Challenging Current Paradigms Related to Cardiomyopathies

ARE CHANGES IN THE Ca2+ SENSITIVITY OF MYOFILAMENTS CONTAINING CARDIAC TROПONIN C MUTATIONS (G159D AND L29Q) GOOD PREDICTORS OF THE PHENOTYPIC OUTCOMES?*

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Two novel mutations (G159D and L29Q) in cardiac troponin C (CTnC) associate their phenotypic outcomes with dilated (DCM) and hypertrophic cardiomyopathy (HCM), respectively. Current paradigms propose that sarcomeric mutations associated with DCM decrease the myofilament Ca2+ sensitivity, whereas those associated with HCM increase it. Therefore, we incorporated the mutant CTnCs into skinned cardiac muscle in order to determine if their effects on the Ca2+ sensitivities of tension and ATPase activity coincide with the current paradigms and phenotypic outcomes. The G159D-CTnC decreases the Ca2+ sensitivity of tension and ATPase activation and reduces the maximal ATPase activity when incorporated into regulated actomyosin filaments. Under the same conditions, the L29Q-CTnC has no effect. Surprisingly, changes in the apparent G159D-CTnC Ca2+ affinity measured by tension in fibers do not occur in the isolated CTnC, and large changes measured in the isolated L29Q-CTnC do not manifest in the fiber. These counterintuitive findings are justified through a transition in Ca2+ affinity occurring at the level of cardiac troponin and higher, implying that the true effects of these mutations become apparent as the hierarchical level of the myofilament increases. Therefore, the contractile apparatus, representing a large cooperative machine, can provide the potential for a change (G159D) or no change (L29Q) in the Ca2+ regulation of contraction. In accordance with the clinical outcomes and current paradigms, the desensitization of myofilaments from G159D-CTnC is expected to weaken the contractile force of the myocardiunm, whereas the lack of myofilament changes from L29Q-CTnC may preserve diastolic and systolic function.

Cardiomyopathies are diseases of the myocardium that often lead to cardiac remodeling to compensate for deficiencies in cardiac output (1). In the case of dilated cardiomyopathy (DCM),2 heart failure is characterized by a systolic dysfunction (i.e. reduced ejection fraction), whereas hypertrophic (HCM) and restrictive cardiomyopathies are characterized as having diastolic dysfunctions (i.e. impaired relaxation) (2). In many cases, the cardiac contractile dysfunction is attributed to inherited sarcomeric gene mutations. The functional effects of more than 40 thin filament mutations associated with cardiomyopathies assessed in vitro suggest that these mutations affect the Ca2+-responsiveness of the myofilament in the absence of CTnI phosphorylation. As the number of in vitro-characterized mutations continues to grow, a developing paradigm emerges that associates decreases in the myofilament Ca2+ sensitivity with DCM (3–7) and associates increases with HCM (4, 6–12) and restrictive cardiomyopathy (13–17). This suggests that distinct effects on the Ca2+-dependent processes of the myofilament are critical determinants of the severity and molecular pathologies of these diseases.

The first cardiac troponin C (CTnC) mutation (E59D/D75Y) was found in an explanted heart from an adult male who died from idiopathic DCM (18); the second mutation (L29Q) was found in a living 60-year-old male diagnosed with HCM, despite having preserved diastolic and systolic function (19); and the third (G159D) was found by linkage analysis and cosegregation studies in more than three affected families with DCM (20, 21). The lack of affected relatives and cosegregation studies associated with the L29Q and E59D/D75Y CTnC mutations may or may not confirm these mutations as etiological causes for the diseases; therefore, the functional and biochemical effects of these mutations must be studied in order to predict the physiological consequences of these mutations. As such, our investigations will relate the changes in apparent CTnC Ca2+ affinity to the myofilament response and determine if these mutations coincide with the developing paradigm. The effects of the E59D/D75Y mutation were previously reported to coincide with the aforementioned paradigms (18, 22, 23); there-

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1 The abbreviations used are: DCM, dilated cardiomyopathy; CDTA, trans-1,2-cyclohexane-N,N',N''-tetraacetic acid; CMF, cardiac myofibril (s); CTn, cardiac troponin; CTnIA, cardiac troponin reconstituted from CTnCIA, CTnI, and CTnT; CTnC, cardiac troponin C; CTnC(B4)IA, CTnC with one IAANS molecule covalently attached to Cys48; CTnC(B4), CTnC with two IAANS molecules covalently attached to Cys48; CTnI, cardiac troponin I; CTnT, cardiac troponin T; HCM, hypertrophic cardiomyopathy; IAANS, 2-(4'-iodoacetamido)anilino)-naphtalene-6-sulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; pCa, −log[Ca2+]; RTF, regulated thin filament; Tm, tropomyosin; WT, wild type.
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fore, this paper will focus on the L29Q and G159D CTnC mutations.

Cardiac muscle contraction and relaxation is regulated by the intracellular Ca\(^{2+}\) concentration via the thin filament regulatory protein, CTnC. During systole, the rise in free calcium promotes Ca\(^{2+}\) binding to CTnC. This leads to the translocation of CTnI and tropomyosin (Tm) away from the outer domain of the actin filaments, allowing for subsequent cross-bridge interaction and the generation of tension. With the decline of Ca\(^{2+}\) during diastole, Ca\(^{2+}\) dissociates from CTnC, and the inhibitory actions of CTnI and Tm are restored (see Refs. 24 and 25 for a review). CTnC contains three metal ion binding sites: two C-terminal sites that bind Ca\(^{2+}\) and Mg\(^{2+}\) ("Ca\(^{2+}\) construct") competitively (\(K_{Ca} = 1.4 \times 10^{-7}\ \text{M}^{-1}\); \(K_{Mg} \approx 1.0 \times 10^{-5}\ \text{M}^{-1}\)) and one N-terminal, Ca\(^{2+}\)-specific regulatory site (\(K_{Ca} = 2.5 \times 10^{-5}\ \text{M}^{-1}\)) that is responsible for transmitting the Ca\(^{2+}\) binding signal to the rest of the thin filament and switching on contraction (26, 27).

The effects of the L29Q and G159D mutations are of particular interest, because previous studies show that the apparent regulatory site Ca\(^{2+}\) afﬁnity (as measured by tension and ATPase activity) is unaffected unless CTnI (Ser\(^{25}\)/Ser\(^{24}\)) is phosphorylated (28–30). These studies identiﬁed a blunted protein kinase A-phosphorylated response as an underlying molecular pathology for these mutations. However, these ﬁndings represe nt challenges to the developing paradigm, because in the presence of phosphorylated CTnI, the myoﬁlament Ca\(^{2+}\) afﬁnities are similarly affected for both mutations yet lead to different effects on the structural opening of the CTnC N-lobe (31) and phonotypic outcomes (19, 20). In addition, the clinical data from the only L29Q proband results in a seemingly benign phenotype coinciding with the effects of other DCM-associated thin ﬁlament mutations (6, 34–36). Surprisingly, the isolated G159D mutation signiﬁcantly decreases the Ca\(^{2+}\) decline (30), increase (32), or not affect (33) the myoﬁlament Ca\(^{2+}\) afﬁnity (as measured by IAANS ﬂuorescence) are seemingly unaffected, possibly explaining the benign Ca\(^{2+}\) sensitivity.

Our results show that in the absence of phosphorylated CTnI, skinned cardiac muscle preparations reconstituted with L29Q CTnC are all unaffected, possibly explaining the benign Ca\(^{2+}\) afﬁnity outcome observed from this mutation. In contrast, the G159D mutation signiﬁcantly decreases the Ca\(^{2+}\) afﬁnities of tension and cardiac myoﬁbrillar ATPase activation, coinciding with the effects of other DCM-associated thin ﬁlament mutations (6, 34–36). Surprisingly, the isolated G159D CTnC structure and apparent regulatory site CA\(^{2+}\) afﬁnity (as measured by IAANS ﬂuorescence) are seemingly unaffected, whereas the structure and Ca\(^{2+}\) afﬁnity of L29Q CTnC are signiﬁcantly altered. The disparity of measurements between the isolated CTnC and skinned muscle have led us to pursue the key intermolecular interactions that dictate the ﬁnal Ca\(^{2+}\) afﬁnity of tension in the muscle ﬁber. Our results show that at the level of CTn, the G159D CTnC structure and Ca\(^{2+}\) afﬁnity deviates from the WT, whereas the L29Q CTnC structure and Ca\(^{2+}\) afﬁnity begin to converge with the WT. Therefore, the contractile apparatus, representing a large cooperative machine, can provide the potential for a change (G159D) or no change (L29Q) in the myoﬁlament Ca\(^{2+}\) afﬁnity if any of the proteins are mutated. Finally, our results show that the changes in myoﬁlament Ca\(^{2+}\) afﬁnity arising from these mutations do coincide with the developing paradigms and are indicative of the phenotypic outcomes.

EXPERIMENTAL PROCEDURES

Mutation, Expression, and Purification of CTnC—The CTnC sequence was derived from a human cardiac cDNA library and subcloned into the pET3-d vector with its sequence veriﬁed. Human CTnC mutants were generated by following Strat- agee’s guidelines for using the QuikChange site-directed mutagenesis kit. All mutant CTnCs were sequenced to verify the correct sequences prior to expression and puriﬁcation. Standard methods previously used in this laboratory were utilized for expression and puriﬁcation of the different CTnC mutants (37).

Fluorescence Labeling of CTnC—Monocysteine CTnC derivatives were engineered using cDNAs previously cloned for G159D, L29Q, and WT CTnC by substituting Cys\(^{35}\) for Ser in order to direct speciﬁc IAANS incorporation to Cys\(^{84}\) (denoted -CTnC\((C84)_{A}\)). The CTnCs were also doubly labeled with IAANS at Cys\(^{35}\) and Cys\(^{84}\) (denoted -CTnC\((C35,84)_{A}\)). Fluorescent incorporation and subsequent puriﬁcation of labeled CTnC followed previous methods (38).

Puriﬁcation of Tropomyosin—Porcine cardiac Tm was puriﬁed from the ammonium sulfate precipitate obtained in the course of native cardiac tropomyosin subunit preparations (39). Brieﬂy, the Tm was puriﬁed by isoelectric precipitation followed by ion exchange chromatography (Q-Sepharose). Puriﬁed Tm was dialyzed against 5 mM NH\(_{4}\)(CO\(_{3}\))\(_{2}\), lyophilized, and stored at −20 °C.

Formation of Cardiac Tropomin Complexes—cDNAs cloned in our laboratory from human cardiac tissue were used for the expression and puriﬁcation of CTnI (40) and CTnT (39). Formation of ﬂuorescent binary and ternary complexes and non-labeled ternary complexes was carried out using recently established protocols (37, 41) in the presence of 1.25 mM Mg\(^{2+}\).

Preparation of IAANS-labeled Regulated Thin Filaments—Regulatory complexes (actin-Tm-CTn) were prepared by mixing F-actin isolated from rabbit skeletal acetone powder (42) and Tm in a 7:1 molar ratio in a solution containing 60 mM KCl, 0.1 mM CaCl\(_{2}\), 2 mM MgCl\(_{2}\), 1 mM ATP, pH 7.0, on ice. The ﬁnal concentration of actin was ~0.8 mg/mL. After homogenization in a glass homogenizer, the F-actin-tropomyosin complex was combined with preformed IAANS-labeled CTn complex (tropomyosin/CTn = 1 mol/mol) in a solution containing 100 mM MOPS, 75 mM KCl, 1.25 mM MgCl\(_{2}\), 2 mM EGTA, 4 mM nitro- triacetic acid (standard buffer conditions for our ﬂuorometric titrations). After homogenization, the mixture was allowed to sit at room temperature for 10 min. The homogenate was centrifuged for 1 h at 150,000 \( \times g \), and the pellet resuspended in standard ﬂuorescence buffer. The solution was centrifuged and resuspended again in order to avoid free Tm-CTn and free CTn trapped in the pellet. Regulated thin ﬁlaments (RTFs) were ﬁltered through a 0.45-µm ﬁlter and stored on ice ready to use for experiments.

Determination of Apparent Ca\(^{2+}\) Affinities—Labeled proteins (isolated CTnC and binary and ternary complex) were dialyzed exhaustively in standard ﬂuorescence buffer (see above). The isolated CTnCs were dialyzed in the absence of...
Mg$^{2+}$ to prevent dimerization (43); therefore, Mg$^{2+}$ was added just before the titration with Ca$^{2+}$. All solutions were prepared at 21 °C. Steady state fluorescence measurements were made with a Jasco 6500 spectrophotometer. IAANS fluorescence was excited at 330 nm, and emission was monitored at 450 nm as incremental amounts of CaCl$_2$ were added to a 2.0-ml mixture containing labeled proteins. The final concentrations of isolated CTnC, binary complex, ternary complex, and RTFs in each experiment were 0.25 μM, 0.5 μM, 0.5 μM, and 0.053 mg/ml, respectively. The concentration of free Ca$^{2+}$ was calculated for actual titration conditions using the computer program pCa Calculator (44). This program corrected for dilution effects attributed to the incremental addition of Ca$^{2+}$. The data were fitted to the Hill equation with the software suite of SigmaPlot 10.0. The Ca$^{2+}$ affinities are reported as pCa$_{50}$ (−log[Ca$^{2+}$] at which 50% of maximal response is observed) values ± S.D. The following modified Hill equation was used to fit biphasic binding curves,

\[
Y = S_1 \cdot \frac{[Ca^{2+}]^n}{(K_{d1}^{n} + [Ca^{2+}]^n)} + S_2 \cdot \frac{[Ca^{2+}]^n}{(K_{d2}^{n} + [Ca^{2+}]^n)} + [Ca^{2+}]^p
\]  

(Eq. 1)

where Y represents the fluorescence intensity, $S_1$ and $S_2$ are the percentage contributions of each class of site to the fluorescence, $K_{d1}$ and $K_{d2}$ are the macroscopic dissociation constants for both classes of sites, and $n_1$ and $n_2$ are the respective Hill coefficients.

Reconstituted Myofilament ATPase Assays—Whole porcine cardiac myosin was isolated from left ventricles (45); F-actin, tropomyosin, and recombinant human CTn were prepared as described above. The ATPase assays were performed in a 96-well flat bottom plate using 0.1-ml reaction volumes. Preformed human cardiac Tn complexes were added (≤20 μl) to the 96 wells to achieve concentrations ranging from 0.0 to 2.0 μM in the final reaction mixture. F-actin, myosin, and tropomyosin were homogenized on ice in a glass tube and allowed to come to room temperature before the addition to each well (≤20 μl). The final concentrations of F-actin, myosin, and Tm in each well were 3.5, 0.6, and 1.0 μM, respectively. Two different ATPase buffers were prepared to adjust the final concentrations of all salts for high (pCa 4) and low (pCa > 9) Ca$^{2+}$ conditions. The remainder of the 0.1-ml reaction volume comprised 50 μl of high or low Ca$^{2+}$ ATPase buffer, double-distilled H$_2$O, and 4 μl of ATP (3.14 mM, pH 7) to initiate the ATPase reaction. Methods for solving the free and bound metal ion equilibria were provided for by the computer program pCa Calculator (44). The final reaction conditions contained 50 mM KCl, 1.0 mM free Mg$^{2+}$, 2.5 mM MgATP$^{2-}$, 1.0 mM dithiothreitol, 20 mM MOPS, 1 mM EGTA (in low Ca$^{2+}$-ATPase buffer) or 0.23 mM CaCl$_2$ (in high Ca$^{2+}$-ATPase buffer), pH 7, at 25 °C. The ATPase reaction was initiated by the addition of ATP in a row-wise fashion and immediately mixed with a 12-channel pipettor 4–5 times and allowed to incubate at 25 °C for 20 min; thereafter, stopping the reaction by adding cold trichloroacetic acid (4.75% final concentration). Each row was activated and deactivated in 30-s intervals. After sedimenting the precipitate by centrifugation, the supernatants were transferred to a new plate, and the liberated inorganic phosphate concentration in the supernatant was determined according to the method of Fiske and Subbarow (46). The ATPase rates were measured by single time points that were predetermined to be linear with time.

Preparation of Skinned Cardiac Myofibers—Porcine cardiac myofibers (CMF) were isolated form porcine left ventricles, as described by Solaro et al. (47), substituting MOPS for imidazole. All CMF preparations were stored in 50% glycerol (v/v) at −20 °C. CDTA treatment of cardiac myofibers were performed by the methods of Morimoto and Ohtsuki (48) with the following modifications; CTnC extraction occurred in 5.0 mM CDTA, 5.0 mM dithiothreitol, pH 8.4, adjusted with Tris-base (solid) for 15 min, followed by centrifugation. This procedure was repeated four more times using 30 min CDTA incubations. CDTA was removed by suspending the pellet in wash buffer containing 10.0 mM MOPS, 10.0 mM KCl, 0.5% glycerol at pH 7 followed by centrifugation. These washes were repeated two more times in the absence of glycerol. CTnC reconstitution was performed by mixing CDTA-treated myofibers (1.5–3.0 mg/ml) in wash buffer with recombinant CTnC (−13 μM final concentration) on ice for 30 min. Unbound CTnC was removed by centrifugation, and the supernatant was discarded. Pellets were resuspended in wash buffer (without glycerol). This wash cycle was repeated and the final precipitate resuspended in wash buffer (without glycerol), stored on ice, and used for experiments.

Ca$^{2+}$-activated Myofibrillar ATPase Assays—0.1-ml reaction mixtures were carried out in a 96-well plate. First, 50 μg (≤46-μl volume) of myofibrils in wash buffer (without glycerol) were aliquoted per well. The remainder of the 0.1-ml reaction volume comprised double-distilled H$_2$O, ATPase buffers (50 μl) that have different amounts of calcium added to achieve the desired free calcium concentration, and 4 μl of ATP (3.14 mM, pH 7), which is added to initiate the ATPase reaction at 25 °C. The reactions were terminated 5 min later by the addition of trichloroacetic acid, and the inorganic phosphate was measured as above. Methods for solving the free and bound metal ion equilibria in our solutions were provided for by the computer program pCa Calculator (44). The final reaction condition contained 10$^{-4}$-10$^{-6}$ M free Ca$^{2+}$, 1 mM free Mg$^{2+}$, 2.5 mM MgATP$^{2-}$, 2 mM EGTA, 4 mM nitrilotriacetic acid, 20 mM MOPS, 1.0 mM dithiothreitol, 80 mM ionic strength (adjusted with KCl), pH 7, at 25 °C. The data were fitted to the Hill equation using the software suite of SigmaPlot 10.0.

Skinned Fiber Preparations—Skinned fibers were prepared using established protocols (37) with the following modifications. Muscle fibers were isolated from porcine left ventricular papillary muscle; pCa solutions were generated using the pCa Calculator program (44); and endogenous CTnC was extracted with 5 mM CDTA, pH 8.4 (adjusted with solid Tris-base) until the fibers developed <20% of the initial force (residual force) in pCa 4.0 solution. CDTA-treated fibers were reconstituted with CTnC (WT and mutant), and the Ca$^{2+}$ dependence of force generation and maximal recovered force (in the pCa 4.0) was measured. Data were analyzed using the following equation,

\[
\text{Change in force(%)} = 100 \times \frac{[Ca^{2+}]^n}{([Ca^{2+}]^n + [Ca^{2+}-50])}
\]

(Eq. 2)
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**Figure 1.** The effects of CTnC mutations associated with cardiomyopathies on the Ca$^{2+}$ sensitivity and maximum tension in skinned muscle fibers. Shown are the Ca$^{2+}$ dependences of tension from porcine muscle fibers reconstituted with WT and G159D CTnC (A) and L29Q CTnC (B). A comparison of the relative tensions from muscle fibers after CDTA treatment in pCa 4.0 (residual tension; white), and after CTnC reconstitution in pCa 4.0 (black) and 5.8 (gray). 100% relative tension indicates the maximal tension (in pCa 4.0) of fibers before CDTA treatment. All CTnC concentrations were 0.5 mg/ml. Data are summarized in Table 1.

**TABLE 1**
Summary of pCa-tension relationships of skinned cardiac fibers reconstituted with mutant CTnCs

| CTnC-reconstituted fiber | pCa$_{50}$$^a$ | $\Delta$pCa$_{50}$$^b$ | n$_{H}$ | Residual tension | Restored tension |
|--------------------------|----------------|-----------------|--------|-----------------|-----------------|
| WT1                      | 5.69 ± 0.05    | 4.07 ± 0.68     | 21.4 ± 3.5 | 74.9 ± 8.2     |
| L29Q                     | 5.70 ± 0.01    | 3.89 ± 0.18     | 19.4 ± 6.3 | 66.0 ± 5.6     |
| WT2                      | 5.70 ± 0.04    | 2.26 ± 0.29     | 12.3 ± 2.5 | 66.1 ± 4.6     |
| G159D                    | 5.62 ± 0.02$^c$| -0.08$^c$       | 2.23 ± 0.10 | 73.4 ± 7.7     |

$^a$ The pCa$_{50}$ values were obtained by fitting the data in the range from pCa 7 to 4.5.

$^b$ $\Delta$pCa$_{50}$ = pCa$_{50}$ of mutant CTnC fiber - pCa$_{50}$ of WT CTnC fiber.

$^c$p < 0.05, unpaired t test versus WT.

where [Ca$^{2+}_{50}$] represents the free Ca$^{2+}$ concentration that produces 50% force, and n is the Hill coefficient (37, 49).

**Statistical Analysis**—Data are expressed as the average of ≥5 experiments ± S.D. Significant differences were determined using an unpaired Student’s t test (Sigma Plot version 8.0), with significance defined as follows: *, p < 0.05; #, p < 0.001.

**RESULTS**

Effect of CTnC Mutations on the Ca$^{2+}$ Sensitivity of Tension—Skinned papillary muscle fibers were treated with CDTA to remove the endogenous porcine CTnC, followed by reconstituting them back with the recombinant CTnCs (WT, G159D, and L29Q). Fig. 1A shows that reconstituting fibers with G159D CTnC significantly decreases the Ca$^{2+}$ sensitivity of tension by −0.08 pCa units. This is made evident as a rightward shift in the curve with respect to the WT. Moreover, this mutant can generate less tension in the Ca$^{4.0}$ than the inability to incorporate the CTnC into the muscle fibers.

A comparison of the relative tensions from muscle fibers after CDTA treatment in pCa 4.0 (residual tension; white), and after CTnC reconstitution in pCa 4.0 (black) and 5.8 (gray). 100% relative tension indicates the maximal tension (in pCa 4.0) of fibers before CDTA treatment. All CTnC concentrations were 0.5 mg/ml. Data are summarized in Table 1.

**Effect of CTnC Mutations on Cardiac Myofibrillar and Regulated Actomyosin ATPase Activities—CMF isolated from porcine left ventricles were treated with CDTA followed by reconstitution with recombinant CTnCs. Fig. 2A shows the Ca$^{2+}$ dependence of ATPase activity from the WT, G159D, and L29Q CTnC-reconstituted CMF. The maximal output for each mutant and WT CTnC were normalized to 100% in Fig. 2A to better illustrate the ability of the G159D reconstituted CMF to reduce the Ca$^{2+}$ sensitivity of ATPase activity by −0.08 pCa units and the inability of the L29Q mutant to generate statistical...
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**TABLE 2** Summary of cardiac myofibrillar and regulated actomyosin ATPase activity data from Fig. 2

|             | WT | L29Q | G159D |
|-------------|----|------|-------|
| pCa (8)     | 5.95 ± 0.03 | 5.97 ± 0.02 | 5.87 ± 0.03 |
| Percentage activation (−Ca²⁺) | 9.4% ± 0.9% | 9.1% ± 0.9% | 8.5% ± 0.8% |
| Fold activation | 4.19 ± 0.15 | 4.27 ± 0.22 | 4.55 ± 0.22 |
| Minimum activity (−Ca²⁺) | 0.088 ± 0.011 | 0.091 ± 0.014 | 0.096 ± 0.015 |
| Maximum activity (−Ca²⁺) | 0.535 ± 0.021 | 0.528 ± 0.031 | 0.485 ± 0.034 |
| Percentage activation (−Ca²⁺) | 26.8 ± 3.3 | 28.4 ± 4.3 | 29.1 ± 4.4 |
| Percentage activation (−Ca²⁺) | 162.5 ± 6.3 | 160.4 ± 9.5 | 143.5 ± 5.9 |

* Values obtained from fitting cardiac myofibrillar data in the range of pCa 8 to 4.5.
* p < 0.05; unpaired t test versus WT.
* Minimum and maximum specific ATPase activities from CMF in pCa 8 (−Ca²⁺) and pCa 4.5 (+Ca²⁺), respectively (nmol of Pi/mol of myosin) × s⁻¹.
* Fold CMF ATPase activation = maximum activity/minimum activity (−fold activation of CDTA-treated CMF − 1.59 ± 0.27).
* Minimum and maximum specific ATPase activities of reconstituted actomyosin when 2.0 μM CTn is present in pCa 9 (−Ca²⁺) and pCa 4 (+Ca²⁺), respectively (mol of Pi/mol of myosin) × s⁻¹.
* Percentage of ATPase activation of reconstituted actomyosin in pCa 9 (−Ca²⁺) and pCa 4 (+Ca²⁺). 100% activation is defined as the percentage of ATPase activation in the presence of F-actin, Tm, and myosin only.

FIGURE 2. The effects of skinned cardiac myofibrils and regulated actomyosin filaments reconstituted with CTnC mutants. A, Ca²⁺ sensitivity of ATPase activity from skinned cardiac myofibrils reconstituted with the G159D and L29Q CTnC. B, a comparison of the CDTA-treated and CTnC-reconstituted myofibrillar ATPase activities in pCa 8.0 (black) and pCa 4.5 (gray) solutions. C, effect of CTnC mutations on the activation (+Ca²⁺) and inhibition (−Ca²⁺) of regulated actin-Tm-activated myosin ATPase activity. CTn containing G159D, L29Q, or WT CTnC was mixed with actin, Tm, and myosin to reconstitute the regulated actomyosin filaments. The protein concentrations used are as follows: 3.5 μM F-actin, 1.0 μM Tm, 0.6 μM myosin, and 0–20 μM CTnC. At all concentrations of CTn (0–20 μM), the specific ATPase activities are measured in the presence of 0.1 mM free Ca²⁺ (+Ca²⁺) or 1.0 mM EGTA (−Ca²⁺). All experiments were done in the presence of 1.0 mM free Mg²⁺. Data are summarized in Table 2.

Table differences from the WT controls (ΔpCa = +0.02, p > 0.05). Fig. 2B shows that after CDTA treatment, the myofibrillar ATPase activity in pCa 4.5 is nearly inactivated (1.59-fold activation), whereas, after the mutant CTnCs are reconstituted, the maximal myofibrillar ATPase is activated −4-fold and statistically insignificant in comparison with the WT (Fig. 2B and Table 2).

Preformed cardiac troponin complexes reconstituted with WT, G159D, or L29Q CTnC were mixed with F-actin, tropo-

myosin, and myosin in order to determine if the different CTnC mutations can affect the ability to inhibit or activate the regulated actomyosin ATPase activity. The ATPase activity of the unregulated filaments (i.e. in the absence of CTnC) is defined as 100%. Fig. 2C shows that in the absence of Ca²⁺ (−Ca²⁺), the ATPase activities of regulated actomyosin filaments containing the G159D and L29Q mutants are statistically insignificant in comparison with the WT (−27%) at all CTn concentrations. However, in pCa 4.0 solution (+Ca²⁺), the incorporation of G159D CTnC into regulated actomyosin filaments significantly lowers the ATPase activation (144%) in comparison with the WT counterpart (163%) when CTn is sufficiently present to saturate the thin filament. Table 2 summarizes the values obtained in Fig. 2.

Effect of Metal Ion Binding on Fluorescence from IAANS-labeled CTnC. The WT, G159D, and L29Q CTnC were used to generate monocysteine mutants to direct specific incorporation of the IAANS fluorophore onto residue Cys⁸⁴ (CTnC(C⁸⁴)IA), which solely reports metal ion binding to the regulatory site of CTnC(C⁸⁴)IA in isolation and in binary complex (38). In addition, both cysteine residues (positions 35 and 84) were labeled with IAANS (CTnCIA), which reports metal ion binding to the Ca²⁺-specific and Ca²⁺-Mg²⁺ sites in isolated CTnCIA and to the Ca²⁺-specific site within CTnC (38, 51).

Fig. 3A represents the Ca²⁺-dependent changes in fluorescence arising from the WT, G159D, and L29Q CTnCIA in the presence (inset) and absence of Mg²⁺. As the free Ca²⁺ increases from pCa 8.0 to 3.6, the graphs are biphasic, representing metal ion binding to two classes of binding sites. Therefore, the pCa⁵₀ and Hill values obtained with the CTnCIA configuration were fitted to a two-site Hill equation and are summarized in Table 3. As the free Ca²⁺ increases from pCa 8.0 to 6.6 in the absence of Mg²⁺, the isolated WT CTnCIA fluorescence decreases −11.3% with a pCa⁵₀ of 7.04, indicating Ca²⁺ binding to the Ca²⁺-Mg²⁺ sites. In the presence of Mg²⁺ (inset), the WT CTnCIA Ca²⁺-Mg²⁺ sites decrease their fluorescence intensity (−4.8%) with a concomitant increase in affinity for Ca²⁺ by +0.08 pCa units. Increasing the concentration of free Ca²⁺ from pCa 6.6 to 3.8 leads to an increase in the
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FIGURE 3. The Ca\(^{2+}\)-dependent changes in fluorescence arising from IAANS-labeled CTnC mutants associated with cardiomyopathy. A, a comparison of the Ca\(^{2+}\)-dependent changes in fluorescence from the double-labeled L29Q and G159D CTnCIA. B, the Ca\(^{2+}\)-dependent changes in fluorescence from the monolabeled L29Q and G159D CTnC(C84)IA. Incremental amounts of Ca\(^{2+}\) were added to 0.2 ml of labeled CTnCs (0.25 \(\mu\)M) in the presence and absence of 1.25 mM Mg\(^{2+}\) (inset). Fluorescence changes are expressed as a percentage of the total change. Data are summarized in Table 3.

Fluorescence intensity for the WT CTnCIA protein, indicating Ca\(^{2+}\) binding to the Ca\(^{2+}\)-specific (regulatory) site with a Ca\(_{50}\) value of 5.23 \((\text{Mg}^{2+})\) and 4.94 \((\text{Mg}^{2+})\).

Upon Ca\(^{2+}\) binding to the Ca\(^{2+}\)-Mg\(^{2+}\) sites of isolated L29Q CTnCIA (Fig. 3A), there are large decreases in fluorescence in the absence \((-72.5\%)\) and presence \((-28.1\%)\) of Mg\(^{2+}\); however, the fitted Ca\(_{50}\) values for the Ca\(^{2+}\)-Mg\(^{2+}\) sites are unaffected in comparison with the WT counterpart. Moreover, the Ca\(_{50}\) value for the regulatory site is markedly reduced in the absence of Mg\(^{2+}\) \((\Delta\text{Ca}_{50} = -0.15)\) and, to a lesser extent, in the presence of Mg\(^{2+}\) \((\Delta\text{Ca}_{50} = -0.06)\) when compared with the WT counterpart. Upon Ca\(^{2+}\) binding to the Ca\(^{2+}\)-Mg\(^{2+}\) sites of G159D CTnCIA, the Ca\(_{50}\) values are reduced in the presence \((\Delta\text{Ca}_{50} = -0.19)\) and absence \((\Delta\text{Ca}_{50} = -0.17)\) of Mg\(^{2+}\). However, from pCa 6.6 to 3.8, the G159D CTnCIA regulatory site affinity is unaffected \((\Delta\text{Ca}_{50} = \pm 0.03)\). Table 3 summarizes the fitted values obtained in Fig. 3A.

TABLE 3

| Preparation | [Mg\(^{2+}\)] | High affinity binding | Low affinity binding |
|-------------|--------------|-----------------------|---------------------|
|             | \(p\text{Ca}_{50}\) \(n_{11}\) \(p\text{Ca}_{50}\) \(n_{11}\) |
| WT-CTnC(C84)IA \(a\) | 5.18 \pm 0.05 | 0.939 | 5.02 \pm 0.05 | 0.969 |
| G159D-CTnC(C84)IA \(b\) | 4.84 \pm 0.01 | 0.843 | 4.92 \pm 0.02 | 0.896 |
| L29Q-CTnC(C84)IA \(c\) | 6.68 \pm 0.02 | 0.724 | 6.53 \pm 0.07 | 0.724 |
| L29Q-CTnC(C84)IA \(d\) | 6.39 \pm 0.03 | 0.996 | 6.48 \pm 0.06 | 1.212 |
| WT-CTnCIA | 7.04 \pm 0.12 | 0.791 | 6.32 \pm 0.16 | 0.791 |
| G159D-CTnCIA \(e\) | 7.12 \pm 0.03 | 0.849 | 6.49 \pm 0.04 | 0.820 |
| G159D-CTnCIA | 6.87 \pm 0.09 | 0.820 | 7.12 \pm 0.04 | 0.820 |
| L29Q-CTnCIA \(f\) | 6.99 \pm 0.10 | 0.724 | 7.14 \pm 0.06 | 0.724 |
| L29Q-CTnCIA | 7.06 \pm 0.06 | 0.996 | 7.28 \pm 0.01 | 0.996 |

* The contaminating Mg\(^{2+}\) levels were less than 1.0 \(\mu\)M in experiments lacking Mg\(^{2+}\). In the presence of 1.25 mM Mg\(^{2+}\), the free concentration was estimated to be \(-0.6\) ms.
* The pCa\(_{50}\) values were obtained by fitting only the data in the range from pCa 6.4 to 3.8 from each individual curve to the Hill equation and averaging them as a group to obtain the S.D.
* The pCa\(_{50}\) values were obtained by fitting the data in the range from pCa 8.0 to 3.6 from each individual curve to a two-site Hill equation and averaging them as a group to obtain the S.D.
* \(p < 0.05\), unpaired \(t\) test versus WT.

Fig. 3B represents the calcium-dependent changes in fluorescence from isolated WT, L29Q, and G159D CTnC(C84)IA in the absence and presence \((\text{inset})\) of Mg\(^{2+}\). As the free Ca\(^{2+}\) increases from pCa 6.8 to 3.6, the WT CTnC(C84)IA fluorescence intensity increases monophasically, indicating Ca\(^{2+}\) binding to the regulatory site. The pCa\(_{50}\) values are 5.18 \((-\text{Mg}^{2+})\) and 4.84 \((\text{Mg}^{2+})\). In this same pCa range, the G159D CTnC(C84)IA regulatory site affinity is unaffected. This is made evident by the WT and G159D CTnC(C84)IA curves overlapping each other in the presence \((\text{inset})\) and absence of Mg\(^{2+}\). Alternatively, upon Ca\(^{2+}\) binding to L29Q CTnC(C84)IA, the fluorescence intensity unexpectedly increases biphasically, revealing that this labeled protein is sensitive to Ca\(^{2+}\) binding to the two classes of sites \((\text{in} + \text{Mg}^{2+} \text{or} -\text{Mg}^{2+})\). Therefore, the L29Q CTnC(C84)IA structural effects differ from the previous CTnCs tested herein and may preclude its comparison with the WT control. Despite the inconsistencies between the L29Q and WT CTnC(C84)IA proteins, these data were fitted with a two-site Hill equation in order to calculate the pCa\(_{50}\) values of both classes of sites \((\text{summarized in Table 3})\). These results show that when fitted, the pCa\(_{50}\) for the L29Q CTnC(C84)IA regulatory site increases \(+0.12\) pCa units \((\text{Mg}^{2+})\) when compared with the WT counterpart.

Effect of Ca\(^{2+}\) Binding on Fluorescence from IAANS-labeled CTnC in Complex—WT, L29Q, and G159D CTnC(C84)IA were mixed with recombinant CTnI to form fluorescent binary complexes. Fig. 4A shows the Ca\(^{2+}\)-dependent changes in fluorescence arising from the WT and mutant binary CTnC(C84)IA complexes. As the free Ca\(^{2+}\) rises from pCa 7.6 to 4.8, the WT binary complex increases its fluorescence intensity 1.24-fold with a pCa\(_{50}\) of 6.24. The results from the G159D binary complex are statistically insignificant in comparison with the WT, whereas the fluorescence intensity of the L29Q binary complex increases 1.33-fold with a significant reduction in Ca\(^{2+}\) affinity \((\Delta\text{Ca}_{50} = -0.19)\). Table 4 summarizes the pCa\(_{50}\) and Hill values calculated from the data obtained in Fig. 4A.
FIGURE 4. The Ca\(^{2+}\)-dependent changes in fluorescence arising from IAANS-labeled CtnC mutants in complex. Shown is the effect of thin filament proteins on the Ca\(^{2+}\) dependence of fluorescence from L29Q and G159D mutations in CtnC(C84)IA binary complexes (A), CtnCIA ternary complexes (B), and CtnC(C84)IA regulated thin filaments (C). Incremental amounts of Ca\(^{2+}\) were added to 2.0 ml of labeled regulatory complexes in the presence of 1.25 mM Mg\(^{2+}\). The protein concentrations used were as follows: 0.5 \(\mu\)M binary complex, 0.5 \(\mu\)M ternary complex, and 0.053 mg/ml regulated thin filaments. Fluorescence changes are expressed as a percentage of the total change. Data are summarized in Table 4.

WT, L29Q, and G159D CtnCIA were mixed with recombinant Ctnl and CtnT to generate fluorescent cardiac troponin complexes (CtnIA). Fig. 4B illustrates the Ca\(^{2+}\)-dependent changes in fluorescence arising from CtnIA complexes. In the pCa range of 8.8 to 5.2, Ca\(^{2+}\) binding to the WT and mutant CtnIA is accompanied by a 1.4-fold decrease in fluorescence intensity. The G159D CtnIA Ca\(^{2+}\) affinity is significantly reduced (\(\Delta pCa_{50} = -0.15\)) in comparison with WT CtnIA, whereas the changes in Ca\(^{2+}\) affinity arising from L29Q CtnIA are not as significant (\(\Delta pCa_{50} = -0.07, p < 0.05\)) (see Table 4). In accordance with previous studies (37), the addition of Ca\(^{2+}\) to binary CtnCIA and ternary CtnC(C84)IA complexes yielded no change in fluorescence (data not shown).

WT, L29Q, and G159D CtnC(C84)IA were mixed with recombinant Ctnl and CtnT to form Ctn(C84)IA complexes. These preformed complexes were mixed with F-actin and Tm to generate fluorescent RTFs. Fig. 4C shows the Ca\(^{2+}\) dependence of fluorescence from IAANS-labeled RTFs. The WT RTF increases its fluorescence intensity 1.62-fold upon increasing the free Ca\(^{2+}\) from pCa 8.0 to 4.5 with a pCa\(_{50}\) of 6.12. In the same pCa range, the G159D RTF fluorescence intensity increases to a lesser extent (1.48-fold) with a concomitant reduction in Ca\(^{2+}\) affinity (\(\Delta pCa_{50} = -0.15\)). Alternatively, the L29Q RTF Ca\(^{2+}\) affinity and fluorescence intensities are statistically insignificant in comparison with the WT control (\(\Delta pCa = +0.05, p > 0.05\)). Table 4 summarizes the pCa\(_{50}\) and Hill values that are derived from the data in Fig. 4.

**DISCUSSION**

Since the heart is a dynamic pump, thoracic imaging techniques are essential in identifying the specific systolic and diastolic dysfunctions underlying the pathologies of familial cardiomyopathies. However, different individuals affected by the same mutation can show variable penetrance (e.g., graded wall thickening or dilation), suggesting that cardiomyopathies are complex multifactorial diseases. Therefore, steady state measurements have the benefit of observing the influence of these mutations on contractility in the absence of long-term neurohumoral and autonomic stimulation. This has led to the in vitro investigation of numerous thin filament mutations linked to cardiomyopathies and the discovery of their characteristic effects on the Ca\(^{2+}\)-dependent processes of contraction in the absence of Ctnl phosphorylation. Within the context of RTF mutations, a unifying paradigm emerges that associates decreases in the myofilament Ca\(^{2+}\) sensitivity with DCM-linked mutations and increases with HCM- and restrictive cardiomyopathy-linked mutations (4, 7, 14). However, does this imply that insignificant changes in the myofilament Ca\(^{2+}\) sensitivity arising from a mutation are going to be associated with a benign phenotype? This poses a difficult challenge to the developing paradigm, because these mutations are rare polymorphisms, making it difficult to determine if the clinical outcome is primarily due to a disease causing mutation in the myocardium or physiological adaptations to conditions such as hypertension, diabetes, obesity, and alcoholism, to name a few (2).

Echocardiographic examination of the only L29Q CtnC proband shows that the septal and free ventricular walls are concentrically hypertrophied (15 mm; cut-off = 13 mm) (2, 19). Notably, in the absence of any cardiomyopathy, many clinical studies strongly correlate concentric hypertrophy with hypertension (52, 53). Moreover, the amount of left ventricle hypertrophy may be overestimated in the L29Q proband, because the authors did not adjust the left ventricle mass to body surface...
area or mass indices in order to assess the possible impact of obesity and hypertension (54, 55). Despite the mild hypertrophy, cardiac catheterization showed a normal ejection fraction with preserved diastolic heart function (19). Based upon the available clinical data and current paradigms, we hypothesized that if the L29Q mutation is benign, then significant changes in the Ca\(^{2+}\) sensitivity should not arise in the skinned muscle. When we assessed the effects of L29Q CTnC on tension and myofibrillar ATPase activation, neither the Ca\(^{2+}\) sensitivities nor the maximal generating capabilities were significantly different from the WT controls. Moreover, regulated actomyosin reconstituted with preformed CTn containing the recombinant L29Q mutant did not affect the ability to inhibit or maximally activate the ATP hydrolysis at all levels of CTn. By taking into account the clinical pathology of the L29Q proband, the authors concluded that the L29Q mutation may “simply be a rare polymorphism without any phenotypical relevance” (19), in which case, our in vitro results would coincide with their findings. Therefore, the combined clinical and in vitro data should be weighed equally in order to determine more accurately the potential severity of specific mutations. Under this premise, the E244D CTnT mutation associated with HCM would be considered a benign polymorphism, because it does not affect the Ca\(^{2+}\) regulation in vitro (56, 57) or present with any cardiac dysfunction in the only proband (58).

Two reports have shown that the L29Q mutation can alter the myofilament Ca\(^{2+}\) sensitivity in a reconstituted S1-ATPase system (30) and in the skinned rat cardiomyocyte (32). Unexpectedly, the first investigation reports a paradigm contradiction, because the HCM-associated L29Q mutation decreases the myofilament Ca\(^{2+}\) sensitivity (30). However, their use of skeletal actin, Tm, and myosin may disrupt specific cardiac myofilament interactions that govern the Ca\(^{2+}\) regulation of contraction (or ATPase activity).

The second study shows that L29Q CTnC is able to increase the apparent CTnC Ca\(^{2+}\) affinity in the isolated state and in the skinned rat cardiomyocyte (32). Although in our hands, the changes in the Ca\(^{2+}\) sensitivities measured in fibers and cardiac myofibrils reconstituted with L29Q CTnC are statistically insignificant with respect to the WT, we do observe tendencies to increase the Ca\(^{2+}\) sensitivity of tension, myofibrillar ATPase activity, and RTF fluorescence (summarized in Table 5). It is expected that the different protein expression profiles among higher mammals and lower rodents will affect the biophysical properties that govern Ca\(^{2+}\) binding to CTnC. Furthermore, the cardiomyocyte system is a more sensitive technique to measure changes in Ca\(^{2+}\) affinity that could otherwise go undetected at the scale of the skinned fiber.

### Table 4

**Comparison of pCa\(_{50}\) and Hill coefficients used to fit the data obtained in Fig. 4**

|                | WT          | G159D       | L29Q        |
|----------------|-------------|-------------|-------------|
| pCa\(_{50}\)   |             |             |             |
| Binary (CTnC(C84)\(_{IA}\))\(^a\) | 6.24 ± 0.02  | 6.68 ± 0.02 | 6.12 ± 0.03 |
| Ternary (CTnC(C84)\(_{IA}\))\(^b\) | −0.94       | −0.94       | −0.94       |
| Regulated thin filament (CTnC(C84)\(_{IA}\))\(^c\) | 1.11        | 1.22        | 1.22        |

\(^a\) Binary values obtained by fitting the data in the range of pCa 7.7 to 4.5.

\(^b\) p < 0.001 unpaired t test versus WT.

\(^c\) Ternary values obtained by fitting the data in the range of pCa 8.7 to 4.9.

\(^d\) Regulated thin filament values obtained by fitting the data in the range of pCa 7.6 to 4.2.

### Table 5

**Summary of mutant CTnC regulatory site ∆pCa\(_{50}\) values from various preparations**

|                | WT pCa\(_{50}\) | G159D ∆pCa\(_{50}\) | L29Q ∆pCa\(_{50}\) |
|----------------|----------------|---------------------|-------------------|
| Isolated (CTnC\(_{IA}\))\(^a\) | 5.18          | +0.02               | −0.15             |
| Isolated (CTnC(C84)\(_{IA}\))\(^b\) | 5.23          | −0.02               | +0.12             |
| Binary (CTnC(C84)\(_{IA}\))\(^c\) | 6.24          | +0.05               | −0.19             |
| Ternary (CTnC\(_{IA}\)) | 6.68          | −0.15\(^d\)        | −0.17\(^d\)       |
| Regulated thin filament (CTnC(C84)\(_{IA}\)) | 6.12          | −0.15\(^d\)        | +0.05             |
| Cardiac myofibrils | 5.95          | −0.08\(^e\)        | +0.02             |
| Skinned fiber | 5.70\(^b\)    | −0.08\(^e\)        | +0.01             |
| Skinned fiber | 5.69\(^b\)    |                     |                   |

\(^a\) ∆pCa\(_{50}\) = Mutant pCa\(_{50}\) − WT pCa\(_{50}\) values are calculated from Tables 1–4.

\(^b\) Values are given in the absence of Mg\(^{2+}\).

\(^c\) p < 0.001 unpaired t test versus WT.

\(^d\) p < 0.005; unpaired t test versus WT.

\(^e\) Mean pCa\(_{50}\) of control fibers associated with G159D.

\(^f\) Mean pCa\(_{50}\) of control fibers associated with L29Q.

Measuring the structural and Ca\(^{2+}\) dependent changes from the isolated L29Q (-CTnC\(_{IA}\) and -CTnC(C84)\(_{IA}\)) proteins confirms that this mutation has the ability to alter the regulatory site binding properties and N- and C-domain intramolecular interactions. Interestingly, the L29Q CTnC\(_{IA}\) reports a significant decrease in affinity at the regulatory site, whereas the L29Q CTnC(C84)\(_{IA}\) protein unexpectedly reports Ca\(^{2+}\) binding to two classes of sites with an increased affinity at the regulatory site (Fig. 3). Although these results suggest that the combined effects of the L29Q mutation and IAA NS fluorophore on Cys\(^{84}\) may dramatically perturb the structure of isolated L29Q CTnC(C84)\(_{IA}\), it also provides the opportunity to determine if the addition of different myofilament proteins and their interactions have the ability to reverse these processes. Formation of L29Q ternary complexes shows that the changes in Ca\(^{2+}\) affinity are smaller and become insignificant in the RTF when compared with the WT counterparts (see Table 5). The above results show that the RTF, representing a highly cooperative system can restore the Ca\(^{2+}\) affinity of L29Q CTnC to that of the WT via myofilament intermolecular interactions and can explain why significant changes in the Ca\(^{2+}\) sensitivity of tension do not arise.

With regard to G159D CTnC in the skinned muscle, this mutation reduces the Ca\(^{2+}\) sensitivity of tension and myofibrillar ATPase activation (Table 5) without affecting the capability to generate maximal output (pCa ≤ 4.5). Despite the inability of this mutant to reduce the maximal output, the myofilament Ca\(^{2+}\) desensitization is expected to recruit fewer strongly attached cross-bridges at submaximal Ca\(^{2+}\) concentrations, leading to the reduction of both ATPase activation and the subsequent generation of tension (1, 3, 59). Since muscle relaxation is directly correlated with Ca\(^{2+}\) dissociation from the
CTnC regulatory site (60), mutations that directly (or indirectly) decrease the calcium affinity of CTnC would increase the rate of cardiac muscle relaxation. Therefore, an improvement in muscle relaxation would facilitate the entry of blood from the atria into the ventricles by providing less resistance to stretch (i.e. fewer attached cross-bridges from previous systole), which can result in higher end diastolic dimensions. In support of this argument, independent studies have shown that the reconstitution of AK210-CTnT (associated with DCM) into skinned fibers also reduces the myofilament Ca\(^{2+}\) sensitivity without affecting the maximal tension (3, 36). Moreover, the AK210-CTnT mutation is still able to recapitulate the DCM phenotype (e.g. reduced ejection fraction and dilation) in a knock-in mouse model (3).

Previous investigations have reported that the G159D mutation does not alter the Ca\(^{2+}\) sensitivity of tension or ATPase activation in the absence of phosphorylated CTnI. These reports show that ~50% (28) to ~68% (29) of the endogenous CTnC is replaced by the mutant in comparison with the ~86% that is exchanged in our fibers. Therefore, an increase in the ratio of incorporated mutant to endogenous CTnC along the thin filament may explain why changes in the Ca\(^{2+}\) sensitivity arise in our system. In addition, the pCa\(_{50}\) values for ATPase activity reported by Biesiadecki et al. (29) suggests that small significant changes in the myofilament Ca\(^{2+}\) sensitivity may not be uncovered by the Ca\(^{2+}\)-buffering capacities of their solutions. Therefore, we employed the combinatorial use of nitrilotriacetic acid and EGTA to improve the Ca\(^{2+}\)-buffering capabilities of our solutions, which has previously been shown to increase the sensitivity and reproducibility of the experiment (44).

Considering the indirect two-way communication between cross-bridge attachment and Ca\(^{2+}\) binding to CTnC, it is expected that a perturbation due to a CTnC mutation can affect the Ca\(^{2+}\) sensitivity of contraction through the following mechanisms: 1) direct modification of the regulatory site; 2) modifying CTnC intramolecular interactions; and/or 3) modifying CTnC intermolecular interactions. Measuring the shifts in the Ca\(^{2+}\) sensitivity and maximum tension (or ATPase) is not sufficient to suggest the mechanism(s). Therefore, covalently attached fluorophores on CTnC were used to identify key intra- and intermolecular interactions that dictate the final Ca\(^{2+}\) sensitivity of tension in the muscle fiber.

The isolated G159D (-CTnC\(_{4\alpha}\) and -CTnC(C844\(_{1\alpha}\)) regulatory site apparent affinities are statistically insignificant in comparison with their respective WT counterparts. Therefore, the existence of mechanism 1 may be excluded. Nevertheless, a reduction in the affinity for the Ca\(^{2+}\)-Mg\(^{2+}\) sites (± Mg\(^{2+}\)) indicates that intramolecular interactions within the C-domain are altered (mechanism 2). Measuring the Ca\(^{2+}\) dependent changes in G159D binary complexes did not uncover the changes seen in the Ca\(^{2+}\) sensitivity of tension or ATPase. Rather, within the ternary complex, significant reductions in the regulatory site Ca\(^{2+}\) affinity begin to emerge (ΔpCa = −0.15), which persist up to the level of the RTF and, to a lesser extent, in the skinned fiber (summarized in Table 5). Since the CTnC C-lobe is “rigidly” integrated into the IT arm of CTn (61), the effect of the G159D mutation on the Ca\(^{2+}\)-Mg\(^{2+}\) sites may destabilize these interactions. This implies that a CTnC C-domain mutation can indirectly affect the N-domain through altered CTnC and CTnT interactions, which becomes apparent at the level of CTn (i.e. mechanisms 2 and 3).

The cardiac contractile apparatus, representing a large cooperative machine, can provide the potential for changes (G159D) or no change (L29Q) in the myofilament Ca\(^{2+}\) sensitivity if any of the proteins are mutated. The true effects of the mutations become apparent as the hierarchical level of the myofilament increases. This is illustrated by the tendency of the RTF (in the absence of myosin) to retain its regulatory role and best indicate changes (+ or −) in the apparent CTnC Ca\(^{2+}\) affinity that are measured in the skinned muscle. Considering that the in vitro characterization of over 40 RTF mutations assessed (in the absence of CTn phosphorylation) have led to the emergence of current paradigms related to familial cardiomyopathies, we measured the various Ca\(^{2+}\)-dependent processes of contraction to determine if these mutations challenge or support the current paradigms. Our results indicate that the myofilament Ca\(^{2+}\) desensitization arising from G159D CTnC and the lack of effects arising from L29Q CTnC do coincide with the current paradigms and are indicative of their respective clinical outcomes.

REFERENCES

1. Fatkin, D., and Graham, R. M. (2002) *Physiol. Rev.* 82, 945–980
2. Griffin, B. P., and Topol, E. J. (2004) *Manual of Cardiovascular Medicine*, 2nd Ed., pp. 101–142, Lippincott Williams and Wilkins, Philadelphia
3. Du, C. K., Morimoto, S., Nishii, K., Minakami, R., Ohta, M., Tadao, N., Lu, Q. W., Wang, Y. Y., Zhan, D. Y., Mochizuki, M., Kitaj, S., Miwa, Y., Takahashi-Yanaga, F., Iwamoto, T., Ohtsuki, I., and Sasaguri, T. (2007) *Circ. Res*. 101, 185–194
4. Gomes, A. V., and Potter, J. D. (2004) *Ann. N. Y. Acad. Sci.* 1015, 214–224
5. Rajan, S., Ahmed, R. P., Jagatheesan, G., Petrashevskaya, N., Boivin, G. P., Urboniene, D., Arteaga, G. M., Wolska, B. M., Solano, R. J., Liggert, S. B., and Wieczorek, D. F. (2007) *Circ. Res*. 101, 205–214
6. Chang, A. N., Harada, K., Ackerman, M. J., and Potter, J. D. (2005) *J. Biol. Chem.* 280, 34343–34349
7. Robinson, P., Griffiths, P. J., Watkins, H., and Redwood, C. S. (2007) *Circ. Res*. 101, 1266–1273
8. Gomes, A. V., Harada, K., and Potter, J. D. (2005) *J. Mol. Cell. Cardiol.* 39, 754–765
9. Karibe, A., Tobacman, L. S., Strand, J., Butters, C., Back, N., Bachinski, L. L., Arau, A. E., Ortiz, A., Roberts, R., Homsher, E., and Fananapazir, L. (2001) *Circulation* 103, 65–71
10. Heller, M. J., Nili, M., Homsher, E., and Tobacman, L. S. (2003) *J. Biol. Chem.* 278, 41742–41748
11. Takahashi-Yanaga, F., Morimoto, S., Harada, K., Minakami, R., Shiraishi, F., Ohta, M., Lu, Q. W., Sasaguri, T., and Ohtsuki, I. (2001) *J. Mol. Cell Cardiol.* 33, 2095–2107
12. Chandra, M., Tschirgi, M. L., and Tardiff, J. I. (2005) *Am. J. Physiol. Heart Circ. Physiol*. 289, H2112–H2119
13. Davis, J., Wen, H., Edwards, T., and Metzger, J. M. (2007) *Circ. Res*. 100, 1494–1502
14. Gomes, A. V., Liang, J., and Potter, J. D. (2005) *J. Biol. Chem.* 280, 30909–30915
15. Kobayashi, T., and Solaro, R. J. (2006) *J. Biol. Chem.* 281, 13471–13477
16. Yumoto, F., Lu, Q. W., Morimoto, S., Tanaka, H., Kono, N., Nagata, K., Ojima, T., Takahashi-Yanaga, F., Miwa, Y., Sasaguri, T., Nishita, K., Takeshima, H., and Ohtsuki, I. (2005) *Biochem. Biophys. Res. Commun.* 338, 1519–1526
17. Pinto, J. R., Parvatiyar, M. S., Jones, M. A., Liang, J., and Potter, J. D. (2008) *J. Biol. Chem.* 283, 2156–2166
18. Liao, R., Gwathmey, J. K., and Wang, C. (1998) *Circulation* 98, 1–625
Effects of L29Q and G159D-CTnC on the Contractile Apparatus

19. Hoffmann, B., Schmidt-Traub, H., Perrot, A., Osterziel, K. J., and Gessner, R. (2001) *Hum. Mutat.* 17, 524
20. Mogensen, J., Murphy, R. T., Shaw, T., Bahl, A., Redwood, C., Watkins, H., Burke, M., Elliott, P. M., and McKenna, W. J. (2004) *J. Am Coll. Cardiol.* 44, 2033–2040
21. Kaski, J. P., Burch, M., and Elliott, P. M. (2007) *Cardiol. Young* 17, 675–677
22. Dweck, D., Gomes, A. V., and Potter, J. D. (2005) *Biochem. J.* 382, 317–320
23. Lim, C. C., Yang, H., Yang, M., Wang, C. K., Shi, J., Berg, E. A., Pimentel, D. R., Gwathmey, J. K., Hajjar, R. J., Helmes, M., Costello, C. E., Huo, S., and Liao, R. (2008) *Biochem. Biophys. J.* 94, 3577–3589
24. Gordon, A. M., Homsher, E., and Regnier, M. (2000) *Physiol. Rev.* 80, 853–924
25. Li, M. X., Wang, X., and Sykes, B. D. (2004) *J. Muscle Res. Cell Motil.* 25, 559–579
26. Potter, J. D., and Gergely, J. (1975) *J. Biol. Chem.* 250, 1421–1427
27. Holroyde, M. J., Robertson, S. P., Johnson, J. D., Solaro, R. J., and Potter, J. D. (1980) *J. Biol. Chem.* 255, 11688–11693
28. Preston, L. C., Ashley, C. C., and Redwood, C. S. (2007) *Biochem. Biophys. Res. Commun.* 357, 27–32
29. Biesiadcki, B. J., Kobayashi, T., Walker, J. S., Solaro, R. J., and de Tombe, P. P. (2007) *Circ. Res.* 100, 1486–1493
30. Schmidtmann, A., Lindow, C., Villard, S., Heuser, A., Mugge, A., Gessner, R., Granier, C., and Jaquet, K. (2005) *FEBS J.* 272, 6087–6097
31. Dong, W. J., Xing, J., Ouyang, Y., An, J., and Cheung, H. C. (2008) *J. Mol. Cell Cardiol.* 46, 259–262
32. Liang, B., Cheng, F., Qu, Y., Pavlov, D., Gillis, T. E., Tikunova, S. B., Davis, J. P., and Tibbits, G. F. (2008) *Physiol. Genomics* 33, 257–266
33. Baryshnikova, O. K., Li, M. X., and Sykes, B. D. (2008) *J. Mol. Biol.* 375, 735–751
34. Lu, Q. W., Morimoto, S., Harada, K., Du, C. K., Takahashi-Yanaga, F., Miwa, Y., Sasaguri, T., and Ohtsuki, I. (2003) *J. Mol. Cell Cardiol.* 35, 1421–1427
35. Venkatraman, G., Gomes, A. V., Kerrick, W. G., and Potter, J. D. (2005) *J. Biol. Chem.* 280, 17584–17592
36. Morimoto, S., Lu, Q. W., Harada, K., Takahashi-Yanaga, F., Minakami, R., Ohta, M., Sasaguri, T., and Ohtsuki, I. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 913–918
37. Szczesna, D., Guzman, G., Miller, T., Zhao, J., Farokhi, K., Ellemerberger, H., and Potter, J. D. (1996) *J. Biol. Chem.* 271, 8381–8386
38. Putkey, J. A., Liu, W., Lin, X., Ahmed, S., Zhang, M., Potter, J. D., and Kerrick, W. G. (1997) *Biochemistry* 36, 970–978
39. Potter, J. D. (1982) *Methods Enzymol.* 85, 241–263
40. Zhang, R., Zhao, J., and Potter, J. D. (1995) *J. Biol. Chem.* 270, 30773–30780
41. Szczesna, D., Zhang, R., Zhao, J., Jones, M., Guzman, G., and Potter, J. D. (2000) *J. Biol. Chem.* 275, 624–630
42. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol.* 85, 164–181
43. Jaquet, K., and Heilmeyer, L. M., Jr. (1987) *Biochem. Biophys. Res. Commun.* 145, 1390–1396
44. Dweck, D., Reyes-Alfonso, A., Jr., and Potter, J. D. (2005) *Anal. Biochem.* 347, 303–315
45. Morimoto, S., and Ohtsuki, I. (1987) *J. Biol. Chem.* 262, 13627–13635
46. Gulati, J., Sonnenblick, E., and Babu, A. (1991) *J. Physiol.* 441, 305–324
47. Johnson, J. D., Collins, J. H., Robertson, S. P., and Potter, J. D. (1980) *J. Biol. Chem.* 255, 9635–9640
48. Lilly, L. S. (2006) *Pathophysiology of Heart Disease: A Collaborative Project of Medical Students and Faculty*, 4th Ed., Lippincott Williams and Wilkins, Philadelphia
49. de Simone, G., Kitzman, D. W., Chinali, M., Oberman, A., Hopkins, P. N., Rao, D. C., Arnett, D. K., and Devereux, R. B. (2005) *Eur. Heart J.* 26, 1039–1045
50. de Simone, G., Kizer, J. R., Chinali, M., Roman, M. J., Bella, J. N., Best, L. G., Lee, E. T., and Devereux, R. B. (2005) *Am. J. Hypertens.* 18, 191–196
51. Palacci, V., de Simone, G., Arnett, D. K., Bella, J. N., Kitzman, D. W., Oberman, A., Hopkins, P. N., Province, M. A., and Devereux, R. B. (2001) *Am J. Cardiol.* 88, 1163–1168
52. Harada, K., and Potter, J. D. (2004) *J. Biol. Chem.* 279, 14488–14495
53. Yanaga, F., Morimoto, S., and Ohtsuki, I. (1999) *J. Biol. Chem.* 274, 8806–8812
54. Watkins, H., McKenna, W. J., Thierfelder, L., Suk, H. J., Anan, R., O’Donoghue, A., Spirito, P., Matsumori, A., Moravec, C. S., Seidman, J. G., and Seidman, C. E. (1995) *N. Engl. J. Med.* 332, 1058–1064
55. Fabiato, A. (1981) *J. Gen. Physiol.* 78, 457–497
56. Miller, T., Szczesna, D., Housmans, P. R., Zhao, J., de Freitas, F., Gomes, A. V., Culbreath, L., McCue, J., Wang, Y., Xu, Y., Kerrick, W. G., and Potter, J. D. (2001) *J. Biol. Chem.* 276, 3743–3755
57. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) *Nature* 424, 35–41