Physiol Genomics 33: 257–266, 2008.
First published February 19, 2008; doi:10.1152/physiolgenomics.00154.2007.

Familial hypertrophic cardiomyopathy-related cardiac troponin C mutation L29Q affects Ca^{2+} binding and myofilament contractility

Bo Liang,[1,2] Franca Chung,[1,2] Yang Qu,[1,2] Dmitri Pavlov,[1,2] Todd E. Gillis,[3] Svetlana B. Tikunova,[4] Jonathan P. Davis,[4] and Glen F. Tibbits[1,2]

[1]*Cardiac Membrane Research Laboratory, Kinesiology, Simon Fraser University, Burnaby;* [2]*Cardiovascular Sciences, Child & Family Research Institute, Vancouver, British Columbia;* [3]*Integrative Biology, University of Guelph, Guelph, Ontario, Canada; and* [4]*Department of Physiology and Cell Biology, The Ohio State University, Columbus, Ohio*

Submitted 13 July 2007; accepted in final form 8 February 2008

Liang B, Chung F, Qu Y, Pavlov D, Gillis TE, Tikunova SB, Davis JP, Tibbits GF. Familial hypertrophic cardiomyopathy-related cardiac troponin C mutation L29Q affects Ca^{2+} binding and myofilament contractility. *Physiol Genomics* 33: 257–266, 2008. First published February 19, 2008; doi:10.1152/physiolgenomics.00154.2007.—The cardiac troponin C (cTnC) mutation, L29Q, has been found in a patient with familial hypertrophic cardiomyopathy. We previously showed that L29, together with neighboring residues, Asp2, Val28, and Gly30, plays an important role in determining the Ca^{2+} affinity of site II, the regulatory site of mammalian cardiac troponin C (McTnC). Here we report on the Ca^{2+} binding characteristics of L29Q McTnC and D2N/V28I/L29Q/G30D McTnC (NIQD) utilizing the $Phe^{27} \rightarrow Trp$ (F27W) substitution, allowing one to monitor Ca^{2+} binding and release. We also studied the effect of these mutants on Ca^{2+} activation of force generation in single mouse cardiac myocytes using cTnC replacement, together with sarcomere length (SL) dependence. The Ca^{2+}-binding affinity of site II of L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ was ~1.3- and ~1.9-fold higher, respectively, than that of $McTnC^{F27W}$. The Ca^{2+} disassociation rate from site II of L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ was not significantly different than that of control ($McTnC^{F27W}$). However, the rate of Ca^{2+} binding to site II was higher in L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ relative to control (~1.5-fold and ~2.0-fold respectively). The Ca^{2+} sensitivity of force generation was significantly higher in myocytes reconstituted with L29Q McTnC (~1.4-fold) and NIQD McTnC (~2-fold) compared with those reconstituted with McTnC. Interestingly, the change in Ca^{2+} sensitivity of force generation in response to an SL change (1.9, 2.1, and 2.3 μm) was significantly reduced in myocytes containing L29Q McTnC or NIQD McTnC. These results demonstrate that the L29Q mutation enhances the Ca^{2+}-binding characteristics of cTnC and that when incorporated into cardiac myocytes, this mutant alters myocyte contractility.

cardiac thin filament; myocardial contractility

HYPERTROPHIC CARDIOMYOPATHY, an inherited cardiac disorder, is characterized by interventricular septum and left ventricular wall hypertrophy, myofibrillar disarray, and sudden cardiac death (43). Familial hypertrophic cardiomyopathy (FHC) can be caused by mutations in at least 10 different cardiac sarcomeric genes, including those encoding sarcomeric proteins on both thick and thin filaments (34, 37, 42). A salient finding in genetic analyses of an inherited condition is that a single clinical entity could be the result of mutations in multiple genes. Similarly, different mutations within a single gene can give rise to surprisingly diverse clinical conditions. Although the clinical phenotypes of the cardiomyopathies vary, two common features are present in most cardiomyopathy patients: altered Ca^{2+} sensitivity of force development and impaired energy metabolism (2, 3, 20). Several groups have postulated that myofilament Ca^{2+} sensitivity is the underlying mechanism of these cardiomyopathies. In fact, it has been hypothesized that all FHC-associated tropomyosin and troponin mutations increase myofilament sensitivity, and, conversely, dilated cardiomyopathy-associated mutations are associated with decreased Ca^{2+} sensitivity (8, 19, 20), however, these relationships remain controversial. In addition, mutations in other sarcomeric proteins, such as the regulatory myosin light chain (E22K), which are associated with FHC, have frequently been shown to increase the Ca^{2+} sensitivity of force generation (39). However, the mechanistic link between changes in myofilament Ca^{2+} sensitivity and the etiology of FHC is unknown.

Myofilament Ca^{2+} sensitivity plays an important role in the determination of striated muscle contractility. There are myriad factors contributing to this parameter, including, for example, TnI phosphorylation (and therefore any β-adrenergic agonist), expression of different TnT isoforms (5, 7), temperature, pH, and sarcomere length (SL) (21, 29). Despite being of both clinical and scientific importance, the molecular mechanisms that regulate myofilament Ca^{2+} sensitivity are not well understood. Theoretically, the possible mechanisms modifying myofilament Ca^{2+} sensitivity may include altered Ca^{2+} binding to the N-domain of troponin C (TnC) and modified kinetics of cross-bridge cycling (21, 35). Ca^{2+} binding to cardiac TnC (cTnC) changes its structure, and the signal is further transmitted to other subunits of the contractile apparatus via TnC/TnI interaction, which initiates cardiac muscle contraction. Therefore, enhanced Ca^{2+} sensitivity could be achieved by stabilizing the Ca^{2+}-dependent interaction between the N-domain of cTnC (cNTnC) and the regulatory region of cTnI or by increasing the Ca^{2+}-binding affinity of cNTnC.

Our previous studies have shown that the Ca^{2+} sensitivity of force generation in salmonid cardiac myofibrils is >10-fold greater than in those isolated from mammalian hearts when measured at the same temperature (9, 18). We demonstrated that one reason for the high Ca^{2+} sensitivity of trout cardiac myofibrils is the cTnC homolog that they express. ScTnC has 13 sequence differences from that of mammalian cTnC (McTnC) and has a Ca^{2+} affinity more than twofold greater than McTnC (17). By making a series of mutations, we have identified that the residues responsible for the high Ca^{2+} affinity of ScTnC are within the NH_2 terminus: Asn2, Ile28, Gln29, and Asp30. When the corresponding residues, Asp2, Val28, Leu29, and

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

Address for reprint requests and other correspondence: G. F. Tibbits, Cardiac Membrane Research Lab., Simon Fraser Univ., Burnaby, BC, V5A 1S6, Canada (e-mail: tibbits@sfu.ca).

Gly30 in McTnC, were mutated to those of the trout-equivalent amino acids, the Ca^{2+}-binding affinities of these McTnC mutants [D2N/V28I/L29Q/G30D (NIQD) McTnC] increased significantly up to the level of that of ScTnC (15). Furthermore, cTnC replacement studies using single rabbit cardiac myocytes demonstrated that NIQD cTnC increases the Ca^{2+} sensitivity of force generation by about twofold compared with myocytes containing either native rabbit cTnC or recombinant McTnC (15). However, how the replacement of these residues in McTnC with the salmonid equivalents affects the kinetics of Ca^{2+} binding to the cTnC regulatory site (site II) is not known. The finding of one of these mutations, L29Q, in a patient with FHC (24) made the further study of L29Q and its nearby NH_2-terminal mutations of McTnC compelling.

In the present paper we investigated the effects of the L29Q and NIQD McTnC mutations of McTnC on the Ca^{2+} binding and exchange at the regulatory site II of McTnC in vitro. For this purpose we utilized the F27W reporter, allowing us to monitor the fluorescence changes during the Ca^{2+} titration of McTnC ($McTnC^{F27W}$) and its mutants. In addition to our in vitro experiments, we studied cardiac myocyte contractile properties by using single skinned cardiac myocytes, in which the endogenous TnC was successfully replaced with the L29Q and NIQD McTnC mutants. The results shed more light on the structure-function relationship of cTnC and provide greater insight into the mechanisms of cardiac diseases caused by sarcomeric protein mutations.

METHODS

Protein mutagenesis and purification. The related cDNA constructs were synthesized by PCR according to methods similar to those previously described (17). We used bovine cTnC as being representative of McTnC. $McTnC^{F27W}$, L29Q $McTnC^{F27W}$, D2N/V28I/L29Q/G30D $McTnC^{F27W}$ (NIQD $McTnC^{F27W}$), L29Q McTnC, D2N/V28I/L29Q/G30D McTnC (NIQD McTnC), and wild-type McTnC (WT McTnC) were constructed from bovine cTnC plasmid and $ScTnC^{F27W}$ from salmonid cTnC plasmid by using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The plasmids for $McTnC^{F27W}$, McTnC, and their mutants were transformed into *Escherichia coli* BL21 (DE3) pLysS cells (Novagen, San Diego, CA). Expression and purification of the proteins were carried out as described previously (15, 17). The identities of all cTnC mutants were confirmed by NH_2-terminal microsequencing and amino acid analysis. The purities of the isolated proteins as well as their atomic masses were determined by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry.

Determination of Ca^{2+}-binding affinities. All of the steady-state fluorescence measurements were carried out as previously described (16, 17) by using a PerkinElmer (Wellesley, MA) LS50B spectrofluorometer with the cuvette maintained at 21.0 ± 0.1°C. Briefly, the fluorescence emissions of $cTnC^{F27W}$ and its mutants were measured during Ca^{2+} titration by using an excitation wavelength of 276 nm and an emission wavelength of 330 nm. The Ca^{2+}-dependent component of the fluorescence change was determined by subtracting the fluorescence at basal [Ca^{2+}] from all other measurements and then expressing the resultant values as percentages of the maximum fluorescence. The Ca^{2+}-binding affinities were reported as $K_{f1/2}$ ([Ca^{2+}] at half-maximal fluorescence). The solutions used in these studies were identical to those previously described (17). The concentration of MOPS free acid and its sodium salt required to equal 50 mM and achieve a pH 7.0 at 21°C was calculated, and then actual pH was measured and adjusted to 7.0 at 21°C.

Determination of Ca^{2+} dissociation rate constants. Ca^{2+} dissociation rate constants (k_{off}) were measured at 15°C and 5°C as previously described (44) by using a stopped-flow apparatus with a dead time of 1.5 ms (model SX. 18 MV, Applied Photophysics, Leatherhead, UK). The samples were excited at 285 nm using a 150W xenon arc source, and the tryptophan emission was monitored through a UV-transmission black glass filter (UG1) from Oriel (Stratford, CT). The data were fit using a computer program that utilizes the nonlinear Levenberg-Marquardt algorithm written by P. J. King (Applied Photophysics). Each k_{off} value represents an average of at least three separate experiments with each averaging at least five traces fit with a single exponential equation (variance $<8\times10^{-4}$). The buffer used for all fluorescence stopped-flow measurements was: (in mM) 10 MOPS, 90 KCl, 3 Mg^{2+}, 1 DTT, pH 7.0, at 15 and 5°C separately.

Estimation of Ca^{2+} association rate constants. The $cTnC^{F27W}$ and its mutants were exposed to artificial Ca^{2+} transients (ACTs) to estimate the rate of Ca^{2+} association to the regulatory Ca^{2+}-binding site (k_{on}) (10, 44). ACTs of various amplitudes and durations were generated in a stopped-flow apparatus by rapidly mixing one solution of known Ca^{2+} concentration with another solution with a known concentration of the Ca^{2+} chelator, EGTA. For each $cTnC^{F27W}$ protein, its ACTs were generated by rapidly mixing the buffer containing 2 μM protein with 1 mM EGTA to allow for the free Ca^{2+} concentration to be varied from 10 to 5,000 μM. Before mixing, the NH_2 terminus of cTnC was in its apo state. After mixing and prior to Ca^{2+} removal from the protein and chelating free Ca^{2+} with EGTA, the NH_2 terminus of the $cTnC^{F27W}$ was transiently occupied by Ca^{2+}. For each ACT, the percentage of transient occupancy trace of each $cTnC^{F27W}$ protein was determined at selected time points, typically ≥2 ms after mixing is completed. Each transient occupancy trace is an average of three separate experiments, with each averaging at least 10 separate traces.

k_{on} were then estimated using a computer program KSIM version 1.1 (N. C. Millar, UCLA School of Medicine, Los Angeles, CA) as previously described (44). Equilibrium dissociation constants (K_d) were then calculated from k_{off} and k_{on}.

Skinned cardiomyocyte preparation. Ventricular myocytes were obtained by enzymatic digestion of hearts from C57BL/6 female mice (3–4 mo old). The cell isolation method used was modified slightly from that described by Huang et al. (27). In brief, mice weighing 30–35 g were heparinized (intravenous 1,000 IU/kg), then euthanized by intravenous injection of Thiopental (60–80 mg/kg body wt). The hearts were excised and immersed in an ice-cold, nominally Ca^{2+}-free solution (in mM: 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 5 $MgSO_4$, 50 taurine, 20 glucose, 10 HEPES, pH 7.2). The heart was then cannulated via the aorta and perfused in a retrograde fashion at a constant perfusion speed of 3 ml/min for 4–5 min. After all the blood had been washed out, the heart was perfused at 37°C for an additional 30 min with a nominally Ca^{2+}-free solution containing 0.12–0.16 mg/ml collagenase (Yakult, Tokyo, Japan), then for all additional 10 min with storage solution (in mM: 120 $C_3H_5KNO_4$, 5 $MgCl_2$, 20 taurine, 1 EGTA, 10 glucose, 10 HEPES, pH 7.4) containing 0.1 mg/ml type XIV protease (Sigma, Oakville, ON, Canada). The ventricle was minced in storage solution containing 1% BSA, and then the cell suspension was filtered through a 100 μm nylon mesh. The pellet was washed twice using storage buffer, and the cardiac myocytes were subsequently skinned by suspending the cell pellet in relaxing solution (in mM: 5.70 $MgCl_2$, 6.31 Na_2ATP, 2 EGTA, 156 potassium propionate, 10 BES, 1 DTT, 0.1 leupeptin, 0.1 PMSF, pH 7.0) containing 0.3% Triton-X for 4 min. The pellet was washed twice with a cold relaxing solution. Finally, the skinned myocytes were resuspended in relaxing solution and kept on ice during the day of the experiment.

Determination of the Ca^{2+} sensitivity of force generation and length dependence of myofilament Ca^{2+} sensitivity. The apparatus used to measure SL and force from skinned cardiac myocytes and for rapid solution changes has been described previously (15). Briefly, a single cardiac myocyte was attached to a force transducer and a

servomotor, both of which were controlled by micromanipulators. The myocyte was gravity-superfused using a special three-barrel pipette attached to a fast-step switcher (Warner Instruments, Hamden, CT) for quick solution change. The experimental temperature-controlled chamber was mounted on a stage of a Nikon (Mississauga, ON, Canada) Diaphot inverted microscope. Myocytes were imaged using a Pulnix (Sunnyvale, CA) charge-coupled device camera, and the SLs were measured by fast Fourier transform analysis of the digitized striated images of the attached cell. Figure 1*A* shows a mouse myocyte attached to a force transducer and a servo motor in preparation for cTnC extraction-reconstitution and force-pCa relationship determination. Endogenous cTnC was extracted from the cell by exposing the myocytes to an extracting solution (in mM: 5 K_2EDTA, 1 DTT, 0.1 leupeptin, 0.1 PMSF, 20 Tris, pH 7.2) for 5 min at 15°C. The skinned myocyte was then reconstituted by 30 min incubation in relaxing solution containing 10 μM of the recombinant McTnC; representative force traces are shown in Fig. 1*B*. Maximal Ca^{2+}-activated force (F_{max}) at pCa 4.5 prior to extraction was used in subsequent calculations for determining the force loss and recovery due to cTnC extraction and reconstitution. F_{max} was measured at the beginning, middle, and end of the experiment to assess the rundown of the preparation, and the data from the myocytes were not included in the final data analysis if the force declined by >10% in a successive test contraction at maximum activation. Data from myocytes that showed altered sarcomere pattern after TnC extraction and reconstitution were also discarded. The steady-state force-pCa relationship was determined by measuring the Ca^{2+}-activated force (F) with SL set to 2.1 μm, as the reconstituted myocyte was exposed to a series of solutions with varied Ca^{2+} concentration (pCa 8.0–4.5). The length-dependent Ca^{2+} sensitivity was determined in a separate experiment by carrying out two force-pCa measurements for each myocyte: one with the SL of the myocytes set to 1.9 μm, and another with the SL set to 2.3 μm.

Data analysis. Each force-pCa curve was fitted to the Hill equation using Origin 6.0:

$$\frac{F}{F_{max}} = \frac{[Ca^{2+}]^{n_H}}{K^{n_H} + [Ca^{2+}]^{n_H}},$$

where n_H is the Hill coefficient and K represents the Ca^{2+} sensitivity of force generation, which corresponds to the $[Ca^{2+}]$ required for generating half-maximal force. The data were analyzed statistically using Student's *t*-test and one-way repeated-measures analysis of variance (one-way ANOVA). The values of $[Ca^{2+}]$ at half-maximal force ($K_{F1/2}$) were reported as means ± SD in μM. Two means were considered to be significantly different when the *P* value was <0.05.

RESULTS

Steady-state Ca^{2+}-binding affinity measurements. The Ca^{2+}-induced increase in Trp fluorescence occurring upon Ca^{2+} binding to the regulatory site of $ScTnC^{F27W}$, $McTnC^{F27W}$, L29Q $McTnC^{F27W}$, and NIQD $McTnC^{F27W}$ is shown in Fig. 2. The Ca^{2+} titration curves of $ScTnC^{F27W}$ and NIQD $McTnC^{F27W}$ were virtually superimposable indicating equivalent Ca^{2+}-binding affinity. The data validate that N2, I28, Q29, and D30 are responsible for the high Ca^{2+} affinity of ScTnC. The titration curves of L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ were shifted to the left of that for $McTnC^{F27W}$. As seen from Table 1, $McTnC^{F27W}$ exhibited a half-maximal Ca^{2+}-dependent increase in its Trp fluorescence ($K_{f1/2}$) at 3.7 ± 0.2 μM, whereas L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ had $K_{f1/2}$ values of 2.8 ± 0.3 and 2.0 ± 0.1 μM, respectively. The Ca^{2+}-binding affinities of L29Q $McTnC^{F27W}$ and NIQD $McTnC^{F27W}$ were ~1.3- and ~1.9-fold, respectively, higher than that of $McTnC^{F27W}$. The results demonstrate that L29Q and NIQD McTnC mutations significantly increased the Ca^{2+}-binding affinity of McTnC (Table 1).

k_{off} measurements. To study the effects of L29Q and NIQD McTnC mutations on Ca^{2+} binding and exchange at the regulatory site II of cTnC, fluorescence stopped-flow technique was used to determine the Ca^{2+} dissociation rate of $cTnC^{F27W}$ and related mutants. Previous studies using this technique had confirmed that the Trp fluorescence of $cTnC^{F27W}$ mutants can accurately report the rate of Ca^{2+} dissociation from the regulatory site of cTnC (14). Figure 3 shows the time course of the Trp fluorescence decrease upon Ca^{2+} removal from cTnC by EGTA. k_{off} of the $cTnC^{F27W}$ proteins ranged from 1,134 ± 89 s^{-1} to 1,188 ± 63 s^{-1} at 15°C (Fig. 3*A*) and 796 ± 23 s^{-1} to 811 ± 29 s^{-1} at 5°C (Fig. 3*B*). The results demonstrated that the sequence differences between McTnC and ScTnC and the

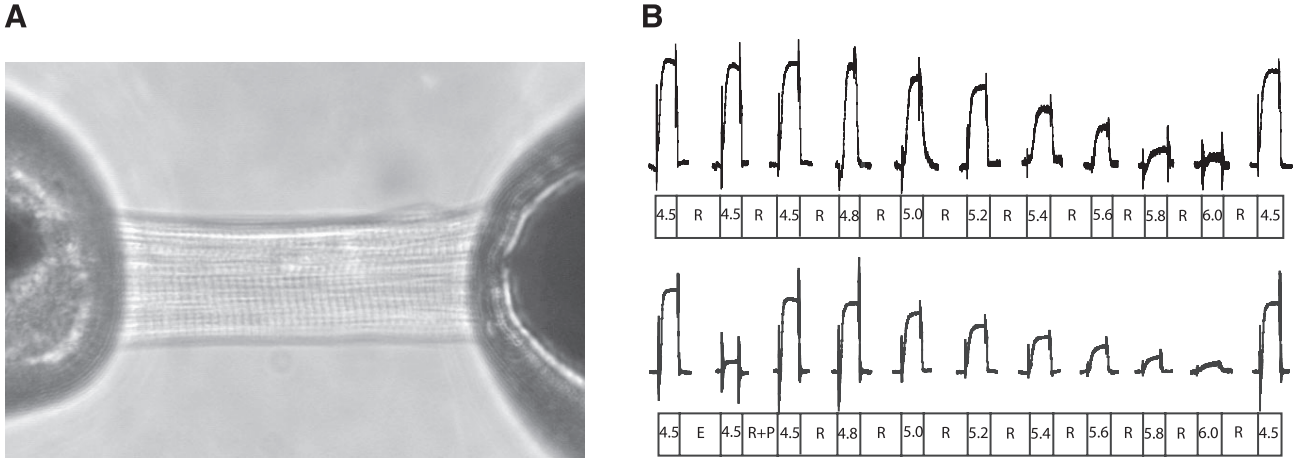

Fig. 1. Representative force traces of a single skinned cardiac myocyte activation using the cardiac troponin C (cTnC) extraction-reconstitution protocol. *A*: mouse myocyte glued to a force transducer on one end and a servo motor (used to control the sarcomere length) on the other end. The image of the myocyte was captured by a charge-coupled device camera and digitized by a frame grabber. The sarcomere length was measured online using a computer program written in LabView (National Instruments) as explained in detail in METHODS. *B*, *top*: force traces of a representative myocyte with endogenous TnC (control). *Bottom*: force traces of a representative myocyte with wild-type mammalian cardiac troponin C (WT McTnC) replaced. R, exposed to relaxing solution; E, exposed to extraction solution; R+P, incubated in relaxing solution containing WT McTnC, D2N/V28I/L29Q/G30D McTnC (NIQD) McTnC, or L29Q McTnC. The numbers represent $[Ca^{2+}]$ of activation solution in pCa units.

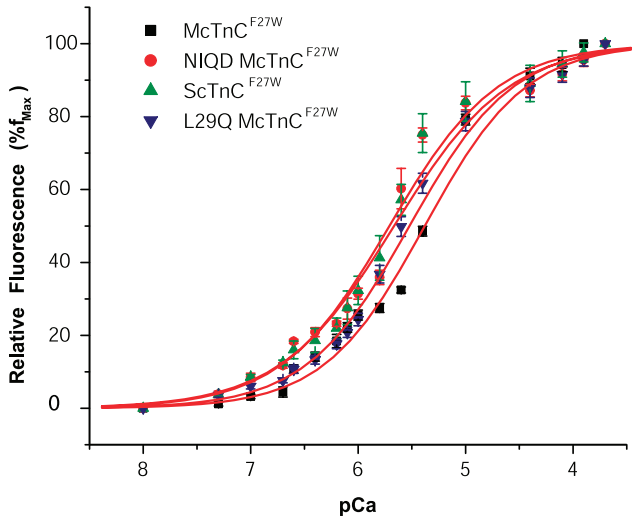

Fig. 2. Comparison of the steady-state Ca^{2+} binding curves of ScTnC^{F27W} (n = 8), NIQD McTnC^{F27W} (n = 8), L29Q McTnC^{F27W} (n = 8), and McTnC^{F27W} (n = 8). Shown are the changes in the reporter Trp fluorescence as a function of [Ca^{2+}] in: ScTnC^{F27W}, McTnC^{F27W}, L29Q McTnC^{F27W}, and NIQD McTnC^{F27W} as determined in a spectrofluorometer at 21°C, pH 7.0. Data are normalized with respect to the maximal fluorescence (f_{Max}) of each Ca^{2+} titration. See Table 1 for the $K_{f1/2}$ values.

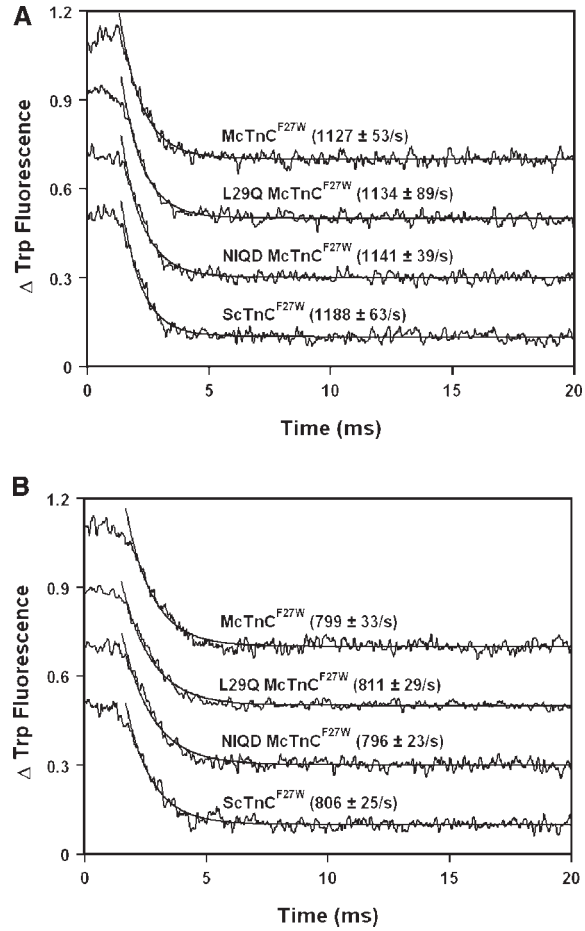

Fig. 3. Rates of Ca^{2+} dissociation from cTnC^{F27W} and its mutants. Cardiac troponin C (2 μM), containing one of McTnC^{F27W}, L29Q McTnC^{F27W}, NIQD McTnC^{F27W}, or ScTnC^{F27W}, in the presence of 500 μM Ca^{2+}, was mixed rapidly with 10 mM EGTA. The fluorescence was monitored through a UV-transmission black glass filter (UG1). All of the kinetic traces were triggered at *time 0*, and the first ~1.6 ms of premixing is shown. The Ca^{2+} dissociation rate constant (k_{off}) values from data fitting are presented as means ± SD. The traces have been displaced vertically to allow for comparison. *A*: measured at 15°C. *B*: measured at 5°C.

residue substitutions in McTnC did not significantly affect the rate of Ca^{2+} release from the regulatory Ca^{2+}-binding site of cTnC at a given temperature.

Ca^{2+} association rate constant estimation. Because the k_{off} was too fast to experimentally determine the Ca^{2+} association rate (k_{on}) by standard pseudo-first order methods in our stopped-flow apparatus (28), we estimated k_{on} by exposing the cTnC^{F27W} proteins to various ACTs of increasing amplitude and duration at 15°C. The experiments were carried out in the presence of 3 mM Mg^{2+} to ensure complete occupation of the COOH terminus binding site of cTnC^{F27W} by Mg^{2+}. Because of the difference in binding affinity for Mg^{2+} and Ca^{2+} to the NH_2-terminal binding site of cTnC, the simulations of the experimental data can represent true Ca^{2+} association rates (44). A solution containing 2 μM ScTnC^{F27W}, McTnC^{F27W}, L29Q McTnC^{F27W}, or NIQD McTnC^{F27W} and 1 mM EGTA was separately mixed with equal volumes of solutions containing 10, 50, 200, or 5,000 μM Ca^{2+}. The results were reported as the time course of Ca^{2+} binding and subsequent dissociation over the time subjected to different ACTs (Fig. 4). The Ca^{2+} k_{on} values could then be estimated since they are directly related to the relative transient Ca^{2+} occupancy of the cTnC. Each individual ACT trace was fitted using the KSIM program, as described previously (44). Figure 5 compares the time course of the decrease in Trp fluorescence of McTnC^{F27W} and its mutants in the presence of 1 mM EGTA upon mixing with 50 μM Ca^{2+}. The k_{on} of ScTnC^{F27W}, McTnC^{F27W}, L29Q McTnC^{F27W}, and NIQD McTnC^{F27W} were 2.1 ± 0.2, 1.0 ± 0.1, 1.5 ± 0.2, and 2.0 ± 0.2 × 10^8 $M^{-1}s^{-1}$, respectively, indicating that the L29Q and NIQD had k_{on} significantly greater than McTnC (Table 1).

K_d were calculated from the k_{off} and k_{on} values. The calculated K_d values for L29Q and NIQD McTnC^{F27W} were 8.0 ± 1.2 μM and 5.7 ± 0.6 μM, respectively (Table 1). The

Table 1. *Summary of Ca^{2+} binding properties and Ca^{2+} sensitivity*

	k_{on}, 10^8 $M^{-1}\cdot s^{-1}$	k_{off}, s^{-1}	K_d, μM	$K_{f1/2}$, μM	$K_{F1/2}$, μM
WT McTnC	1.0±0.1	1,127±53	11.3±1.3	3.7±0.2	4.1±0.5
L29Q McTnC	1.5*± 0.2	1,134±89	8.0*± 1.2	2.8*± 0.3	3.0*± 0.5
NIQD McTnC	2.0*± 0.2	1,141±39	5.7*± 0.6	2.0*± 0.1	2.1*± 0.3
ScTnC	2.1*± 0.2	1,188±63	5.7*± 0.6	2.0*± 0.4	N/A

Values are presented as means ± SD. K_d, $K_{f1/2}$ represent the Ca^{2+} binding affinity calculated from the k_{on} and k_{off} values and determined by steady-state Trp fluorescence titration, respectively. $K_{F1/2}$ represents the Ca^{2+} sensitivity of force in skinned cardiomyocytes reconstituted with wild-type mammalian cardiac troponin C (WT McTnC), L29Q McTnC, or D2N/V28I/L29Q/G30D McTnC (NIQD) McTnC. *Compared with WT McTnC, $P < 0.01$. NA, not available.

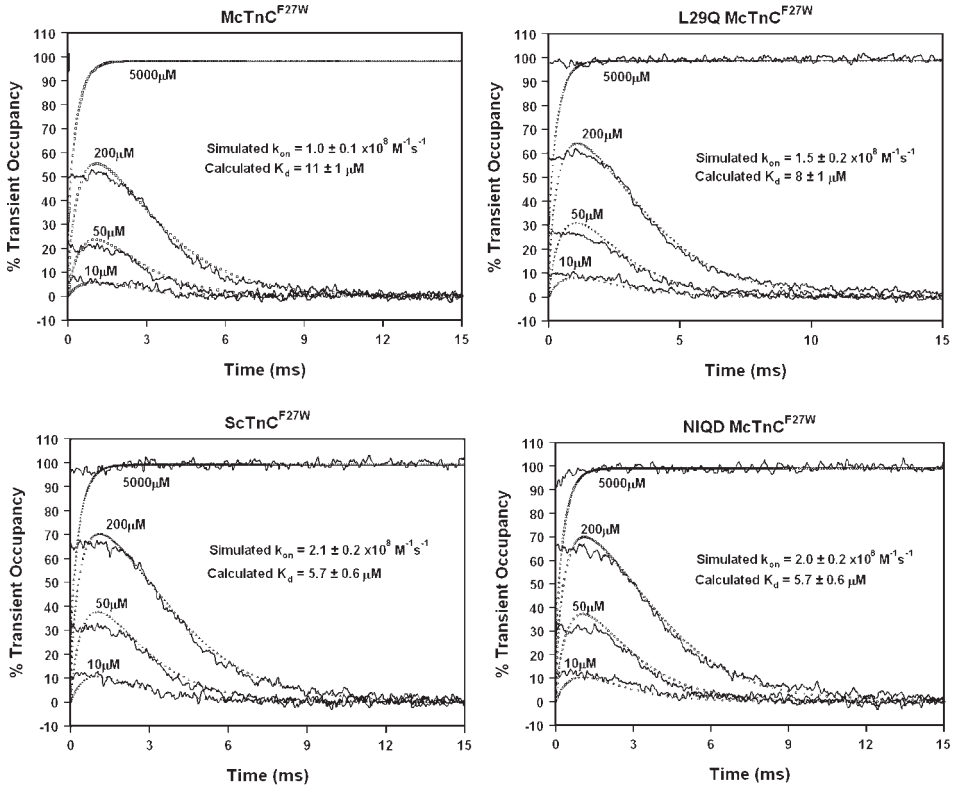

Fig. 4. Percentage of Ca^{2+} occupancy of the regulatory site of $cTnC^{F27W}$ and its mutants during ACTs of different amplitude and duration. Shown are the time courses of the decrease in Trp fluorescence when $cTnC^{F27W}$ or its mutants (2 μM, in the presence of 1 mM EGTA in 10 mM MOPS, 90 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.0, at 15°C) was rapidly mixed with increasing free Ca^{2+} ranging from 10 to 5,000 μM in the same buffer. Each trace represents an average of 3 separate experiments, each averaging at least 10 separate traces. The specific value of $[Ca^{2+}]$ before mixing for each ACT is shown under the trace. The $cTnC^{F27W}$ transient occupancy was simulated using the program KSIM and is shown (□) overlaying the experimental data. The initial [EGTA] was set to 500 μM, and the initial $[Ca^{2+}]$ was set to values ranging from 5 to 2,500 μM to mimic the experimental data after mixing. The simulated Ca^{2+} association rate constant (k_{on}) and the equilibrium dissociation constant (K_d), calculated from k_{on} and k_{off}, are presented as means ± SD.

affinities determined from the K_d values of L29Q and NIQD $McTnC^{F27W}$ were 1.4- and 1.9-fold, respectively, higher than that of $McTnC^{F27W}$ (K_d 11.3 ± 1.3 μM), and in agreement with the affinities determined by steady-state fluorescence measurements (~1.3- and 1.9-fold, respectively, higher than that of $McTnC^{F27W}$).

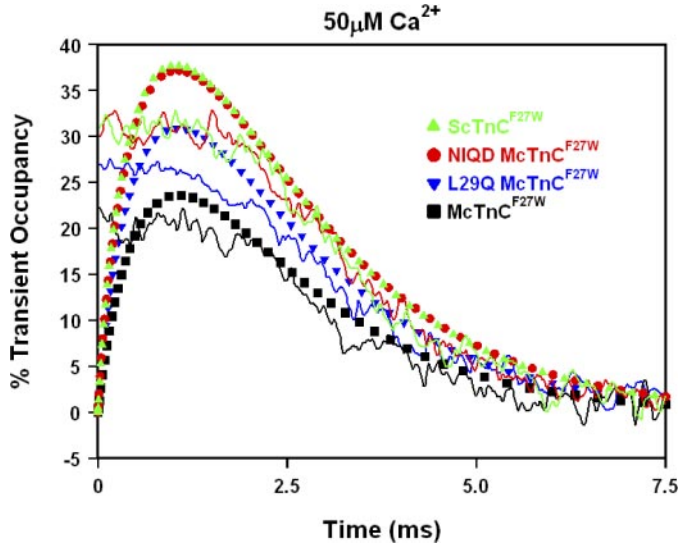

Fig. 5. Comparison of transient occupancy of the regulatory site of $cTnC^{F27W}$ and its mutants. The time course of the decreases in Trp fluorescence is shown when $cTnC^{F27W}$ and its mutants (in the presence of 1 mM EGTA) were rapidly mixed with 50 μM Ca^{2+}. The results from computer simulations for $McTnC^{F27W}$ (■), L29Q $McTnC^{F27W}$ (▼, blue), NIQD $McTnC^{F27W}$ (●, red) and $ScTnC^{F27W}$ (▲, green) (as described in METHODS) are shown overlaying the experimental data.

The steady-state Ca^{2+}-binding affinity measurements and Ca^{2+}-binding kinetics measurements were carried out at our laboratories at SFU and OSU, respectively, using the same batch of proteins. Despite the fact that differences in experimental methods and conditions between the labs resulted in predictable differences in the absolute values, the relative differences between the various constructs observed from two labs are strikingly similar. Both experimental approaches clearly demonstrate that L29Q McTnC and NIQD McTnC mutants significantly increased Ca^{2+}-binding affinity of McTnC.

Ca^{2+} sensitivity of force generation measurements. The Ca^{2+} sensitivity of force generation was measured by determining the force-pCa relationship of skinned cardiac myocytes before and after the endogenous cTnC was replaced with L29Q McTnC, NIQD McTnC or WT McTnC. Because all of the in vitro experiments described previously used the F27W reporter, we did control experiments in which the reporter was included in all constructs used in the single fiber reconstitution experiments. Since there were no significant differences between proteins containing the F27W reporter and those that did not (data not shown), cTnCs without the reporter were used in these experiments. Figure 1*B* illustrates the mechanical behavior of a representative skinned myocyte in which the endogenous cTnC was extracted (E), and then the fiber was reconstituted (R) with WT McTnC. After the endogenous cTnC was extracted, the maximal force generated was <15% of the control isometric force (maximal force before cTnC extraction), indicating an effective extraction. When reconstituted with L29Q McTnC, NIQD McTnC, or WT McTnC, the maximal force of the skinned myocyte recovered to ~80% of the control level. There were no significant differences in the

recovery of the maximal force amplitude (data not shown). These results suggest that there were no significant differences in how L29Q McTnC, NIQD McTnC, and WT McTnC recombinant proteins were incorporated into the skinned myocytes. The loss of force due to cTnC extraction and the level of force recovery following reconstitution of the myocytes with recombinant cTnC are similar to the values previously reported by us (15) and others using comparable protocols (22, 36).

Force-pCa relationships obtained with L29Q McTnC, NIQD McTnC, or WT McTnC at an SL of 2.1 μm are summarized in Fig. 6. In a series of control experiments, force-pCa relationships were also determined with F27W L29Q McTnC, F27W NIQD McTnC, and F27W "WT" McTnC. There were no significant differences in the $K_{F1/2}$ values between each of the constructs used in these experiments and its F27W counterpart (data not shown). In addition, the force-pCa curves of the myocytes containing either endogenous cTnC or recombinant WT McTnC were very similar, and there were no significant differences in the $K_{F1/2}$ ($P > 0.05$). These results demonstrate that the cTnC replacement procedure had minimal affect on the Ca^{2+} sensitivity of force generation. There were no significant differences in the Hill coefficients between the various cTnC constructs (n_H was 2.13–2.32, data not shown). The force-pCa curves generated by the myocytes reconstituted with L29Q or NIQD McTnC were shifted to the left of those of myocytes reconstituted with WT McTnC, indicating an increase in Ca^{2+} sensitivity. The $K_{F1/2}$ values for L29Q McTnC and NIQD McTnC were 3.0 ± 0.5 and 2.1 ± 0.3 μM, respectively. Compared with a $K_{F1/2}$ value of 4.1 ± 0.5 μM for WT McTnC, the Ca^{2+} sensitivities for L29Q McTnC and NIQD McTnC were increased 1.4- and 2.0-fold, respectively. These results demonstrate that the L29Q McTnC and NIQD McTnC mutants significantly increased Ca^{2+} sensitivity of force generation in skinned cardiac myocytes at an SL of 2.1 μm (Table 1).

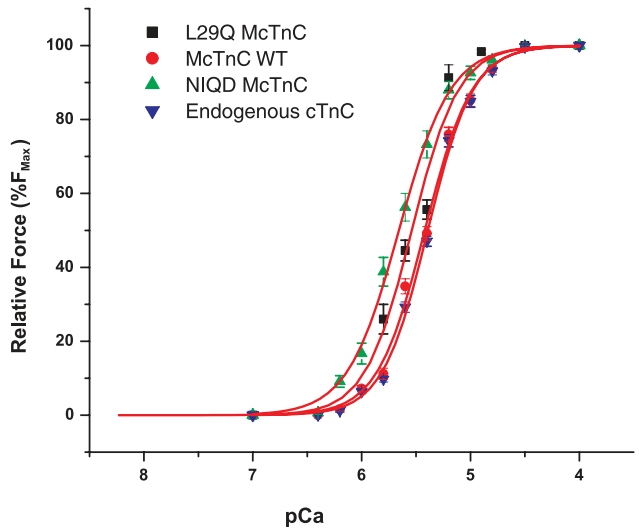

Fig. 6. Comparison of the force-pCa relationships of skinned mouse cardiac myocytes containing endogenous cTnC or McTnC WT and its mutants at sarcomere length 2.1 μm. The data are expressed as normalized force, which was calculated as a fraction of the force generated at pCa 4.5. Data points are means ± SD of 6–8 myocytes at 15°C, pH 7.0, and were fit by the Hill equation. The derived [Ca^{2+}] at half-maximal fluorescence ($K_{F1/2}$) values were: 3.8 ± 0.4 μM for endogenous cTnC, 4.1 ± 0.5 μM for WT McTnC, 3.0 ± 0.5 μM for L29Q McTnC, and 2.1 ± 0.3 μM for NIQD McTnC.

Length dependence of myofilament Ca^{2+} sensitivity measurements. To study further the effects of L29Q and NIQD McTnC on the contractile properties of cardiac muscle, we measured the changes in length-dependent Ca^{2+} sensitivity of single skinned cardiac myocytes reconstituted with WT McTnC, L29Q, or NIQD McTnC. In control myocytes (with endogenous cTnC), the force-pCa relationship was shifted to the left when SL was increased from 1.9 to 2.3 μm. The $K_{F1/2}$ decreased from 4.6 ± 05 to 2.7 ± 0.2 μM ($\Delta K_{F1/2} = 1.9 \pm 0.3$ μM), indicating a significant length-dependent Ca^{2+} sensitivity increase (Ca^{2+} sensitivity increased 1.7-fold as SL increased from 1.9 to 2.3 μm). When endogenous cTnC was replaced with WT McTnC, the force-pCa curve shift was very similar to that of the nonreconstituted myocytes (Fig. 7), and the $K_{F1/2}$ decreased from 4.8 ± 0.3 to 2.9 ± 0.3 μM, as the SL was increased from 1.9 to 2.3 μm. There was no significant difference in $K_{F1/2}$ between endogenous cTnC and McTnC (Table 2), indicating again that the cTnC replacement procedure had minimal effect. For L29Q or NIQD McTnC substitution, the leftward shift of the force-pCa curve at longer SLs was significantly less than that for WT McTnC substitution (Fig. 7). The $\Delta K_{F1/2}$ values of L29Q and NIQD McTnC constructs were 1.2 ± 0.3 and 0.7 ± 0.3 μM, respectively, notably different from that of WT McTnC (2.0 ± 0.1 μM, Table 2). These results demonstrate that both L29Q and NIQD McTnC significantly reduce the length-dependent Ca^{2+} sensitivity of skinned cardiac myocytes.

At SL 1.9 and 2.3 μm, the $K_{F1/2}$ values for L29Q McTnC and NIQD McTnC were significantly lower than that of WT McTnC (Table 2), indicating the Ca^{2+} sensitivities for L29Q McTnC and NIQD McTnC are higher than that of WT McTnC at both of the SLs. (~1.3- and ~1.7-fold at SL 1.9 μm, ~1.2- and ~1.5-fold at SL 2.3 μm, higher than that of WT McTnC, for L29Q McTnC and NIQD McTnC, respectively).

These data and the data collected at 2.1 μm SL (Table 1) demonstrate that replacing endogenous cTnC with NIQD McTnC or L29Q McTnC increased Ca^{2+} sensitivity of force generation at all three SLs tested, but significantly reduced length-dependent myofilament Ca^{2+} sensitivity in mouse skinned cardiac myocytes. In Fig. 8 the Ca^{2+} sensitivity of force generation is plotted against SL for the myocytes reconstituted with WT McTnC or the two mutant McTnCs. It should be noted that while the correlation coefficients of the linear regressions are excellent (0.84–0.99), it does not necessarily imply that this relationship is linear given the number of data points. This figure demonstrates that an increase in SL caused a greater increase in Ca^{2+} sensitivity in myocytes containing McTnC than in those containing either L29Q McTnC or NIQD McTnC. This difference is reflected in the slope of the lines for each data set. Additionally, it appears that the myocytes containing NIQD McTnC were less affected by a change in SL than those containing L29Q McTnC.

DISCUSSION

Mutations in cardiac thin filament proteins are responsible for ~25% of total FHC cases (33, 43). Most of the FHC-related thin filament mutations are found in TnT, TnI, and tropomyosin. L29Q is the first mutation in cTnC found in a patient with FHC. It should be noted that the role of this mutation in the etiology of FHC remains unclear because this is the only

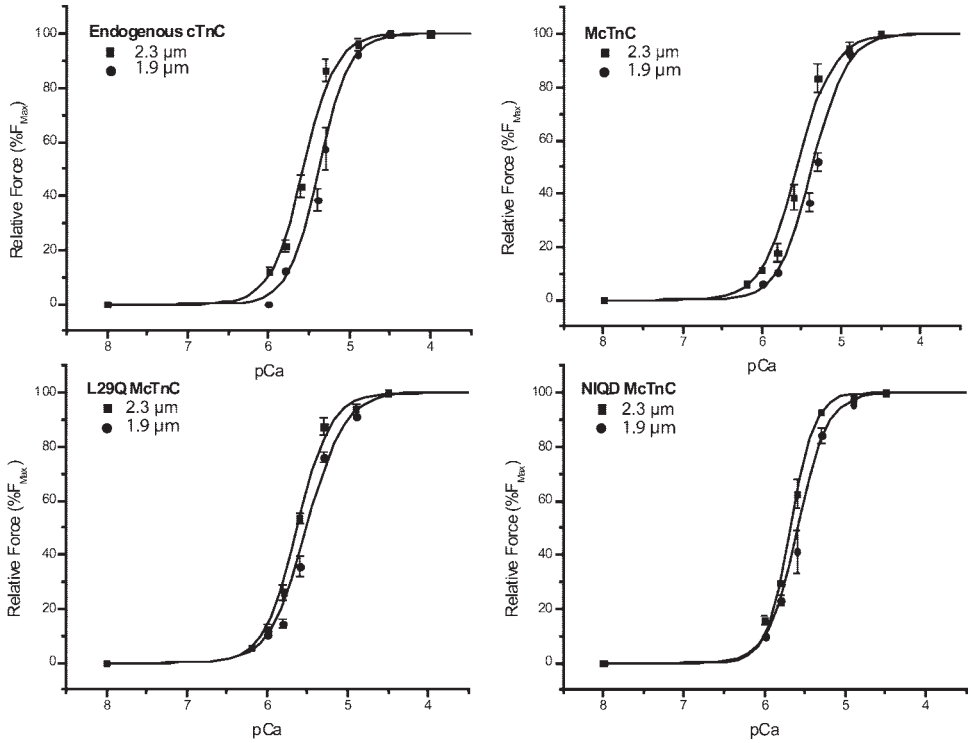

Fig. 7. Changes of force-pCa relationship as sarcomere length (SL) was increased from 1.9 to 2.3 μm in skinned cardiac myocytes reconstituted with WT McTnC, L29Q McTnC, or NIQD McTnC. The data are expressed as normalized force, which was calculated as a fraction of the force generated at pCa 4.5 at each SL. Data points are means ± SD of 5–6 myocytes at 15°C, pH 7.0, and the lines represent best fits using the Hill equation. See Table 2 for the derived $K_{F1/2}$ values and Hill coefficients.

reported case of this mutation to date and more experimentation is required to link this mutation to FHC. However, the data in this study clearly show that the L29Q mutation is not benign and is likely to have a significant impact on contractility. The present data demonstrate that both L29Q and NIQD mutations in McTnC significantly increased Ca^{2+}-binding affinity of McTnC by increasing the rate of Ca^{2+} association to the regulatory site II of McTnC, and correspondingly, increased the Ca^{2+} sensitivity of force generation in single skinned cardiac myocytes. Moreover, the length-dependent difference in Ca^{2+} sensitivity was reduced significantly in the myocytes reconstituted with L29Q McTnC or NIQD McTnC. These results provide the first in situ evidence that the FHC-related L29Q mutation and its nearby NH_2-terminal mutations, NIQD, affect Ca^{2+}-binding kinetics and cardiac myofilament contractility. These observations are consistent with the notion that the etiology of FHC caused by the mutations in thin filament regulatory proteins involves increasing myofilament Ca^{2+} sensitivity.

TnC has eight α-helices (designated as A through H) associated with four Ca^{2+}-binding sites and an extra α-helix at the NH_2 terminus (N-helix). Ca^{2+} binding to the regulatory sites of skeletal TnC causes helices B and C to move away from helices N, A, and D, exposing a hydrophobic patch. The regulatory N-domain switches from a "closed" to an "open" conformation. Ca^{2+} binding to the single regulatory site of cTnC does not cause similar hydrophobic exposure because of the defunct site 1, and also because the N-domain remains in a relatively "closed" conformation (41). In cardiac muscle, both Ca^{2+} binding to the regulatory site of cTnC and cTnI are required to stabilize the "open" conformation of cNTnC (12, 32, 41). A study by Tikunova and Davis (44) demonstrated that the substitution of any of the hydrophobic amino acids at positions 20, 44, 45, 48, and 81 by a single glutamine resulted in an increase in Ca^{2+} affinity in the NH_2 terminus of TnC. These authors postulated that the glutamine substitution reduces the hydrophobic contact between helices B and C with N, A, and D in the apo state, thereby destabilizing the protein and facilitating the opening of the TnC molecule during Ca^{2+} activation and increasing the Ca^{2+} binding affinity (44). In McTnC, Leu29 is located in the transition region of helix A within the nonfunctional Ca^{2+}-binding loop of McTnC. The glutamine insertion at this location (L29Q) could therefore decrease the stability of the helix, thereby making it easier for

Table 2. *$K_{F1/2}$ and Hill coefficients as a function of sarcomere length increase from 1.9 to 2.3 μm*

	$K_{F1/2}$, μM			Hill Coefficient		
	1.9 μm	2.3 μm	Δ %	1.9 μm	2.3 μm	Δ %
Endogenous cTnC (n = 6)	4.6±0.5	2.7±0.2	170	2.4±0.1	2.5±0.1	104
WT McTnC (n = 5)	4.8±0.3	2.9±0.3	166	2.4±0.1	2.5±0.1	104
NIQD McTnC (n = 6)	2.8±0.4*	2.0±0.1*	144	2.5±0.1	2.7±0.1†	108
L29Q McTnC (n = 6)	3.7±0.4*	2.5±0.3	148	2.4±0.2	2.6±0.1	108

Unless indicated otherwise, values are presented as means ± SD. The effect of change in sarcomere length (from 1.9 to 2.3 μm) on myofilament Ca^{2+} sensitivity and the Hill coefficient is expressed as percentage change. Compared with WT McTnC, *$P < 0.05$; compared with 1.9 μm sarcomere length, †$P < 0.05$.

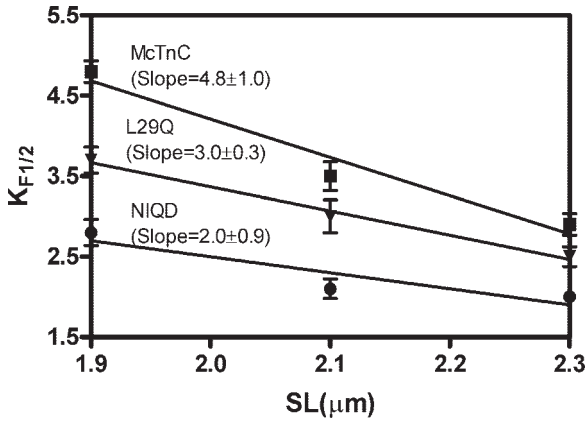

Fig. 8. Ca^{2+} sensitivity of force generation as a function of SL for myocytes containing WT McTnC and the 2 mutant McTnCs. The $K_{F1/2}$ values from the experiments shown in Fig. 6 (for SL 2.1 μm) and Fig. 7 (for SLs 1.9 and 2.3 μm) are plotted as a function of SL. The data were fit to a linear regression line (r^2 for McTnC, L29Q McTnC and NIQD McTnC are 0.96, 0.99, and 0.84, respectively) and the resultant slopes for each construct are indicated on the graph.

helices B and C to move away from helices N, A, and D. This would increase the size of the hydrophobic surface area exposed upon activation, as well as facilitate Ca^{2+} binding to regulatory site II of cTnC. This proposal is supported by work of Blumenschein et al. (6), who used nuclear magnetic resonance spectroscopy to study the backbone dynamics of the regulatory domain of salmonid cardiac TnC (ScNTnC aa 1–89). Their results demonstrate that site I of ScNTnC is more flexible than site II, resulting in a slightly more open structure. The N-domain of NIQD McTnC is only one amino acid different from ScNTnC, suggesting that NIQD McTnC is likely to be structurally very similar to ScTnC, especially since both TnCs contain the glutamine at residue 29. The amino acids substitutions responsible for the higher Ca^{2+} affinity in ScTnC may increase the molecular flexibility and decrease the compactness of either a selected area or the overall protein structure. This would help decreasing the energy required for Ca^{2+} activation and would result in higher Ca^{2+} affinity. In the present study, the Ca^{2+} affinity of L29Q McTnC is intermediate between McTnC and NIQD McTnC. These results support our hypothesis that L29Q McTnC and NIQD McTnC increased the Ca^{2+} binding affinities and affected the Ca^{2+} binding kinetics by causing similar structural changes as in ScTnC.

The Ca^{2+} dissociation rate from TnC is an important factor determining muscle relaxation rate. An increase in Ca^{2+}-binding affinity may result in impaired relaxation because Ca^{2+} would dissociate at a comparatively slow rate. The present study demonstrated that there was very little variation in the k_{off} values of the $cTnC^{F27W}$ proteins determined at both 5 and 15°C, suggesting that L29Q and NIQD mutations do not affect Ca^{2+} dissociation from the regulatory binding site when only cTnC is present in vitro. As a consequence, the changes in Ca^{2+}-binding affinities observed between the different constructs were, probably, solely due to changes in the rate of Ca^{2+} binding to the TnC regulatory site (k_{on}). Since the changes in k_{on} could potentially reflect a perturbation of the TnC apo state, the increase in k_{on} can be interpreted as a decrease in the energy required for the conformational changes of the protein (44). The data obtained are consistent with the idea that NH_2-terminal mutations of McTnC increase the Ca^{2+} affinity by destabilizing the apo structures of the NH_2-terminal domain and facilitating the helices movement induced by Ca^{2+} binding.

In the tightly coupled multicomponent cardiac regulatory system structural changes in one part of it may often cause allosteric structural and functional changes either in other connected parts or in the whole system. Various FHC-related mutations in thin filaments' regulatory proteins, for example, increase Ca^{2+} sensitivity of ATPase activity and force development by increasing the Ca^{2+} binding affinity to cTnC. Another example of such allosteric effects is an effect of phosphorylation of cTnI at two NH_2-terminal serine residues (Ser-22 and -23), which decreases Ca^{2+} sensitivity of the muscle by causing a decrease in Ca^{2+} binding to the regulatory site on cTnC (1, 31, 38, 47). These authors found that an interaction between the NH_2-terminal extension of cTnI and the NH_2-terminal regulatory domain of cTnC may affect the conformation of the regulatory domain of cTnC by shifting the equilibrium toward an "open" structure. That area of troponin subunits interaction, therefore, seems to be especially interesting. In the present study, the NH_2-terminal mutations of McTnC, L29Q, and NIQD are located in the region of cTnC that interacts with cTnI during Ca^{2+} activation (13, 40, 47). Ca^{2+} sensitivity of force generation and ATPase activity can be influenced by increased TnC Ca^{2+} binding affinity (23, 25, 26, 30, 45) The present data demonstrate that the changes in Ca^{2+}-binding affinity of L29Q McTnC and NIQD McTnC mutants may influence the changes in Ca^{2+} sensitivity sensitivities of force generation and, therefore, cause a global affect to the muscle contractile properties. Similarly, an increase in Ca^{2+} binding affinities resulting from the affected conformational changes in the regulatory domain of McTnC should in turn affect Ca^{2+} sensitivity of force generation. By replacing endogenous cTnC with related McTnC mutants, we demonstrated that L29Q McTnC and NIQD McTnC significantly increased the Ca^{2+} sensitivity of force generation in single skinned mouse cardiac myocytes. The changes of Ca^{2+} sensitivities of force generation parallel the changes of affinities of Ca^{2+} binding to related isolated cTnC mutants, suggesting that L29Q McTnC and NIQD McTnC increase the Ca^{2+} sensitivity of contractile apparatus mainly by the enhancement of the Ca^{2+} binding affinity. Indeed, to fully understand the relationship between the Ca^{2+} sensitivity and Ca^{2+} binding found in the present study, more information on Ca^{2+} binding directly in skinned cardiac myocytes is required.

Schmidtmann et al. (40) measured the Ca^{2+}-dependent actomyosin subfragment 1 (actoS1)-ATPase activity and the sliding velocity of thin filaments reconstituted with L29Q cTnC mutant and cTnI, dephosphorylated or phosphorylated at serine 22/23 in an in vitro motility assay. These authors reported a small (~0.1 pCa unit) but significant reduction in Ca^{2+} sensitivity when they reconstituted thin filaments with human L29Q cTnC and dephosphorylated cTnI, whereas a significant increase was observed when thin filaments were reconstituted with biphosphorylated cTnI. Two recent studies lend credibility to the hypothesis that the L29Q mutation in cTnC affects its ability to interact with TnI particularly in its phosphorylated state (4, 11). The study by Baryshnikova et al. (4), who used nuclear magnetic resonance (NMR) and the

NH_2-terminal L29Q cTnC, concluded that the mutation affected the interactions of TnC with TnI but not Ca^{2+}. The deviations in results likely arise from differences in experimental methods and conditions. Compared with the studies on single skinned cardiac myocytes, ATPase and in vitro motility assays are excellent techniques for defining the possible molecular interactions but lack the geometric and mechanical constraints imposed on the relationship between the proteins within the sarcomere. In addition, using NMR precludes the use of Ca^{2+} chelators such as EGTA, and therefore one cannot accurately assess the free [Ca^{2+}] in the low μM range. Therefore, we submit that the Ca^{2+} sensitivities determined in these experiments using single skinned cardiac myocytes held at a constant SL are likely to be more precise and physiologically relevant.

It has been shown that changes in sarcomere structural geometry can significantly affect the protein interactions by bringing the sarcomere components together or moving them apart (21). Length-dependence of myofilament Ca^{2+} sensitivity is an important property of cardiac muscle and is recognized as being a critical component of the Frank-Starling relationship in the heart. The mechanisms underlying length dependence of myofilament Ca^{2+} sensitivity are still not fully understood (14). Studies on skinned cardiac muscle fibers have shown that the Ca^{2+} sensitivity of force generation increases with SL increase, and this effect is associated with an increase in Ca^{2+} binding affinity of site II on cTnC (25, 46). Force-induced changes in cTnC conformation have been linked to the force-induced changes in the Ca^{2+} binding affinity of cTnC, and correspondingly, to the changes in Ca^{2+} sensitivity of force. Both force and activation-dependent changes in cTnC structure are SL dependent (36). These results support the notion that an increase in Ca^{2+} binding to cTnC may be one of the key factors involved in the length-dependent increase in Ca^{2+} sensitivity of contractile apparatus. Another observation of the present study is that the dependence of myofilament Ca^{2+} sensitivity on sarcomere length was less pronounced in the skinned cardiac myocytes reconstituted with L29Q or NIQD McTnC. It is possible, therefore, to conclude from the present study that the limited SL-dependent enhancement of Ca^{2+}-binding affinity of NIQD and L29Q McTnC was due to elevated Ca^{2+} affinities of their regulatory sites at short SL. This reduction effect in the length dependence of myofilament Ca^{2+} sensitivity could influence the ability of the heart to regulate ventricular output in response to changes in ventricular filling and, therefore, result in cardiac dysfunction.

The difference in Ca^{2+} sensitivity between myocytes containing McTnC and those containing NIQD and L29Q McTnC mutants was the largest at SL 1.9 μm compared with those stretched to 2.1 and 2.3 μm (Fig. 8). This implies that the contribution of cTnC affinity to overall Ca^{2+} sensitivity of the contractile element was greater at shorter SL. This possibly means that the stretch-induced increase in Ca^{2+} sensitivity in the cardiac myocytes containing the mutants was more likely due to improved sarcomere geometry (favoring the formation of strong Ca^{2+}-dependent cross-bridges) than to changes in cTnC Ca^{2+} affinity. The relative difference in Ca^{2+} sensitivity between myocytes containing McTnC and NIQD McTnC was greater than between those containing McTnC and L29Q McTnC at SL 2.3 μm. This effect was possibly due to a smaller stretch-induced (when SL was increased from 1.9 to 2.3 μm) augmentation in Ca^{2+} sensitivity of myocytes containing NIQD McTnC compared with those containing L29Q. Since NIQD McTnC has a higher Ca^{2+} affinity than L29Q McTnC, we suggest that there may be a limit to which the Ca^{2+} affinity of cTnC can be increased as a result of an increase in SL. Further work needs to be done to test this hypothesis.

In summary, we utilized the $Phe^{27} \rightarrow Trp$ substitution to study Ca^{2+} binding and exchange with the regulatory site II of cardiac troponin C. We found that the FHC-related cardiac troponin C mutations, L29Q McTnC and NIQD McTnC (which mimics ScTnC), significantly increase the Ca^{2+}-binding affinity of regulatory site II of cTnC. We demonstrated by replacing endogenous cTnC with related McTnC mutants in single skinned cardiac myocytes that L29Q McTnC and NIQD McTnC significantly increased the Ca^{2+} sensitivity of force generation and depressed the length dependence of myofilament Ca^{2+} sensitivity. This suggests that increased Ca^{2+} sensitivity of cTnC might be one of the key factors of the cTnC-related FHC pathogenesis. The results also demonstrate that the residues 2, 28, 29, and 30 of McTnC are important for determining Ca^{2+} sensitivity of mammalian cardiomyocytes, which makes them potential targets for pharmacological modification to enhance the Ca^{2+} sensitivity of cardiac tissue in pathologies involving poor cardiac contractility.

It is necessary to stress again that Ca^{2+} sensitivity determined by using single skinned cardiac myocytes better represents the physiological conditions. Indeed, to fully understand the relationship between the Ca^{2+} sensitivity, Ca^{2+} binding, and SL that we attempted to describe in the present study, more information on Ca^{2+} binding directly in skinned cardiac myocytes is required. Further studies of the effects of L29Q and NIQD mutations in McTnC on cardiac muscle structure and function using a transgenic animal model are necessary to further understand the relationship between the increase in myofilament sensitivity and the etiology of FHC.

ACKNOWLEDGMENTS

We acknowledge the generous support of the Heart and Stroke Foundation of BC and Yukon to G. F. Tibbits. Y. Qu is supported by a postdoctoral fellowship from the Heart and Stroke Foundation of Canada. S. B. Tikunova was supported by from the National Heart, Lung, and Blood Institute Grant 1K99HL-087462-01. J. P. Davis was supported by a grant from the American Heart Association. G. F. Tibbits is the recipient of a Tier I Canada Research Chair.

REFERENCES

1. **Abbott MB, Dong WJ, Dvoretsky A, DaGue B, Caprioli RM, Cheung HC, Rosevear PR.** Modulation of cardiac troponin C-cardiac troponin I regulatory interactions by the amino-terminus of cardiac troponin I. *Biochemistry* 40: 5992–6001, 2001.
2. **Ahmad F, Seidman JG, Seidman CE.** The genetic basis for cardiac remodeling. *Annu Rev Genomics Hum Genet* 6: 185–216, 2005.
3. **Ashrafian H, Redwood C, Blair E, Watkins H.** Hypertrophic cardiomyopathy: a paradigm for myocardial energy depletion. *Trends Genet* 19: 263–268, 2003.
4. **Baryshnikova OK, Li MX, Sykes BD.** Modulation of cardiac troponin C function by the cardiac-specific N-terminus of troponin I: influence of PKA phosphorylation and involvement in cardiomyopathies. *J Mol Biol* 375: 735–751, 2008.
5. **Biesiadecki BJ, Chong SM, Nosek TM, Jin JP.** Troponin T core structure and the regulatory NH2-terminal variable region. *Biochemistry* 46: 1368–1379, 2007.
6. **Blumenschein TM, Gillis TE, Tibbits GF, Sykes BD.** Effect of temperature on the structure of trout troponin C. *Biochemistry* 43: 4955–4963, 2004.

7. **Brotto MA, Biesiadecki BJ, Brotto LS, Nosek TM, Jin JP.** Coupled expression of troponin T and troponin I isoforms in single skeletal muscle fibers correlates with contractility. *Am J Physiol Cell Physiol* 290: C567–C576, 2006.
8. **Chang AN, Harada K, Ackerman MJ, Potter JD.** Functional consequences of hypertrophic and dilated cardiomyopathy-causing mutations in alpha-tropomyosin. *J Biol Chem* 280: 34343–34349, 2005.
9. **Churcott CS, Moyes CD, Bressler BH, Baldwin KM, Tibbits GF.** Temperature and pH effects on Ca^{2+} sensitivity of cardiac myofibrils: a comparison of trout with mammals. *Am J Physiol Regul Integr Comp Physiol* 267: R62–R70, 1994.
10. **Davis JP, Tikunova SB, Walsh MP, Johnson JD.** Characterizing the response of calcium signal transducers to generated calcium transients. *Biochemistry* 38: 4235–4244, 1999.
11. **Dong WJ, Xing J, Ouyang Y, An J, Cheung HC.** Structural kinetics of cardiac troponin C mutants linked to familial hypertrophic and dilated cardiomyopathy in troponin complexes. *J Biol Chem* 283: 3424–3432, 2008.
12. **Dong WJ, Xing J, Villain M, Hellinger M, Robinson JM, Chandra M, Solaro RJ, Umeda PK, Cheung HC.** Conformation of the regulatory domain of cardiac muscle troponin C in its complex with cardiac troponin I. *J Biol Chem* 274: 31382–31390, 1999.
13. **Finley N, Abbott MB, Abusamhadneh E, Gaponenko V, Dong W, Gasmi-Seabrook G, Howarth JW, Rance M, Solaro RJ, Cheung HC, Rosevear PR.** NMR analysis of cardiac troponin C-troponin I complexes: effects of phosphorylation. *FEBS Lett* 453: 107–112, 1999.
14. **Fuchs F, Martyn DA.** Length-dependent Ca^{2+} activation in cardiac muscle: some remaining questions. *J Muscle Res Cell Motil* 26: 199–212, 2005.
15. **Gillis TE, Liang B, Chung F, Tibbits GF.** Increasing mammalian cardiomyocyte contractility with residues identified in trout troponin C. *Physiol Genomics* 22: 1–7, 2005.
16. **Gillis TE, Marshall CR, Xue XH, Borgford TJ, Tibbits GF.** Ca^{2+} binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. *Am J Physiol Regul Integr Comp Physiol* 279: R1707–R1715, 2000.
17. **Gillis TE, Moyes CD, Tibbits GF.** Sequence mutations in teleost cardiac troponin C that are permissive of high Ca^{2+} affinity of site II. *Am J Physiol Cell Physiol* 284: C1176–C1184, 2003.
18. **Gillis TE, Tibbits GF.** Beating the cold: the functional evolution of troponin C in teleost fish. *Comp Biochem Physiol A Mol Integr Physiol* 132: 763–772, 2002.
19. **Gomes AV, Potter JD.** Cellular and molecular aspects of familial hypertrophic cardiomyopathy caused by mutations in the cardiac troponin I gene. *Mol Cell Biochem* 263: 99–114, 2004.
20. **Gomes AV, Potter JD.** Molecular and cellular aspects of troponin cardiomyopathies. *Ann NY Acad Sci* 1015: 214–224, 2004.
21. **Gordon AM, Homsher E, Regnier M.** Regulation of contraction in striated muscle. *Physiol Rev* 80: 853–924, 2000.
22. **Gulati J, Sonnenblick E, Babu A.** The role of troponin C in the length dependence of Ca^{2+}-sensitive force of mammalian skeletal and cardiac muscles. *J Physiol* 441: 305–324, 1991.
23. **Harada K, Potter JD.** Familial hypertrophic cardiomyopathy mutations from different functional regions of troponin T result in different effects on the pH and Ca^{2+} sensitivity of cardiac muscle contraction. *J Biol Chem* 279: 14488–14495, 2004.
24. **Hoffmann B, Schmidt-Traub H, Perrot A, Osterziel KJ, Gessner R.** First mutation in cardiac troponin C, L29Q, in a patient with hypertrophic cardiomyopathy. *Hum Mutat* 17: 524, 2001.
25. **Hofmann PA, Fuchs F.** Bound calcium and force development in skinned cardiac muscle bundles: effect of sarcomere length. *J Mol Cell Cardiol* 20: 667–677, 1988.
26. **Hofmann PA, Fuchs F.** Effect of length and cross-bridge attachment on Ca^{2+} binding to cardiac troponin C. *Am J Physiol Cell Physiol* 253: C90–C96, 1987.
27. **Huang J, Hove-Madsen L, Tibbits GF.** Na^{+}/Ca^{2+} exchange activity in neonatal rabbit ventricular myocytes. *Am J Physiol Cell Physiol* 288: C195–C203, 2005.
28. **Johnson JD, Nakkula RJ, Vasulka C, Smillie LB.** Modulation of Ca^{2+} exchange with the Ca^{2+}-specific regulatory sites of troponin C. *J Biol Chem* 269: 8919–8923, 1994.
29. **Kobayashi T, Solaro RJ.** Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu Rev Physiol* 67: 39–67, 2005.
30. **Kreutziger KL, Gillis TE, Davis JP, Tikunova SB, Regnier M.** Influence of enhanced troponin C Ca^{2+}-binding affinity on cooperative thin filament activation in rabbit skeletal muscle. *J Physiol* 583: 337–350, 2007.
31. **Layland J, Solaro RJ, Shah AM.** Regulation of cardiac contractile function by troponin I phosphorylation. *Cardiovasc Res* 66: 12–21, 2005.
32. **Li MX, Spyracopoulos L, Sykes BD.** Binding of cardiac troponin-I147–163 induces a structural opening in human cardiac troponin-C. *Biochemistry* 38: 8289–8298, 1999.
33. **Li MX, Wang X, Sykes BD.** Structural based insights into the role of troponin in cardiac muscle pathophysiology. *J Muscle Res Cell Motil* 25: 559–579, 2004.
34. **Lind JM, Chiu C, Semsarian C.** Genetic basis of hypertrophic cardiomyopathy. *Expert Rev Cardiovasc Ther* 4: 927–934, 2006.
35. **MacIntosh BR.** Role of calcium sensitivity modulation in skeletal muscle performance. *News Physiol Sci* 18: 222–225, 2003.
36. **Martyn DA, Gordon AM.** Influence of length on force and activation-dependent changes in troponin c structure in skinned cardiac and fast skeletal muscle. *Biophys J* 80: 2798–2808, 2001.
37. **Richard P, Villard E, Charron P, Isnard R.** The genetic bases of cardiomyopathies. *J Am Coll Cardiol* 48: A79–A89, 2006.
38. **Robertson SP, Johnson JD, Holroyde MJ, Kranias EG, Potter JD, Solaro RJ.** The effect of troponin I phosphorylation on the Ca^{2+}-binding properties of the Ca^{2+}-regulatory site of bovine cardiac troponin. *J Biol Chem* 257: 260–263, 1982.
39. **Roopnarine O.** Mechanical defects of muscle fibers with myosin light chain mutants that cause cardiomyopathy. *Biophys J* 84: 2440–2449, 2003.
40. **Schmidtmann A, Lindow C, Villard S, Heuser A, Mugge A, Gessner R, Granier C, Jaquet K.** Cardiac troponin C-L29Q, related to hypertrophic cardiomyopathy, hinders the transduction of the protein kinase A dependent phosphorylation signal from cardiac troponin I to C. *FEBS J* 272: 6087–6097, 2005.
41. **Sia SK, Li MX, Spyracopoulos L, Gagne SM, Liu W, Putkey JA, Sykes BD.** Structure of cardiac muscle troponin C unexpectedly reveals a closed regulatory domain. *J Biol Chem* 272: 18216–18221, 1997.
42. **Takeda N.** Cardiomyopathy: molecular and immunological aspects (review). *Int J Mol Med* 11: 13–16, 2003.
43. **Tardiff JC.** Sarcomeric proteins and familial hypertrophic cardiomyopathy: linking mutations in structural proteins to complex cardiovascular phenotypes. *Heart Fail Rev* 10: 237–248, 2005.
44. **Tikunova SB, Davis JP.** Designing calcium-sensitizing mutations in the regulatory domain of cardiac troponin C. *J Biol Chem* 279: 35341–35352, 2004.
45. **Tobacman LS, Lin D, Butters C, Landis C, Back N, Pavlov D, Homsher E.** Functional consequences of troponin T mutations found in hypertrophic cardiomyopathy. *J Biol Chem* 274: 28363–28370, 1999.
46. **Wang YP, Fuchs F.** Length, force, and Ca^{2+}-troponin C affinity in cardiac and slow skeletal muscle. *Am J Physiol Cell Physiol* 266: C1077–C1082, 1994.
47. **Ward DG, Brewer SM, Cornes MP, Trayer IP.** A cross-linking study of the N-terminal extension of human cardiac troponin I. *Biochemistry* 42: 10324–10332, 2003.