed from WT cTnC and cTnI plasmids, respectively, using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). We (Fig. 2A) and others (27, 28) demonstrated that cTnIR21C disrupts the PKA phosphorylation on Ser-23/Ser-24 of cTnI. Thus, in addition to being hyper-contractile during systole, hearts with these mutations may have impaired initiation of diastole during \(\beta\)-adrenergic stimulation.

**Steady-state Fluorescence Measurements—**All steady-state fluorescence experiments were measured using an LS50B luminescence spectrometer (PerkinElmer Life Sciences) at 15 °C as described previously (7, 36). Solution composition for this fluorescence measurement was as follows (in mM): 150 KCl, 20

![Figure 2](image-url)
R146G and R21C cTnI Disrupt PKA Modulation of Contraction

MOPS, 3 MgCl₂, 2 EGTA, and 1 DTT, pH 7.0. Fluorescence signal of 2 ml of cTnI C₃SS₅ or cTnC C₅SS₇ (0.6 μM) was monitored during the titration of micromolar amounts of Ca²⁺ or cTnI variants (WT, Ser(P)-23/Ser(P)-24, S23D/S24D, R146G, R146G/Ser(P)-23/Ser(P)-24, R21C or R21C/S23D/S24D) in the presence of Ca²⁺ (100 μM) at ~530 nm with an excitation wavelength of 490 nm. The concentration of free Ca²⁺ was computed using Maxchelator (41). The Ca²⁺ sensitivity (measured as pCa₅₀, the pCa (pCa = −log [Ca²⁺])) value at half-maximal fluorescence signal change) was collected by fitting the binding curve with the sigmoid Hill equation as described previously (42). The reported values are the means ± S.E. of three to six successive titrations.

Ethical Approval and Tissue Preparation—Animal procedures were conducted in accordance with the National Institutes of Health Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). Rats were housed in the Department of Comparative Medicine, University of Washington, and cared for according to IACUC procedures. Male Sprague-Dawley rats (3 months old, 150–250 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) after initial exposure to isoflurane (3–5% in oxygen). When the rat had no reflexive response, its heart was rapidly excised and dissected in oxygenated physiological saline solution (containing (in mM): 100 NaCl, 24 NaHCO₃, 2.5 KCl, 1 MgSO₄·7H₂O, 1 Na₂HPO₄, and 1 CaCl₂ (43). After this, both ventricles were cut open, and the whole heart was demembranated in skinnng solution containing (in mM): 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 mixture” (Sigma, catalog no. P8340) overnight at 4 °C (44, 45). The heart was then washed three times in the same solution without Triton X-100 and stored at ~20 °C for up to 1 week. Myofibrils from the left ventricles (LV) were used for the mechanical measurements described below.

Solutions—Composition of solution used for mechanical measurements was determined by an iterative algorithm that computes the equilibrium concentration of ions and ligands based on published affinity constants (46). Composition of relaxing solution was as follows (in mM): 200 KCl, 20 MOPS, 5 MgCl₂, 2 EGTA, 1 DTT, 4 ATP, and 1:100 dilution of protease inhibitor mixture on a slow rocker overnight at 4 °C (7). Following exchange, myofibrils were washed with relaxing solution containing 1 mg/ml bovine serum albumin (BSA) twice for 30 min to remove any nonspecifically bound exogenous cTn.

Myofibril Mechanical/Kinetics Measurement—Myofibril mechanical/kinetics measurements were performed on a custom-built setup as described previously (48). Briefly, single or small bundles (~2–4) of cardiac myofibrils were attached between two glass micro-tools forged from borosilicate glass capillary tubes (outer diameter 1.0 mm and inner diameter 0.5 mm, Sutter Instruments, Novato, CA), with the initial sarcomere length set as ~2.3 μm, and perfused with solutions that can be rapidly switched. One of the needles acted as a force transducer, which deflected in a predictable manner upon application of force (48). Needle stiffness was determined by first deflecting the needle with a known amount of force using a galvanometer. Needle deflections were measured under a ×40 lens, and this yielded stiffness in nN μm⁻¹. The stiffness of needles used for the experiments ranged between 5 and 11 nN μm⁻¹. This force transducer needle was positioned over a dual diode system, which records needle displacement and correlates displacement to force development. A second straight needle was attached to the other end of the myofibril and was applied to rapidly shorten and re-stretch the myofibril through a computer interface and a Piezo-controller motor (PZT Servo controller, LVPZT amplifier, Physik Instrumente, Irvine, CA). At the end of each experiment, a calibration curve was performed in which the force transducer needle was moved in 1-μm steps over the range of the diodes using micromanipulators (MP-285, Sutter Instruments, Novato, CA).

A double-barreled borosilicate θ glass pipette (capillary glass tubing outer diameter 2.0 mm and inner diameter 1.4 mm, SEP 0.2 mm, modified in-house to outer diameter of 0.55 mm, Warner Instruments, Hamden, CT) was used to stream low (10⁻⁹ M, pCa 9.0) and high (10⁻⁵ M, pCa 4.0) Ca²⁺-containing solutions to the mounted preparation, and stepping for solution switch over the preparation was controlled by a computerized motor (SF-77B Perfusion Fast Step, Warner Instruments). The solution change was complete in ~10 ms (48, 49).

Activation and relaxation data were collected at 15 °C and fit as described previously (48–50). The kinetics of contractile activation (k₅ act; with rapid increase in Ca²⁺) was obtained from a single-exponential rise to a maximum. A rapid release-re-stretch protocol (a sudden 20% decrease in optimal length followed by rapid stretching back to the original length after 25 ms of unloaded shortening) was applied to measure the rate of force redevelopment (k₅ rel). A slow phase relaxation rate (k₅ rel, slow) was reported as the slope of a regression line fitted to the tension trace and normalized to the tension amplitude, and the slow phase duration (t₅ rel, slow) was measured from the onset of solution change at the myofibril to the shoulder marking the beginning of fast phase. Transition from slow to rapid phase was determined through multiple factors. An apparent change in the slope of the data or a change in the signal-to-noise ratio at a final concentration of ~1 mg/ml were passively exchanged into isolated rat LV myofibrils in a buffer containing the following (in mM): 200 KCl, 20 MOPS, 5 MgCl₂, 2 EGTA, 1 DTT, 4 ATP, and 1:100 dilution of protease inhibitor mixture on a slow rocker overnight at 4 °C (7). Following exchange, myofibrils were washed with relaxing solution containing 1 mg/ml bovine serum albumin (BSA) twice for 30 min to remove any nonspecifically bound exogenous cTn.
was often apparent at the transition. The fast phase relaxation rate \(k_{\text{rel, fast}}\) was measured from a single exponential decay fitted to the data. A \(t_{\text{90}}\) estimation was made in cases where the decay was not well described by a single exponential, and this was converted to a rate \(\tau = \ln(2)/t_{\text{90}}\). Myofibrils that contracted >10% of their optimal length were excluded from the analysis as non-isometric.

**Protein 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 (7). The phosphorylated cTnI was detected using a rabbit polyclonal to cTnI (phospho-Ser-22 + Ser-23, from Abcam, catalog no. ab58545) as primary antibody (1:1000) and goat anti-rabbit 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).

**Statistics**—Comparisons between groups of data were performed using paired or unpaired Student’s t test as appropriate. All reported data are expressed as mean ± S.E. and “n” represents the number of experimental samples in each group. Results with \(p < 0.05\) were considered statistically significant. In this study, the R146G, R21C, WT + PKA, and S23D/S24D data were compared with the WT sets; the R146G + PKA data were compared with WT + PKA sets, and the R21C/S23D/S24D results were compared with S23D/S24D sets.

**Computational Modeling**—The initial structure of the cTnI complex was built up based on the core crystal structure of Takeda *et al.* (51) with the addition of the N terminus of cTnI from the NMR structure provided by Howarth *et al.* (30). To mimic phosphorylation, a bis-phosphomimics model (cTnIR21C/S24D) was constructed by mutating Ser-23/Ser-24 of cTnIR21C to get Ser-23/Ser-24 phosphorylated, we introduced the bis-phosphomimic substitutions S23D/S24D into cTnIR21C. Thus, to determine whether it is the cTnIR21C mutation per se that is altering function or just the inability to get Ser-23/Ser-24 phosphorylated, we introduced the bis-phosphomimic substitutions S23D/S24D into cTnIR21C (cTnIR21C/S23D/S24D) to mimic the effect of PKA phosphorylation. We (7, 8, 25, 57) and others (58–61) previously demonstrated that cTnIR21C can mimic the PKA phosphorylation effects on Ser-23/Ser-24 of cTnI (cTnI Ser(P)-23/Ser(P)-24) both structurally and functionally.

**Steady-state Fluorescence Measurements of \(K_{C-I}\) and \(K_{Ca}\)**—The effects of R146G or R21C mutation ± PKA phosphorylation (or bis-phosphomimic substitutions) on cTnI \(K_{Ca}\) and the binding affinity of cTnC for cTnI \((K_{C-I})\) were determined by steady-state fluorescence measurements using a fluoroprobe IANBD, as described previously (7, 36). IANBD, a sulfhydryl-reactive and environment-sensitive extrinsic fluorophore, has been widely used to study the intra-molecular interactions of proteins, and labeling at Cys-84 of cTnCC35S reflects conformational and environmental changes of NcTnC that arise from Ca\(^{2+}\) binding and/or interaction with cTnI (7, 35, 36, 40). We first measured the conformational changes with Ca\(^{2+}\) binding to cTnI containing either cTnIR146G or cTnIR146G/Ser(P)-23/Ser(P)-24 compared with WT cTnI. As shown in Fig. 3A, cTnIR146G increased (left shift) Ca\(^{2+}\) binding affinity (\(K_{Ca}\)) compared with cTnI containing cTnIWT, in agreement with previous studies (18–20). The Ca\(^{2+}\) sensitivity of the fluorescence intensity (reported as \(pCa_{50}\)) was shifted by 0.24 pCa units, from 7.07 ± 0.03 (cTnI with cTnIWT) to 7.31 ± 0.03 (cTnI with cTnIR146G). Consistent with our previous finding (7), phosphorylation of cTnIWT at
Ser-23 and -24 (cTnISer(P)-23/Ser(P)-24) also reduced $K_{Ca}$, resulting in a 0.31 $pCa$ unit decrease (right shift). Similarly, PKA phosphorylation of cTnIR146G (cTnIR146G/Ser(P)-23/Ser(P)-24) reduced the Ca$^{2+}$ sensitivity ($pCa_{50} = 7.03 \pm 0.03$), resulting in a 0.28 $pCa$ unit decrease (right shift).

In view of the “gatekeeper” role of cTnC-cTnI interaction in translating the Ca$^{2+}$ signal to myofilament proteins to initiate cardiac muscle contraction, we also tested how the cTnIR146G mutation affected $K_{Ca}$. The $K_{Ca}$ was measured by titrating cTnIR146G or cTnIR146G/Ser(P)-23/Ser(P)-24 into cTnCIANBD in the presence of 100 $\mu$M Ca$^{2+}$. Fig. 3B shows the IANBD fluorescence signal change as the concentration of cTnI was increased up to 0.8 $M$ in solutions containing 0.6 $M$ cTnCIANBD. There was no further change in the fluorescence signal beyond 0.6 $M$ cTnI, suggesting strong binding of cTnI to cTnC such that 1:1 binding was achieved. Similar to $K_{Ca}$, cTnIR146G left-shifted $K_{C-I}$ compared with cTnIWT. As we reported previously (7), phosphorylation of cTnIWT reduced $K_{C-I}$. However, this effect was completely eliminated (blunted) for the cTnIR146G mutant.

Consistent with our previous finding (7), with respect to the cTnIWT, cTnIS23D/S24D substitutions also reduced (right-shifted) both Ca$^{2+}$ sensitivity ($pCa_{50} = 6.78 \pm 0.03$) and $K_{C-I}$, similar to phosphorylation of cTnIWT at Ser-23 and -24. As with cTnIR146G, cTnIR21C also increased both $K_{Ca}$ ($pCa_{50} = 7.29 \pm 0.02$) and $K_{C-I}$ compared with cTnIWT (Fig. 3, C and D), and upon introduction of the bis-phosphomimic substitutions at Ser-23/Ser-24 of cTnIR21C (cTnIR21C/S23D/S24D), $K_{Ca}$ also decreased ($pCa_{50} = 7.03 \pm 0.03$), and the effect on $K_{C-I}$ was blunted. Interestingly, this suggests that both mutations, in different regions of cTnI, behave similarly in solution.

Recombinant Troponin (cTn) Complex Exchange Profiles—The native cTn in isolated myofibrils was passively exchanged with recombinant rat cTn containing either cTnIWT, cTnIR146G, or cTnIR21C. 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 (7, 57).

To study the effects of PKA phosphorylation, myofibrils exchanged with cTn containing either cTnIWT or cTnIR146G were incubated with relaxing solution containing 100 units of PKA and DTT for 45 min. For cTnIR21C, PKA effects were studied by exchanging cTn containing cTnIR21C/S23D/S24D and were compared with the cTn containing cTnIS23D/S24D. The phosphorylation profile for cMyBP-C and titin (also phosphorylated by PKA incubation) was not measured; however, they should be similar for each group as paired comparisons of myofibrils containing cTnIR146G or cTnIR21C versus cTnIWT were made from each heart. The extent of cTn phosphorylation in exchanged myofibrils (prior to PKA treatment) was inversely related to the exchange efficiency. The phosphorylation profile is plotted in Fig. 2, B and C. A very small amount of residual phosphorylated cTnI was likely present in every exchange preparation because the exchange efficiency was not 100%. It is clear that the cTn phosphorylation level in exchanged myofibrils was quite low, as recombinant cTnI was not phosphorylated, further confirming
high exchange efficiency. PKA treatment significantly increased the cTn phosphorylation level, resulting in over 90% of cTn phosphorylated, which is consistent with our previous observation (7).

**cTnI-R146G and cTnI-R21C Mutations Effects on Myofibril Contraction**—The effects of mutations on tension development and relaxation kinetics (at 15 °C) were determined from isolated myofibrils from rat LV cardiac muscle exchanged with cTn containing either cTnIR146G or cTnIR21C and compared with the WT-cTn complex. Myofibrils were exposed to continuously flowing solutions that were rapidly switched to provide step increases and decreases in bathing [Ca2+]i, from relaxing solution (pCa 9.0) to either maximal (pCa 4.0) or submaximal (pCa 5.4, pCa 5.6, and pCa 5.8) [Ca2+]i and then back to 9.0. Representative example tension traces for cTnIWT, cTnIR146G, and cTnIR21C exchanged myofibrils during the submaximal [Ca2+]i (pCa 5.4) activation-relaxation protocol are presented in Fig. 4. A summary of tension magnitude and kinetic parameters for rat LV myofibrils exchanged with cTn containing cTnIWT, cTnIR146G, or cTnIR21C is presented in Table 1 and Figs. 5 and 6.

Maximal tension (Tmax) did not differ for myofibrils exchanged with cTnIR146G (69 ± 7 mN/mm2) compared with cTnIWT myofibrils (73 ± 4 mN/mm2, Fig. 5A). Tension was also measured at multiple submaximal Ca2+ levels, and the pCa50 was left-shifted 0.13 pCa units (Fig. 5B), from 5.32 ± 0.02 (cTnIWT myofibrils) to 5.45 ± 0.03 (cTnIR146G myofibrils), demonstrating an increase in the Ca2+ sensitivity of tension, as reported previously by others for the cTnIWT-exchanged samples (3.2 ± 0.3 s⁻¹), kact, did not differ for either the cTnIR146G, (2.7 ± 0.2 s⁻¹) or cTnIR21C (3.0 ± 0.3 s⁻¹)-exchanged myofibrils at pCa 4.0 or any sub-maximal Ca2+ level tested (Table 1). For all myofibrils, kact was significantly slower at sub-maximal Ca2+ levels than during maximal Ca2+ activations, as reported previously for rodent cardiac myofibrils (7, 47), suggesting that the Ca2+ sensitivity of cardiac contraction kinetics is maintained upon introduction of HCM-associated mutations. Once the activation was completed (i.e. tension was in steady state), a rapid release-restretch protocol was applied on myofibrils to measure the rate of tension redevelopment (krel). The krel protocol is designed to measure the rate of myosin cross-bridge attachment and subsequent tension generation (45) when Ca2+ binding to troponin is in near steady state (e.g. the thin filament is already activated). This measurement can help to differentiate the contribution of Ca2+-mediated thin filament activation versus the cross-bridge cycling kinetics to krel. At all measured conditions (cTnIR146G- or cTnIR21C-exchanged myofibrils), krel was faster at both maximal and submaximal Ca2+ levels, as reported previously for cTnIWT-exchanged myofibrils (7), suggesting thin filament activation is rate-limiting for rat cardiac myofibril tension generation from rest (diastole) at 15 °C. Comparing the kact/krel ratio can give an indication of whether thin filament activation kinetics is more rate-limiting to tension development in the R146G or R21C exchanged myofibrils as compared with the WT myofibrils. Fig. 5, E and F, demonstrates that this ratio did not change upon introduction of either mutation, suggesting the activation process is maintained.

**cTnIR146G and cTnIR21C Mutations Affect Myofibril Relaxation**—Rapid deactivation of myofibrils by switching from a maximal or sub-maximal [Ca2+]i solution to relaxing solution (pCa 9.0) induced a biphasic relaxation, an initial linear tension decay followed by a more rapid (fast) exponential decay back to the baseline tension (see example trace in the inset of Fig. 4). The rate of the slow phase relaxation (krel,slow) is thought to be predominantly reflective of the cross-bridge detachment rate (47, 50, 62–65), whereas the duration of slow phase relaxation (trel,slow) may be influenced by the time for the troponin to move back to a “blocked” state (66). For the maximal activations, krel,slow was unaltered for cTnIR146G-treated myofibrils (1.3 ± 0.2 s⁻¹, Fig. 6D) compared with cTnIWT exchanged myofibrils (1.1 ± 0.1 s⁻¹), whereas trel,slow of the cTnIR146G-exchanged myofibrils (105 ± 7 ms, Fig. 6E) was significantly prolonged compared with those of the cTnIWT-treated myofibrils (79 ± 6 ms). Similarly, at pCa 4.0, cTnIR21C-exchanged myofibrils also prolonged trel,slow (100 ± 6 ms, Fig. 6G) and did not affect krel,slow (1.1 ± 0.1 s⁻¹, Fig. 6F). By analyzing the contribution of slow phase on whole relaxation, we found the contributions for the cTnIWT-, cTnIR146G-, and cTnIR21C-exchanged myofibrils at pCa 4.0 were 6, 7, and 7% of the total amplitude, respectively, suggesting cross-bridge detachment was not affected by the mutations. In contrast to the slow phase of relaxation, the much larger, rapid phase of relaxation (krel,fast) was determined by several sarcomeric properties as well as uneven relaxation kinetics between sarcomeres in series (63–65). There was no difference in krel,fast.
R146G and R21C cTnI Disrupt PKA Modulation of Contraction

TABLE 1

Tension generation and relaxation parameters after recombinant rat WT cTn or rat cTn contained either cTnIR146G or cTnIR21C exchange into rat ventricular myofibrils at 15 °C.

Values given are mean ± S.E. Number in parentheses indicates the number of myofibrils. *p < 0.05 versus WT; †p < 0.05 versus WT + PKA; ‡p < 0.05 versus WT + PKA; ††p < 0.05 versus WT + PKA; †‡p < 0.05 versus WT + PKA; †††p < 0.05 versus WT + PKA; †‡†p < 0.05 versus WT + PKA; ††††p < 0.05 versus WT + PKA.

TABLE 1

| Myofibril Batches | pCa | Tension (mN/mm²) | kₐct | kₜ | tₑₛ,slow | kₑₛ,slow | kₑₛ,fast |
|-------------------|-----|-----------------|------|---|---------|----------|----------|
|                   |     |                 |      |   |         |          |          |
| WT                | 4.0 | 73 ± 4 (30)     | 3.2 ± 0.3 (30) | 5.4 ± 0.5 (30) | 79 ± 6 (28) | 1.1 ± 0.1 (28) | 18 ± 2 (29) |
| WT + PKA          | 4.0 | 69 ± 7 (29)     | 2.3 ± 0.2* (29) | 5.6 ± 0.9 (27) | 66 ± 3* (26) | 1.8 ± 0.2* (26) | 21 ± 2 (28) |
| S23D/S24D         | 4.0 | 66 ± 5 (23)     | 2.6 ± 0.2* (23) | 6.1 ± 0.6 (23) | 65 ± 5* (20) | 2.1 ± 0.4* (20) | 22 ± 3 (20) |
| R146G             | 4.0 | 69 ± 7 (27)     | 2.7 ± 0.2* (27) | 4.6 ± 0.6 (27) | 105 ± 7* (25) | 1.3 ± 0.2 (25) | 18 ± 2 (27) |
| R146G + PKA       | 4.0 | 69 ± 10 (23)    | 2.8 ± 0.3 (23)  | 4.9 ± 0.6 (22) | 91 ± 5* (21) | 1.3 ± 0.2* (21) | 18 ± 2 (22) |
| R21C              | 4.0 | 74 ± 6 (23)     | 3.0 ± 0.3 (23)  | 5.6 ± 0.3 (23) | 100 ± 6* (22) | 1.1 ± 0.1 (22) | 19 ± 2 (23) |
| R21C/S23D/S24D    | 4.0 | 70 ± 5 (24)     | 2.7 ± 0.2 (24)  | 5.3 ± 0.4 (23) | 89 ± 7* (23) | 1.2 ± 0.2* (23) | 18 ± 2 (23) |
| WT                | 5.4 | 31 ± 3 (23)     | 1.6 ± 0.2 (23)  | 3.6 ± 0.5 (23) | 84 ± 5 (21) | 2.0 ± 0.3 (21)  | 18 ± 2 (23) |
| WT + PKA          | 5.4 | 18 ± 3* (16)    | 1.4 ± 0.2 (16)  | 3.0 ± 0.4 (15) | 67 ± 3* (14) | 5.9 ± 1.4* (14) | 19 ± 3 (15) |
| S23D/S24D         | 5.4 | 17 ± 3* (13)    | 1.9 ± 0.3 (13)  | 4.2 ± 0.3 (13) | 58 ± 6* (13) | 6.1 ± 1.5* (13) | 20 ± 3 (13) |
| R146G             | 5.4 | 39 ± 4* (17)    | 2.0 ± 0.2 (17)  | 3.4 ± 0.3 (16) | 103 ± 10* (16) | 2.1 ± 0.3 (16) | 18 ± 3 (16) |
| R146G + PKA       | 5.4 | 36 ± 6* (15)    | 1.7 ± 0.2 (15)  | 3.6 ± 0.4 (15) | 87 ± 6* (15) | 2.0 ± 0.3* (15) | 21 ± 2 (15) |
| R21C              | 5.4 | 40 ± 4* (16)    | 1.7 ± 0.2 (16)  | 3.9 ± 0.3 (16) | 90 ± 5 (15) | 1.8 ± 0.2 (15)  | 18 ± 2 (16) |
| R21C/S23D/S24D    | 5.4 | 37 ± 3* (15)    | 1.9 ± 0.2 (15)  | 4.0 ± 0.4 (14) | 88 ± 5* (14) | 2.1 ± 0.2* (14) | 18 ± 3 (14) |

FIGURE 5. Tension (A) and pCa-tension relationship (B) for cTnIR146G-exchanged myofibrils prior to and after PKA treatment. Tension (C) and pCa-tension relationship (D) for cTnIR21C-exchanged myofibrils prior to and after introduction of the bis-phosphomimic mutations. The kₐct/kₜ ratio for cTnIR146G-exchanged myofibrils prior to PKA treatment and after introduction of the bis-phosphomimic mutations. The kₐct/kₜ ratio for cTnIR21C-exchanged myofibrils prior to and after PKA treatment (or introduction of the bis-phosphomimic mutations). *, p < 0.05; †, p < 0.01.

for either cTnIR146G (18 ± 2 s⁻¹) or cTnIR21C (19 ± 2 s⁻¹) versus cTnIRWT (18 ± 2 s⁻¹) exchanged myofibrils. At a more physiological level of Ca²⁺, pCa 5.4, kₑₛ,slow was twice as fast compared with maximal activation (pCa 4.0) for all myofibrils (Table 1), suggesting faster cross-bridge detachment. Akin to pCa 4.0, at sub-maximal Ca²⁺ levels there was no difference in...
k_{rel, slow} or k_{rel, fast} between cTnI^{WT}, cTnI^{R146G}, and cTnI^{R21C}-exchanged myofibrils. However, t_{rel, slow} was also prolonged for the cTnI^{R146G}-exchanged myofibrils (Tables 1 and Fig. 6E). We calculated the times to reach 50% (RT_{50}) and 90% (RT_{90}) relaxation time (RT) and found that only cTnI^{R146G} significantly prolonged the RT_{90} with respect to the cTnI^{WT} exchanged myofibrils.

cTnI-R146G and cTnI-R21C Mutations Blunt the PKA Effects on Myofibril Contraction and Relaxation—We next studied the effects of PKA (or introduction of bis-phosphomimic substitutions at Ser-23/Ser-24 of cTnI) on myofibril contraction and relaxation. Consistent with previous studies (7), we found that after treating the cTnI^{WT}-exchanged myofibrils with PKA, T_{max} (69 ± 7 mN/mm²) was maintained (Fig. 5A), and pCa_{50} was right-shifted 0.2 pCa units to 5.12 ± 0.03, demonstrating reduced Ca^{2+} sensitivity of tension development (Fig. 5B). Akin to the PKA-treated WT-exchanged myofibrils, T_{max} (66 ± 7 mN/mm²) was also maintained (Fig. 5C), and pCa_{50} was also decreased for the S23D/S24D-exchanged myofibrils (Fig. 5D), as we previously reported (7). PKA treatment of cTnI^{WT} myofibrils (or S23D/S24D-exchanged myofibrils) also slowed k_{act} (2.3 ± 0.3 s^{-1}) during maximal Ca^{2+} activation, although the k_{tr} (5.6 ± 0.8 s^{-1}) was unchanged, suggesting PKA phosphorylation affects the kinetics of thin filament activation prior to cross-bridge binding and tension development. This can be clearly seen by calculating the k_{act}/k_{tr} ratio (Fig. 5, E and F) for PKA-treated WT-exchanged myofibril (or S23D/S24D-exchanged myofibrils), which is significantly decreased with
respect to WT-exchanged myofibrils prior to PKA treatment. Following PKA treatment (or introduction of bis-phosphomimetic substitutions), maximal tension was also maintained for cTnIR146G-(69 ± 10 mN/m²) and cTnIR21C/S23D/S24D-(70 ± 5 mN/m²) exchanged myofibrils. In contrast to cTnWT, the Ca²⁺ sensitivity of tension development following PKA phosphorylation was not reduced (blunted) for either cTnIR146G or cTnIR21C (Fig. 5, B and D). Additionally, maximal kₚCa of cTnIR146G (pCa 4.0) did not change for myofibrils exchanged with cTnIR146G following PKA phosphorylation or cTnIR21C-(50 and 50), which can be clearly observed in the plots of kₚCa/kₚr, ratio from Fig. 5, E and F, suggesting the slowing of thin filament activation at maximal Ca²⁺ was also blunted.

One of the main effects of β-adrenergic stimulation on cardiac function is an increase in heart rate, so faster relaxation is crucial to ensure maintained or increased diastolic ventricular filling. Thus, we measured how relaxation rates were affected following PKA treatment of cTn-exchanged myofibrils. As we observed previously (7), PKA treatment of cTnIR146G-(exchanged myofibrils (or cTnIR21C-(exchanged myofibrils) significantly increased kₚCa (1.8 ± 0.2 s⁻¹) and decreased kₚrel,slow (66 ± 2 ms) during maximal Ca²⁺ activation, speeding up the overall relaxation. Moreover, these effects were greater at sub-maximal Ca²⁺ levels where the heart operates. Interestingly, for all PKA-phosphorylated cTnIR146G myofibrils or cTnIR21C/S23D/S24D-(exchanged myofibrils, there were no changes in either the rate (1.3 ± 0.2 s⁻¹ versus 1.2 ± 0.2 s⁻¹) or the duration (91 ± 5 ms versus 89 ± 7 ms) of slow phase relaxation, suggesting the effects of PKA to speed relaxation were blunted for both mutations. Fig. 6, A–C, is a set of example tension traces demonstrating these findings. Additionally, no changes were detected in kₚrel,fast with PKA phosphorylation. We also saw that upon PKA phosphorylation (or introduction of the bis-phosphomimetic substitutions) to cTnIR146G, both RT₀, and RT₁, were significantly decreased. Importantly, these effects were blunted with the introduction of either cTnIR146G or cTnIR21C mutations.

Molecular Dynamics Simulations—We recently reported on differences in cTn dynamics between molecular dynamics models containing the WT versus R146G cTn (8, 25). Here, we studied the dynamics of WT versus R21C cTn in our cTn model for comparison with the R146G model. Triplicate 150-ns MD simulations were compared. The root-mean-square fluctuations (RMSF) versus the protein residue numbers of each subunit were calculated, and the average (± S.D.) RMSF of the cTnC and cTnI subunits for both cTnWT and cTnR21C cTn systems is presented in Fig. 7, A and B. In Fig. 7, we highlight site I (pink) and site II (the Ca²⁺-binding loop, blue) of cTnC, and the inhibitory peptide (green) and switch peptide regions (orange) of cTnI. Similar to the cTnIR146G-(25), fluctuations were comparable for the cTnIR21C cTn model with respect to the WT model throughout most of the residues (average RMSFs of cTnC are 2.8 and 2.9 Å, respectively; and the average RMSFs of cTnI are 2.8 and 2.9 Å, respectively). Most of the regions had no changes larger than the standard deviations. The most pronounced differences to cTnC were seen in site I (cTnC residues 28–38, 2.2 Å for cTnWT versus 2.6 Å for cTnIR21C cTn model) and the linker loop (cTnC residues 80–100, 2.5 Å for cTnWT versus 2.3 Å for cTnIR21C cTn model) connecting the N- and C-terminal lobe of cTnC. The most fluctuating region detected in cTn with both complexes was the N terminus (cTnC residues 1–41), although the helical bundle identified as the I-T arm (cTn residues 42–137) was the most stable region in the cTnI subunits, again suggesting its structural rather than regulatory function.

We next investigated how introduction of bis-phosphomimetic substitutions to Ser-23/Ser-24 of cTnI affected the dynamics of cTn containing the cTnIR21C mutation. Fig. 7, C and D, shows that the average (± S.D.) RMSF of the cTnC and cTnI subunits for cTnIR21C and cTnIR21C/S23D/S24D cTn systems, respectively. Fluctuations were increased slightly in the cTnIR21C/S23D/S24D system (average RMSF 2.8 Å), akin to what we previously reported for WT and cTnIR23D/S24D simulations (8), as well as the cTnIR146G and cTnIR146G/S23D/S24D simulations (25). Previously, we observed a significant change (p < 0.001) in NcTnI upon introduction of bis-phosphomimetic substitutions to the WT model (8). Interestingly, introduction of bis-phosphomimetic substitutions to the cTnIR21C model had very little impact on the stability of the N terminus of cTnI, similar to what we recently observed for cTnIR146G model (25). To better visualize how introduction of the R21C and/or bis-phosphomimetic substitutions influences the subunit interactions among the cTn complexes, 15 snapshots taken every 10 ns during the entire MD simulations were superimposed (Fig. 7, E–G). For clarity, cTnC is shown in blue, cTnI in red, and cTnT in yellow. In contrast to the greater flexibility exhibited for the NcTnI in the cTnIR21C/S23D/S24D cTn model with respect to the WT model (8), the introduction of S23D/S24D to the cTnIR21C model had very little impact on the overall structures. As we discuss below, we speculate that this difference between the WT and R21C model upon introduction of the bis-phosphomimetic substitutions may result in interference of the interaction of NcTnI with other regions of the cTn complex, and thus blunt the effects of PKA normally seen for the WT system.

We next measured the time evolution of distances between the bound Ca²⁺ ion and the six coordinating residues of site II (Asp-65, Asp-67, Ser-69, Thr-71, Asp-73, and Glu-76) versus the course of each 150-ns simulation. Among these six coordinating residues, four (Asp-65, Asp-67, Ser-69, and Thr-71) fluctuated much more and varied for each run in all the four systems. Thr-71 generally did not coordinate, in agreement with the structural data from x-ray crystallography (results not shown) (51). Fig. 8 shows the distances between Ca²⁺ and Ser-69. It is clear that Ser-69 had the most pronounced difference for Ca²⁺ coordination, in agreement with our previous observations (8, 25, 27, 40). The percentage of contact time for Ser-69 varies among different systems. Compared with the WT system (10%), the coordinating time of Ser-69 was increased to 23% in the cTnIR21C system. Although this was not statistically significant (p = 0.3981), it suggests a stronger interaction that could provide stabilization.
This may interpret the increased Ca$^{2+}$ binding affinity of $\text{cTnI}_{21C}$ with respect to the WT observed from the steady-state fluorescence measurements. Interestingly, the contact time was decreased to 17% upon introduction of the phospho-mimic substitutions to $\text{R21C}$, in agreement with the reduction of Ca$^{2+}$ binding affinity ($K_{Ca}$) observed from the steady-state fluorescence measurements. Interestingly, we previously (25) found that compared with the WT system, the coordinating
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The time of Ser-69 in the cTnI<sup>R146G</sup> system was increased to 29%. Upon introduction of the bis-phosphomimic substitutions to cTnI<sup>R146G</sup>, the contact time was decreased to 6%. Thus, the results obtained for cTnI<sup>R146G</sup> were similar to cTnI<sup>R21C</sup> (proliferation of the coordinating time). However, upon introduction of the bis-phosphomimic substitutions, cTnI<sup>R146G</sup> displayed a greater reduction of the coordinating time compared with cTnI<sup>R21C</sup>.

Next, the residue-residue contacts of key regions were monitored over the time course of the entire 450-ns simulations. Fig. 9 shows the corresponding average contact map of residue-residue pairs between 14 hydrophobic residues of NcTnC and cTnI following Ca<sup>2+</sup> binding, we examined the contact stability of the cTnI switch peptide with hydrophobic NcTnC residues. Fig. 10 shows the different contact maps of residue-residue pairs between 14 hydrophobic residues of NcTnC (from left to right: they are 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) and the cTnI switch peptide (cTnI residues 148–164) for the different systems. Because Ca<sup>2+</sup> and the cTnI switch peptide were present at the start of the MD model simulations, and we did not remove Ca<sup>2+</sup> during simulations, we did not expect to see dramatic structural changes in this region. However, a change in the fluctuation of contacts can be considered as an indicator of stability of the cTnC-cTnI interaction associated with activation. As compared with the WT complex, there was little change in contact time upon introduction of the R21C mutant (Fig. 10A). A more dramatic change was seen upon introduction of the bis-phosphomimic residues to Ser-23/Ser-24 of WT complex (cTnI<sup>S23D/S24D</sup>), suggesting decreased interaction between NcTnC hydrophobic residues and cTnI switch peptide upon phosphorylation (Fig. 10B). However, with introduction of the bis-phosphomimic to cTnI-R21C system (Fig. 10C), there was little change in fluctuation for the contacts compared with the cTnI-R21C or WT systems. Together with the contact information for the cTnI intra-subunit interaction (Fig. 9), our MD simulations suggest that phosphorylation of Ser-23/Ser-24 cTnI results in intra-subunit interaction of the cTnI N terminus with the inhibitory peptide, which reduces stability of cTnI switch peptide contacts with the cTnC hydrophobic patch, and that both the R21C and R146G cTnI mutations abrogate this action. In turn, this suggests a potential structure-based mechanism of how these mutations impair PKA regulation of contraction and relaxation.

Discussion

In this study, we tested whether HCM-associated mutations located in either the N terminus or inhibitory peptide of cTnI may disrupt the formation of an interaction between these regions that occurs with Ser-23/Ser-24 phosphorylation by PKA, thus blunting the regulatory effects on cTnI that normally occur during β-adrenergic stimulation. We report here the effects of cTnI<sup>R146G</sup> or cTnI<sup>R21C</sup>±PKA phosphorylation (or bis-phosphomimic substitutions) on K<sub>C</sub>, the contractile properties of isolated rat LV cardiac myofibrils, and the whole troponin structure and dynamics changes. The most significant findings of the current study were as follows: 1) Both mutations
significantly increased $K_{Ca}$ and $K_{C-I}$ compared with the cTnI<sup>WT</sup>. However, although PKA phosphorylation of cTn resulted in a similar reduction of $K_Ca$ for WT and both mutant-containing cTn complexes, the reduction in $K_{C-I}$ seen for cTnI<sup>WT</sup> was eliminated for both mutations. 2) $T_{\text{max}}$ was maintained for both cTnI<sup>R146G</sup>- and cTnI<sup>R21C</sup>-exchanged myofibrils, and the Ca$^{2+}$ sensitivity of tension ($pC_{50}$) was left-shifted. However, although PKA phosphorylation (or bis-phosphomimic substitutions) decreased $pC_{50}$ (0.2 $pC$ units) for WT myofibrils, this effect was blunted for both mutations. 3) PKA phosphorylation of WT myofibrils accelerated the early slow phase relaxation, especially during the sub-maximal Ca$^{2+}$ levels that heart operates in vivo, but most importantly, this effect was blunted for both cTnI<sup>R146G</sup>- and cTnI<sup>R21C</sup>-exchanged myofibrils. 4) MD simulations suggest the mechanism by which cTnI<sup>R146G</sup> and cTnI<sup>R21C</sup> blunt PKA-mediated reduction of $K_{C-I}$, Ca$^{2+}$ sensitivity of tension, and the early phase of relaxation is inhibition of the formation of an intra-subunit
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![Difference contact map of residue-residue pairs between 14 hydrophobic NcTnC residues and the switch peptide of cTnI mostly affected upon introduction of mutation or the phosphomimic mutations. The 14 hydrophobic residues of NcTnC are (from left to right): 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. Color green (value 0) reflects no difference between the two systems; the red end of the spectrum (values above 0) reflects more contacts in WT, WT, or R21C model.](image)

interaction between the N terminus and the inhibitory peptide of cTnI. This is consistently seen for WT-cTn with introduction of the bis-phosphomimetic substitutions of cTnI (S23D/S24D).

**Effects of Mutations on Contractile Activation and Relaxation Kinetics**—Previous studies to determine the effects of cTnI\(^{R146G}\) on the maximal tension production and the Ca\(^{2+}\) sensitivity of tension generation in the cardiac muscle have produced complex and sometimes contradictory results. For example, Takahashi-Yanaga *et al.* (19) reported that the cTnI\(^{R146G}\) resulted in an increase in the Ca\(^{2+}\) sensitivity of force generation and myofilibrillar ATPase activity in skinned muscle fibers. In contrast, using the reconstituted actin-tropomyosin-activated myosin ATPase assay, Lang *et al.* (14), Takahashi-Yanaga *et al.* (19), and Elliot *et al.* (18) reported that cTnI\(^{R146G}\) decreases the maximal ATPase in the presence of Ca\(^{2+}\) and reduces inhibition of actomyosin ATPase activity in the absence of Ca\(^{2+}\). Using the human cTnI\(^{R146G}\)- exchanged into murine myofibrils, Kruger *et al.* (21) reported no change in the Ca\(^{2+}\) sensitivity of tension development; however, a slightly decreased Ca\(^{2+}\) sensitivity was detected in the myofibrils from transgenic cTnI\(^{R146G}\) mice. In this study, compared with WT cTn-exchanged myofibrils, \(T_{max}\) was maintained for cTnI\(^{R146G}\), and cTnI\(^{R21C}\)- exchanged myofibrils, and the Ca\(^{2+}\) sensitivity of tension (\(p_{Ca_{50}}\)) was left-shifted by 0.13 and 0.10 \(p_{Ca}\) units, respectively. This agrees with the steady-state fluorescence measurements that showed both mutations increased \(K_{Ca}\). To understand the structural basis of this, we monitored the time evolution of distances between the Ca\(^{2+}\) ion (site II) and its six coordinating residues over the course of multiple 150-ns MD simulations and found that Ser-69 coordination with Ca\(^{2+}\) was increased with the cTnI\(^{R21C}\) mutation (Fig. 7), in agreement with our previous report for simulations with cTn containing cTnI\(^{R146G}\) (25). This may explain how the Ca\(^{2+}\) sensitivity of contractile activation is left-shifted for both cTnI\(^{R146G}\) and cTnI\(^{R21C}\), in accordance with previous studies on cTnI mutations where Ca\(^{2+}\) binding at site II was stabilized (36).

To better determine how both mutations affect thin filament activation and cross-bridge kinetics, we compared the rapid release-restretch protocol (\(k_{tr}\)) with the Ca\(^{2+}\)-activation protocol (\(k_{act}\)) during the same activation trial. Here, \(k_{act}\) was faster than \(k_{tr}\) for cTnI\(^{WT}\), cTnI\(^{R146G}\), and cTnI\(^{R21C}\)- exchanged rat cardiac myofibrils at all Ca\(^{2+}\) levels we tested, indicating that thin filament activation was rate-limiting for tension generation of the rat myofibrils at 15 °C. This finding is consistent with our previous work in rodent hearts (7, 47) but different from several other references that reported no difference between \(k_{act}\) and \(k_{tr}\) (62, 66). As we have previously discussed (47), the presence of 0.5 mM Pi in our solutions (a level of Pi that is close to the physiological level of Pi present in the heart) can explain this difference, and when a phosphate “mop” is used in activation solutions, the difference is eliminated. The presence of Pi influences the cross-bridge tension generating isomerization specifically, without affecting thin filament activation kinetics (47). Our current results confirm those previous findings in rodents and extend them by demonstrating that a similar effect occurs at both maximal and sub-maximal Ca\(^{2+}\) levels with introduction of either of these putative HCM-associated cTnI mutations.

Rapid, complete Ca\(^{2+}\) removal from cardiac myofibrils (Fig. 3) by a rapid solution switching protocol induces two distinct relaxation phases (biphasic), starting with an initial early slow phase of relaxation and followed by a more rapid (fast) relaxation phase. During the slow phase, isometric conditions are maintained in sarcomeres and the force decays with a linear constant rate, indicating that \(k_{rel,slow}\) primarily reflects the
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Efforts of PKA Phosphorylation on Contractile Activation and Relaxation Kinetics—β-Adrenergic stimulation is a major physiological mechanism to meet the increase in circulatory demand, acting through positive inotropic and lusitropic effects (6). For cTnI, β-adrenergic stimulation results in the phosphorylation of Ser-23/Ser-24 of cTnI by PKA (6). Considering its key role in the heart performance, we studied how both PKA phosphorylation (or with bis-phosphomimic substitutions), the phosphorylation-mediated effects on speed relaxation of the contractile apparatus. To investigate whether “forced phosphorylation” could overcome this, we studied bis-phosphomimic substitutions S23D/S24D of cTnIWT. Our results indicated that even with bis-phosphomimic substitutions, the phosphorylation-mediated effects on speed relaxation (and myofibril relaxation) were still blunted, suggesting that the cTnIR21C mutation per se results in the cardiac dysfunction of modulation by phosphorylation, similar to our results for the cTnIR146G mutation. Considering the similar results for cTnIR146G and cTnIR21C (with/without PKA phosphorylation or bis-phosphomimic substitutions) in both solution biochemistry and myofibril kinetics/mechanics measurements, we wanted to compare their phenotypes in humans, but unfortunately the data are rather sparse. An affected individual with the R145G mutation had ventricular hypertrophy characteristic of severe HCM (12). The missense mutation R21C has been identified in at least two families (69). In one family, a patient had apical hypertrophy after presenting with atrial fibrillation. The patient’s father, three siblings, and an 18-year-old daughter all succumbed to sudden cardiac death. The clinical evaluations of three surviving mutation carriers from this family revealed that one had asymmetrical septal hypertrophy; another had isolated left atrial enlargement, and the third one had normal cardiac dimensions despite an abnormal electrocardiogram. Another family was recently also identified with the R21C mutation. This family has four members with subaortic asymmetrical turnover kinetics of cross-bridge cycling, dominated by the detachment rate (62, 66). The duration of the initial slow phase of relaxation \( t_{rel, slow} \) depends on the Ca\(^{2+}\) activation levels and can be influenced by the Ca\(^{2+}\) binding and, likely, the cTnC-cTnI interaction properties of cTnI. Here, compared with WT myofibrils, \( k_{act, slow} \) was maintained, whereas \( t_{rel, slow} \) was prolonged upon introduction of either cTnIR146G or cTnIR21C mutations at both maximal and submaximal Ca\(^{2+}\) levels. Consistent with previous observations (7), \( k_{act, slow} \) also accelerated at sub-maximal Ca\(^{2+}\) level for all conditions. During submaximal Ca\(^{2+}\) activations, there is less Ca\(^{2+}\) binding to thin filaments (troponin) at any given time, so that it is easier for the thin filament to become deactivated when myosins detach. Moreover, the presence of 0.5 mM P, in our study should exacerbate this effect, as it results in a reduction of the tension-bearing cross-bridges. This may accelerate the detachment of myosin cross-bridges from the thin filament, thus contributing to an increase in the slow phase of relaxation at submaximal Ca\(^{2+}\) levels.

Effects of PKA Phosphorylation on Contractile Activation and Relaxation Kinetics—β-Adrenergic stimulation is a major physiological mechanism to meet the increase in circulatory demand, acting through positive inotropic and lusitropic effects (6). For cTnI, β-adrenergic stimulation results in the phosphorylation of Ser-23/Ser-24 of cTnI by PKA (6). Considering its key role in the heart performance, we studied how both mutations influence the PKA responsiveness. With PKA phosphorylation (or bis-phosphomimic substitutions), \( T_{max} \) did not differ for cTnIWT, cTnIR146G-, and cTnIR21C-exchanged myofibrils. However, although PKA phosphorylation (or with bis-phosphomimetic substitutions) right-shifted \( p_{Ca50} \) (∼0.2 pCa units) for WT myofibrils, this effects was blunted for both mutations (Fig. 4, A and D). In addition, during the maximal Ca\(^{2+}\) activation, the ratio of \( k_{act}/k_{rel} \) decreased from 0.67 ± 0.03 to 0.48 ± 0.05 for WT myofibril upon PKA treatment (or to 0.45 ± 0.05 with introduction of the bis-phosphomimetic substitutions), suggesting a slowed thin filament activation process with PKA phosphorylation. Interestingly, \( k_{act}/k_{rel} \) did not differ with PKA phosphorylation (or bis-phosphomimetic substitutions) in the presence of either cTnIR146G or cTnIR21C, suggesting the slowing of thin filament activation by PKA-mediated cTnI phosphorylation during maximal Ca\(^{2+}\) conditions is blunted. Consistent with previous work in our laboratory (7) and by others (6, 9), we demonstrated that PKA treatment (or with bis-phosphomimetic substitutions) increased the speed of the slow phase of relaxation for WT myofibrils, especially at the submaximal Ca\(^{2+}\) levels that heart operates during a cardiac twitch. Most importantly, we found that this effect of PKA on slow phase relaxation was eliminated (blunted) for both mutations.

It is important to point out that the conditions for PKA phosphorylation and introduction of bis-phosphomimetic substitutions (S23D/S24D) are different in vivo, because cMyBP-C and titin are also targets for PKA phosphorylation during β-adrenergic stimulation (67, 68). So, to determine the specific effect on cTnI phosphorylation, we exchanged recombinant cTnI containing cTnIS23D/S24D into cardiac myofibrils. In steady-state fluorescence studies, we found that cTnIS23D/S24D and PKA-mediated phosphorylation of cTnI (cTnISer(P)-23/Ser(P)-24) resulted in an almost identical effect, a right shift of the \( K_{C-I} \) and \( K_{Ca} \) curves (Fig. 3) compared with cTnIWT. The \( p_{Ca50} \) shift was −0.31 and −0.29 pCa units for the cTn complex containing cTnISer(P)-23/Ser(P)-24 versus cTnIS23D/S24D, respectively. We also saw very similar results on the modulation of thin filament activation and myofibril relaxation for PKA-phosphorylated cTnIWT-exchanged myofibrils and cTnIS23D/S24D-exchanged myofibrils. After treating the cTnIWT-exchanged myofibrils with PKA, \( T_{max} \) was maintained and \( p_{Ca50} \) was right-shifted 0.2 pCa units. Similarly, \( T_{max} \) was maintained, and \( p_{Ca50} \) was also decreased (0.21 pCa units) for the cTnIS23D/S24D-exchanged myofibrils. For relaxation, both PKA treatment of cTnIWT-exchanged myofibrils and cTnIS23D/S24D-exchanged myofibrils significantly increased the rate and decreased the duration of early slow phase relaxation, especially at sub-maximal Ca\(^{2+}\) levels where the heart operates. All the above findings suggest similar functional effects of PKA phosphorylation and cTnIS23D/S24D substitutions in our systems. In this study, the PKA-phosphorylated cTnIR146G data were compared with PKA-phosphorylated cTnIWT sets, and the cTnIR21C/S23D/S24D results were compared with cTnIS23D/S24D sets. Considering the same (similar) functional effects of PKA phosphorylation and cTnIS23D/S24D substitutions, we decided it was fair to compare the PKA-phosphorylated cTnIR146G with the cTnIR21C/S23D/S24D data.

We and others (27, 28) have demonstrated that cTnIR21C disrupts PKA phosphorylation of Ser-23/Ser-24 on cTnI, and this abrogates the effect of β-adrenergic stimulation on cTnI regulation of contraction and relaxation. This may be the actual physiological/pathogenic mechanism of cTnIR21C, where normally PKA-mediated phosphorylation of Ser-23/Ser-24 would speed relaxation of the contractile apparatus. To investigate whether “forced phosphorylation” could overcome this, we studied bis-phosphomimic substitutions S23D/S24D of cTnIR21C. Our results indicated that even with bis-phosphomimic substitutions, the phosphorylation-mediated effects on \( K_{C-I} \) and myofibril relaxation were still blunted, suggesting that the cTnIR21C mutation per se results in the cardiac dysfunction of modulation by phosphorylation, similar to our results for the cTnIR146G mutation. Considering the similar results for cTnIR146G and cTnIR21C (with/without PKA phosphorylation or bis-phosphomimic substitutions) in both solution biochemistry and myofibril kinetics/mechanics measurements, we wanted to compare their phenotypes in humans, but unfortunately the data are rather sparse. An affected individual with the R145G mutation had ventricular hypertrophy characteristic of severe HCM (12). The missense mutation R21C has been identified in at least two families (69). In one family, a patient had apical hypertrophy after presenting with atrial fibrillation. The patient’s father, three siblings, and an 18-year-old daughter all succumbed to sudden cardiac death. The clinical evaluations of three surviving mutation carriers from this family revealed that one had asymmetrical septal hypertrophy; another had isolated left atrial enlargement, and the third one had normal cardiac dimensions despite an abnormal electrocardiogram. Another family was recently also identified with the R21C mutation. This family has four members with subaortic asymmetrical...
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hypothesis and one mutation carrier with normal cardiac dimensions who had to be resuscitated from sudden death. Thus the sparse amount of data available suggest human phenotypes for both mutations may be similar, but more information is needed to conclude this.

To understand the structural molecular level basis of how phosphorylation of cTnI residues Ser-23/Ser-24 changes the behavior of whole cTn complex and thus results in the changes in cardiac function, we performed paralleled MD simulations on WT, cTnIR146G, and cTnIR21C containing cTn and found that introduction of the bis-phosphomimetic substitutions significantly altered the cTnC-cTnI interactions, particularly in the inhibitory-switch peptide regions (8). The most significant finding is that there were no intra-subunit interactions in the WT model in the absence of phosphorylation, but introduction of the bis-phosphomimetic substitutions (S23D/S24D) for the WT model led to the formation of an intra-subunit interaction between the N terminus and the inhibitory peptide regions of cTnI (Fig. 9B) (8). This intra-subunit interaction has been suggested by Solaro and co-workers (11, 30, 32) based on solution biochemical and spectroscopic studies (31). In addition, we also compared our simulation results of bis-phosphomimic cTnI with biochemical studies from other laboratories (70–72). Dong et al. (70) found that bis-phosphorylation resulted in a reduction of the axial ratio of cTnI and the formation of a more compact structure upon phosphorylation using fluorescence studies. Heller et al. (71) reported that bis-phosphorylation induced a dramatic bending of the rod-like cTnI at the N-terminal extension that binds with cTnC, resulting in a significant decrease in the axial ratio of cTnI and the cTn complex overall. Reiffert et al. (72) used surface plasmon resonance to determine that the shape of cTnI changed from an asymmetrical structure to a more symmetrical one upon phosphorylation, which is consistent with the bending that results in a shorter and broader structure. Our simulations also suggest a bending at the N-terminal extension of cTnI and a more compact cTnI structure upon phosphorylation that is consistent with these previous biochemical studies. We further speculate that this intra-subunit interaction may subsequently weaken interactions of the cTnI switch peptide with NcTnC, as demonstrated by increased fluctuation of contacts in MD simulations (Fig. 10B). This would allow stronger interaction between the inhibitory peptide of cTnI and actin and move the equilibrium toward thin filament activation. Interestingly, this intra-subunit interaction no longer formed in simulations with introduction of the cTnIR146G or cTnIR21C to the cTnI complex, demonstrating that both mutations blunted the ability of cTnI phosphorylation to reposition the N-terminal extension to interact the inhibitory peptide region. These findings suggest a structural mechanism that can explain the loss of PKA-mediated modulation of thin filament activation and relaxation of myofibrils that need to occur with increasing heart rate during β-adrenergic stimulation and increased physical activity.

An important caution of using our simulations to explain our experimental data is that recombinant cTnI subunit proteins were made from rat sequences, although the MD simulations were based on the human sequence. For cTnI, there is only one amino acid difference (Ile-119 in human and Met-119 in rat), which is a conservative substitution. For cTnT, there is also only one amino acid difference (Phe-251 in human and His-251 in rat) in the portion included in our computational model (residues 236–285), and this is also a conservative substitution. For cTnC, there are total 13 variants in the portion used for our computational model (residues 1–172). Four of those are located in the N terminus of cTnI (Gly-4, Arg-10, Arg-13, and Ile-19 in human and Glu-4, Gly-10, Glu-13, and Val-19 in rat). One variant is located in the switch peptide of cTnI (Ala-161 in human and Thr-162 in rat). All the other variants are resided in the I-T arm region of cTnI (Leu-53, Leu-61, Ala-75, Glu-84, Ala-86, Ala-91, Ile-114, and Phe-133 in human and Met-54, Met-62, Leu-76, Val-85, Asp-87, Glu-92, Val-115, and Tyr-134 in rat). Among them, G4E, R10G, E84V, A86D, and A91E substitutions change the electrostatic charge properties and size of the amino acid; R13Q changes the charge property of the amino acid; A75V changes the size of the amino acid; and, I19V, L53M, L61M, L114V, F133Y, and A161T are conservative substitutions. There is no evidence to suggest these variants alter structure-function of the cTnI complex, but this has not been studied in any detail. None of these positions has been reported to be associated with disease, which supports the idea that these sequence variants among the two species have little or no effects on the function. Additionally, most results of ATPase assays and the Ca2+-force relationship show consistent results between rodents and human. Some minor differences have been attributed to the different myosin isoforms between the two species. For these reasons, we think it is fair to compare the in vitro findings with the computational results. In the future, it would be interesting to perform the in vitro study based on the human sequence (and/or also conduct the computational modeling based on the rodent sequence) and to compare these results with our current findings to further investigate the disease-unrelated variants among species.

Author Contributions—M. R., J. A. M., and A. D. M conceived this study. Y. C. and V. R. designed the experiment; Y. C., V. R., A. T., D. W., and L. O. performed the experiment and analyzed the data; Y. C. and S. L. built up the computational model and performed the computational study; Y. C. and M. R. wrote the initial draft of the paper. All authors reviewed, edited, and approved the manuscript.

Acknowledgments—We thank Dr. Charles Luo and Luping Xie for preparations of cTnI mutant proteins and protein isolation and Drs. Maria Razumova and Galina Flint for the assistance with rat tissue and solution preparation. We appreciate Drs. John Solaro and Paul Rosevear for structural information of the cTnI N-terminal extension. We appreciate Prof. Rommie Amaro and Dr. Peter Kekenes-Huskey for the support of this work, and Dr. Jordan Klaiman for helpful suggestions on writing the article. We are indebted to Martha Mathiason for the development of data acquisition and analysis software.

References
1. Seidman, C. E., and Seidman, J. G. (2011) Identifying sarcomere gene mutations in hypertrophic cardiomyopathy, a personal history. Circ. Res. 108, 743–750
2. Willott, R. H., Gomes, A. V., Chang, A. N., Parvatiyar, M. S., Pinto, J. R., and Potter, J. D. (2010) Mutations in troponin that cause HCM, DCM, and RCM: what can we learn about thin filament function? J. Mol. Cell. Car
1. Gordon, A. M., Hornscher, E., and Regnier, M. (2000) Regulation of contraction in striated muscle. Physiol. Rev. 80, 853–924

2. Sia, S. K., Li, M. X., Spyraopoulos, L., Gagné, S. M., Liu, W., Putkey, J. A., and Sykes, B. D. (1997) Structure of cardiac muscle troponin C unexpectedly reveals a closed regulatory domain. J. Biol. Chem. 272, 18216–18221

3. Spyraopoulos, L., Li, M. X., Sia, S. K., Gagné, S. M., Chandra, M., Solaro, R. J., and Sykes, B. D. (1997) Calcium-induced structural transition in the regulatory domain of human cardiac troponin C. Biochemistry 36, 12138–12146

4. Zhang, R., Zhao, J., Mandveno, A., and Potter, J. D. (1995) Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. Circ. Res. 76, 1028–1035

5. Rao, V., Cheng, Y., Lindert, S., Wang, D., Oxenford, L., McCulloch, A. D., McCammon, J. A., and Regnier, M. (2014) PKA phosphorylation of cardiac troponin I modulates activation and relaxation kinetics of ventricular myofibrils. Biophys. J. 107, 1196–1204

6. Cheng, Y., Lindert, S., Kekenes-Huskey, P., Rao, V. S., Solaro, R. J., Rosevear, P. R., Amaro, R., McCulloch, A. D., McCammon, J. A., and Regnier, M. (2014) Computational studies of the effect of the S23D/S24D troponin I mutation on cardiac troponin structure dynamics. Biophys. J. 107, 1675–1685

7. Kentish, J. C., McCloskey, D. T., Layland, J., Palmer, S., Leiden, J. M., Martin, A. F., and Solaro, R. J. (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and cross-bridge cycle kinetics in mouse ventricular muscle. Circ. Res. 88, 1059–1065

8. Chandra, M., Ding, W.-J., Pan, B.-S., Cheung, H. C., and Solaro, R. J. (1997) Effects of protein kinase A phosphorylation on signaling between cardiac troponin I and the N-terminal domain of cardiac troponin C. Biochemistry 36, 13305–13311

9. Solaro, R. J., Rosevear, P., and Kobayashi, T. (2008) The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. Biochem. Biophys. Res. Commun. 369, 82–87

10. Kimura, A., Harada, H., Park, J. E., Nishi, H., Satoh, M., Takahashi, M., Hiroi, S., Sasaoka, T., Ohbuchi, N., Nakamura, T., Koyanagi, T., Hwang, T. H., Choo, J. A., Chung, K. S., Hasegawa, A., et al. (1997) Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. Nat. Genet. 16, 379–382

11. Deng, Y., Schmidtmann, A., Redlich, A., Westerdorf, B., Jaquet, K., and Thieleczek, R. (2001) Effects of phosphorylation and mutation R145G on human cardiac troponin I function. Biochemistry 40, 14593–14602

12. Lang, R., Gomes, A. V., Zhao, J., Housmans, P. R., Miller, T., and Potter, J. D. (2002) Functional analysis of a troponin I (R145G) mutation associated with familial hypertrophic cardiomyopathy. J. Biol. Chem. 277, 11670–11678

13. Lindhout, D. A., Li, M. X., Schieve, D., and Sykes, B. D. (2002) Effects of T142 phosphorylation and mutation R145G on the interaction of the inhibitory peptide region of cardiac troponin I with the C-domain of human cardiac troponin C. Biochemistry 41, 7267–7274

14. Westfall, M. V., Borton, A. R., Albaya, F. P., and Metzger, J. M. (2002) Myofilament calcium sensitivity and cardiac disease—insights from troponin I isoforms and mutants. Circ. Res. 91, 525–531

15. Reis, S., Littwitz, C., Preilowski, S., Mühge, A., Stienen, G. J., Pott, L., and Jaquet, K. (2008) Expression of cTnI/R145G affects shortening properties of adult rat cardiomyocytes. Pflügers Arch. 457, 17–24

16. Elliott, K., Watkins, H., and Redwood, C. S. (2000) Altered regulatory properties of human cardiac troponin I mutants that cause hypertrophic cardiomyopathy. J. Biol. Chem. 275, 22069–22074

17. Takahashi-Yanaga, F., Morimoto, S., Harada, K., Minakami, R., Shiraishi, F., Ohta, M., Lu, Q. W., Sasaguri, T., and Ohtsuki, I. (2001) Functional consequences of the mutations in human cardiac troponin I gene found in familial hypertrophic cardiomyopathy. J. Mol. Cell. Cardiol. 33, 2095–2107

18. Wang, D., Robertson, I. M., Lin, M. X., McCully, M. E., Crane, M. L., Luo, Z., Tu, A.-Y., Daggett, V., Sykes, B. D., and Regnier, M. (2012) Structural and functional consequences of the cardiac troponin C L48Q Ca²⁺-sensitizing mutation. Biochemistry 51, 4473–4487

19. Dong, W.-J., Xing, J., Chandra, M., Solaro, J., and Cheung, H. C. (2000) Structural mapping of single cysteine mutants of cardiac troponin I. Pro.
R146G and R21C CtnI Disrupt PKA Modulation of Contraction

teins. 41, 438–447
38. Potter, J. D. (1982) Preparation of troponin and its subunits. Methods Enzymol. 85, 241–263
39. Dong, W.-J., Robinson, J. M., Stagg, S., Xing, I., and Cheung, H. C. (2003) Ca$^{2+}$-induced conformational transition in the inhibitory and regulatory regions of cardiac troponin I. J. Biol. Chem. 278, 8686–8692
40. Wang, D., McCully, M. E., Luo, Z., McMichael, J. T., A. Y.-A., Daggett, V., and Regnier, M. (2013) Structural and functional consequences of cardiac troponin C L57Q and I61Q Ca$^{2+}$-desensitizing variants. Arch. Biochem. Biophys. 535, 68–75
41. Patton, C., Thompson, S., and Epel, D. (2004) Some precautions in using chelators to buffer metals in biological solutions. Cell Calcium 35, 427–431
42. George, S. E., Su, Z., Fan, D., Wang, S., and Johnson, J. D. (1996) The fourth EF-hand of calmodulin and its helix-loop-helix components: impact on calcium binding and enzyme activation. Biochemistry 35, 8307–8313
43. Adhikari, B. B., Regnier, M., Rivera, A. J., Kreutziger, K. L., and Martyn, D. A. (2004) Cardiac length dependence of force and force redevelopment kinetics with altered cross-bridge cycling. Biophys. J. 87, 1784–1794
44. Regnier, M., Rivera, A. J., Chen, Y., and Chase, P. B. (2000) 2-Deoxy-ATP enhances contractility of rat cardiac muscle. Circ. Res. 86, 1211–1217
45. Brenner, B. and Eisenberg, E. (1986) Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. Proc. Natl. Acad. Sci. U.S.A. 83, 3542–3546
46. Fabiato, A. (1988) Computer programs for calculating total free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. Methods Enzymol. 157, 378–417
47. Kreutziger, K. L., Piroddi, N., McMichael, J. T., Tesi, C., Poggesi, C., and Regnier, M. (2011) Calcium binding kinetics of troponin C strongly modulate cooperative activation and tension kinetics in cardiac muscle. J. Mol. Cell. Cardiol. 50, 165–174
48. Colomo, F., Piroddi, N., Poggesi, C., te Kronnie, G., and Tesi, C. (1997) Active and passive forces of isolated myofibrils from cardiac and fast skeletal muscle of the frog. J. Physiol. 500, 555–548
49. Tesi, C., Colomo, F., Nencini, S., Piroddi, N., and Poggesi, C. (2000) The effect of inorganic phosphate on force generation in single myofibrils from rabbit skeletal muscle. Biophys. J. 78, 3081–3092
50. Kreutziger, K. L., Piroddi, N., Scellini, B., Tesi, C., Poggesi, C., and Regnier, M. (2008) Thin filament Ca$^{2+}$-binding properties and regulatory unit interactions alter kinetics of tension development and relaxation in rabbit skeletal muscle. J. Physiol. 586, 3683–3700
51. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Structure of the core domain of human cardiac troponin C in the Ca$^{2+}$-saturated form. Nature 424, 35–41
52. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics. J. Molec. Graph. 14, 33–38
53. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulation liquid water. J. Chem. Phys. 79, 926–935
54. Phillips, J. C., Braun, R., Wang, W., Gambart, J., Tjakhovshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. J. Comput. Chem. 26, 1781–1802
55. MacKerell, A. D., Jr., Banavali, N., and Fopolpe, N. (2000) Development and current status of the CHARMM force field for nucleic acids. Biopolymers 56, 257–265
56. Ryckaert, J.-P., Cicotti, G., and Berendsen, H. J. C. (1977) Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. J. Comput. Phys. 23, 327–341
57. Rao, V. S., Korte, F. S., Razumova, M. V., Feest, E. R., Hsu, H., Irving, T. C., Regnier, M., and Martyn, D. A. (2013) N-terminal phosphorylation of cardiac troponin-I reduces length-dependent calcium sensitivity of contraction in cardiac muscle. J. Physiol. 591, 475–490
58. Finley, N., Abbott, M. B., Abusamhadneh, E., Gaponenko, V., Dong, W., Gasmi-Seabrook, G., Howarth, J. W., Rance, M., Solaro, R. J., Cheung, H. C., and Rosevear, P. R. (1999) NMR analysis of cardiac troponin C-troponin I complexes: effects of phosphorylation. FEBS Lett. 453, 107–112
59. Sakhivel, S., Finley, N. L., Rosevear, P. R., Lorenz, J. N., Gulick, J., Kim, S., VanBuren, P., Martin, L. A., and Robbins, J. (2005) In vivo and in vitro analysis of cardiac troponin I phosphorylation. J. Biol. Chem. 280, 703–714
60. Dohet, C., al-Hillawi, E., Trayer, I. P., and Rüegg, J. C. (1995) Reconstitution of skinned cardiac fibres with human recombinant cardiac troponin-I mutants and troponin-C. FEBS Lett. 377, 131–134
61. Takimoto, E., Soergel, D. G., Janssen, P. M., Stull, L. B., Kass, D. A., and Murphy, A. M. (2004) Frequency- and afterload-dependent cardiac modulation in vivo by troponin I with constitutively active protein kinase a phosphorylation sites. Circ. Res. 94, 496–504
62. Poggesi, C., Tesi, C., and Stehle, R. (2005) Sarcomeric determinants of striated muscle relaxation kinetics. Pflugers Arch. 449, 505–517
63. Kress, M., Huxley, H. E., Faruqui, A. R., and Hendrix, J. (1986) Structural changes during activation of frog muscle studied by time-resolved x-ray diffraction. J. Mol. Biol. 188, 325–342
64. Huxley, A. F., and Simmons, R. M. (1970) Rapid ‘give’ and the tension ‘shoulder’ in the relaxation of frog muscle fibres. J. Physiol. 210, 32P–33P
65. Luo, Y., Davis, J., Tikunova, S. B., Smillie, L. B., and Rall, J. A. (2003) Myofibrillar determinants of rate of relaxation in skinned skeletal muscle fibers. Adv. Exp. Med. Biol. 538, 573–582
66. Tesi, C., Piroddi, N., Colomo, F., and Poggesi, C. (2002) Relaxation kinetics following sudden Ca$^{2+}$ reduction in single myofibrils from skeletal muscle. Biophys. J. 83, 2142–2151
67. Colson, B. A., Bekyarova, T., Locher, M. R., Fitzsimons, D. P., Irving, T. C., and Moss, R. L. (2008) Protein kinase A-mediated phosphorylation of cMyBP-C increases proximity of myosin heads to actin in resting myocardium. Circ. Res. 103, 244–251
68. Yamasaki, R., Wu, Y., McNabb, M., Greaser, M., Labeit, S., and Granzier, H. (2002) Protein kinase A phosphorylates Titin’s cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. Circ. Res. 90, 1181–1188
69. Arad, M., Penas-Lado, M., Monserrat, L., Maron, B. J., Sherrid, M., Ho, C. Y., Barr, S., Karim, A., Olson, T. M., Kaminso, M., Seidman, J. G., and Seidman, C. E. (2005) Gene mutations in apical hypertrophic cardiomyopathy. Circulation 112, 2805–2811
70. Dong, W. J., Chandra, M., Xing, J., She, M., Solaro, R. J., and Cheung, H. C. (1997) Phosphorylation-induced distance change in a cardiac muscle troponin I mutant. Biochemistry 36, 6754–6761
71. Heller, W. T., Finley, N. L., Dong, W. J., Timmins, P., Cheung, H. C., Rosevear, P. R., and Trewella, J. (2003) Small-angle neutron scattering with contrast variation reveals spatial relationships between the three sub-units in the ternary cardiac troponin complex and the effects of troponin I phosphorylation. Biochemistry 42, 7790–7800
72. Reifert, S. U., Jaquet, K., Hellmeyer, L. M., Jr., and Herberg, F. W. (1998) Stepwise subunit interaction changes by mono- and bisphosphorylation of cardiac troponin I. Biochemistry 37, 13516–13525