Published in final edited form as:
J Mol Cell Cardiol. 2011 January ; 50(1): 165–174. doi:10.1016/j.yjmcc.2010.10.025.

Calcium binding kinetics of troponin C strongly modulate cooperative activation and tension kinetics in cardiac muscle

Kareen L. Kreutziger[a,c], **Nicoletta Piroddi**[b], **Jonathan T. McMichael**[a], **Chiara Tesi**[b], **Corrado Poggesi**[b], and **Michael Regnier**[a]

Kareen L. Kreutziger: klk3@u.washington.edu; Nicoletta Piroddi: nicoletta.piroddi@unifi.it; Chiara Tesi: chiara.tesi@unifi.it; Corrado Poggesi: corrado.poggesi@unifi.it; Michael Regnier: mregnier@u.washington.edu

[a] Department of Bioengineering, University of Washington, Box 355061, 3720 15th Avenue NE, Seattle, WA 98195, USA

[b] Dipartimento di Scienze Fisiologiche, Universitá degli Studi di Firenze, Viale G.B. Morgagni, I-50134 Firenze, Italia

Abstract

Tension development and relaxation in cardiac muscle are regulated at the thin filament via Ca^{2+} binding to cardiac troponin C (cTnC) and strong cross-bridge binding. However, the influence of cTnC Ca^{2+}-binding properties on these processes in the organized structure of cardiac sarcomeres is not well-understood and likely differs from skeletal muscle. To study this we generated single amino acid variants of cTnC with altered Ca^{2+} dissociation rates (k_{off}), as measured in whole troponin (cTn) complex by stopped-flow spectroscopy (I61Q cTn > WT cTn > L48Q cTn), and exchanged them into cardiac myofibrils and demebranated trabeculae. In myofibrils at saturating Ca^{2+}, L48Q cTnC did not affect maximum tension (T_{max}), thin filament activation (k_{ACT}) and tension development (k_{TR}) rates, or the rates of relaxation, but increased duration of slow phase relaxation. In contrast, I61Q cTnC reduced T_{max}, k_{ACT} and k_{TR} by 40–65% with little change in relaxation. Interestingly, k_{ACT} was less than k_{TR} with I61Q cTnC, and this difference increased with addition of inorganic phosphate, suggesting reduced cTnC Ca^{2+}-affinity can limit thin filament activation kinetics. Trabeculae exchanged with I61Q cTn had reduced T_{max}, Ca^{2+} sensitivity of tension (pCa_{50}), and slope (n_H) of tension-pCa, while L48Q cTn increased pCa_{50} and reduced n_H. Increased cross-bridge cycling with 2-deoxy-ATP increased pCa_{50} with WT or L48Q cTn, but not I61Q cTn. We discuss the implications of these results for understanding the role of cTn Ca^{2+}-binding properties on the magnitude and rate of tension development and relaxation in cardiac muscle.

Keywords

contraction; troponin C; calcium binding kinetics; cardiac muscle; cooperative activation; relaxation

Corresponding Author: Michael Regnier, Ph.D., mregnier@u.washington.edu, Office: +001-206-616-4325 or +001-206-221-0504, Fax: +001-206-685-3300.
[c]Present Address: Center for Cardiovascular Biology, University of Washington, Box 358050, Brotman 454, 815 Mercer Street, Seattle, WA 98109, USA

DISCLOSURES
None declared.

INTRODUCTION

Activation and relaxation of cardiac myofilaments are controlled by Ca^{2+}-mediated, coordinated interactions between the thin and thick filament proteins. Thin filament activation (or the process by which binding sites for myosin on actin become available) is initiated by Ca^{2+} binding to cardiac troponin C (cTnC).[1] Subsequent interactions between troponin (Tn) subunits result in increased mobility of tropomyosin and increased probability of myosin interaction with actin [1]. Strong myosin binding to actin (i.e. cross-bridges) displaces tropomyosin [2,3] and enhances Ca^{2+} binding to troponin in cardiac muscle[2]. These cooperative events are important for rapid thin filament activation and force development in cardiac muscle. Relaxation involves deactivation of the Tn complex following Ca^{2+} dissociation from cTnC and reduction of strong cross-bridge binding, though the mechanistic details are less well known [4], and it is not known whether this process is cooperative in nature.

The heart operates at sub-maximal levels of Ca^{2+} where the mechanism of cooperative activation may depend heavily upon myofilament protein isoform and structure, even in the absence of adrenergic modulation. For example, we [5–9] and others [10] have demonstrated that, while both Ca^{2+} binding (to Tn) and cross-bridge binding (to actin) are required for complete activation of thin filaments in both skeletal and cardiac muscle, cardiac thin filament activation is relatively more dependent on strong cross-bridge binding at sub-saturating Ca^{2+}. It has been demonstrated in skeletal muscle that the Ca^{2+} binding properties of TnC can affect cooperative activation and tension development and relaxation kinetics [11–15]. Previous studies have also examined the relationships between specific point mutations in cardiac TnC (cTnC) and how they alter cTn Ca^{2+} binding properties [16] and Ca^{2+} sensitivity of tension in cardiac muscle [17]. However, it is not clear how cTnC Ca^{2+} binding properties contribute to the complex cooperative interactions between the thin and thick filaments during thin filament activation in the organized structure of the cardiac sarcomere. Further, it is not known how the properties of cTnC influence the kinetics of tension activation and relaxation or cooperative activation in cardiac muscle. As such, a more detailed understanding of how altered Ca^{2+} binding properties of cTnC can affect mechanical function of cardiac muscle is warranted.

We hypothesize that the Ca^{2+} binding properties of cTnC are a primary determinant of thin filament activation and contractile kinetics in cardiac muscle, as evidenced by steep cooperativity of tension generation and a narrow spread of activation along thin filaments [8]. To examine this, we introduced site-directed mutations in the N-terminus of rat cTnC that increased or decreased cTn Ca^{2+}dissociation rate (k_{off}) [16,18] to study their effects on cooperative activation and the kinetics of tension development and relaxation in demembranated rat ventricular trabeculae and mouse ventricular myofibrils. Cross-bridge binding and cycling was independently augmented using 2 deoxy-ATP (dATP; [7]). The use of demembranated trabeculae allowed multiple activations at varying Ca^{2+} with little degradation of tension, making it possible to study both Ca^{2+} and cross-bridge components of cooperative thin filament activation in the same preparation [8,9,14,15]. The use of

[1]Abbreviations: ANOVA, analysis of variance; BDM, 2,3-butanedione monoxime; cTn, cardiac troponin; cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; dATP, 2-deoxy-ATP; DTT, dithiothreitol; f_{app}, rate of cross-bridges transitioning into tension-generating states; g_{app}, rate of cross-bridges transitioning out of tension-generating states; k_{ACT}, tension activation rate; K_i, inhibition constant; k_{off}, Ca^{2+} dissociation rate; k_{on}, Ca^{2+} association rate; $k_{REL,fast}$, fast phase relaxation rate; $k_{REL,slow}$, slow phase relaxation rate; k_{TR}, tension redevelopment rate; L_0, initial length; n_H, Hill coefficient; MOPS, 3-(N-mopholino) propanesulfonic acid; pCa, log[Ca^{2+}]; pCa_{50}, pCa at half-maximal activation; P_i, inorganic phosphate; SL, sarcomere length; sTnC, skeletal troponin C; T_{max}, maximal tension; Tn, troponin; xcTnC, a cardiac troponin C mutant that does not bind Ca^{2+} at site II; WT, wild type.

myofibril preparations allowed measurements of the rate of contractile activation (k_{ACT}), rate of tension redevelopment (k_{TR}), and the biphasic slow and fast phases of relaxation ($k_{REL,slow}$ and $k_{REL,fast}$, respectively) during the same activation trial, because rapid changes in [Ca^{2+}] throughout these small preparations were not limited by diffusion rates.

We found that activation kinetics (k_{ACT}) were slowed by increased cTn Ca^{2+} k_{off} (I61Q cTnC), and became slower than tension redevelopment kinetics (k_{TR}); this difference was increased with increased inorganic phosphate. The duration of slow phase relaxation was not affected by increased k_{off} (I61Q cTnC), but was prolonged by decreased k_{off} (L48Q cTnC). L48Q cTnC and I61Q cTnC enhanced or reduced (respectively) the Ca^{2+} sensitivity of steady-state tension (pCa_{50}), consistent with the presumed changes in Ca^{2+} affinity of cTn with the mutations. However, both cTn complexes resulted in a large reduction in the slope (n_H) of the steady-state tension-pCa relationship, implicating cTn Ca^{2+} binding as a major component of cooperative thin filament activation in cardiac muscle. This reduction in n_H could not be rescued by strong cross-bridge augmentation. These results differ from our previous findings in fast skeletal muscle [12,14,15] and suggest that Tn Ca^{2+} binding properties are an important component in the differential regulation of tension development and relaxation in the two striated muscle types. Preliminary abstract reports of this work have been previously published [19,20].

MATERIALS AND METHODS

Proteins, Solution Biochemistry, and Exchange

Rat cardiac TnC (cTnC) mutations were introduced by site-directed mutagenesis. Recombinant cTnC, cTnI, and cTnT were extracted and purified from *E. coli* [21]. Whole cTn complexes were formed using WT or mutant rat cTnC and WT rat cTnI and WT rat cTnT (see Supplementary Materials for details).

Ca^{2+} dissociation rate (k_{off}) from whole cTn (containing WT or mutant cTnC) and reconstituted thin filaments was measured at 15 °C using an Applied Photophysics Ltd. (Leatherhead, U.K.) model SX-18MV stopped-flow instrument as previously described [14,22,23]. Ca^{2+} dissociation from whole cTn or reconstituted thin filaments was measured by rapidly mixing the protein with the fluorescing Ca^{2+} chelator Quin-2. Buffer composition, thin filament reconstitution methods, and detection details are included in the Supplementary Material.

Protein exchange into myofilaments was accomplished with passive exchange of cTnC in myofibrils or whole cTn exchange in trabeculae. Native cTnC in myofibrils was replaced with recombinant cTnC through (mass-action) passive exchange at 4 °C overnight in relaxing solution with 0.05 mg mL^{-1} recombinant rat cTnC. No myofibril exchange group developed any Ca^{2+}-independent tension in relaxing solution, indicating complete regulation following the exchange protocol. Maximal tension (T_{max}) was the same for untreated, treated control, and WT cTnC-exchanged myofibrils. Replacement of native cTnC in trabeculae was accomplished by passive (mass action) exchange via incubation of preparations in a high concentration of whole cTn under rigor conditions, as previously described [24]. In a subset of experiments after exchange, an additional incubation in 1 mg mL^{-1} cTnC (WT or mutant) was completed to ensure that all cTn complexes were complete, and this was confirmed by no increase in steady-state tension (data not shown). Reported post-exchange relative T_{max} values (Table 3) are from back-to-back measurements in pCa 4.0 or 3.5 comparing just prior to exchange and immediately following exchange. Additional details are in the online Supplementary Material. Analysis of Variance (ANOVA) was used to compare between groups and when differences were significant, Student's unpaired *t* tests were used with statistical significance set at $P < 0.05$.

Experimental Animals and Tissue Preparations

All animal procedures performed in Italy were conducted in accordance with the official regulations of the European Community Council on Use of Laboratory Animals (Directive 86/609/EEC) and protocols were approved by the Ethical Committee for Animal Experiments of the University of Florence. Male C57 mice were killed by rapid cervical dislocation and myofibrils were prepared as previously described [25] with minor modifications (see Supplementary Material). Myofibrils were prepared by homogenization of 4–8 strips of ventricular tissue in rigor solution on ice, washed free of glycerol, and used for up to 5 days.

All animal procedures performed in the United States were conducted in accordance with the US National Institutes of Health Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Washington (UW) Animal Care Committee. Rats were housed in the Department of Comparative Medicine at UW and cared for in accordance with the UW Institutional Animal Care and Use Committee (IACUC) procedures. Male Sprague-Dawley rats were anesthetized via intraperitoneal injection of pentobarbital (50 mg kg^{-1}). When animals had no reflexive response, the heart was rapidly excised and demembranated right ventricular trabeculae were prepared as previously described [5] (see Supplementary Material for details). Individual trabeculae were dissected from the right ventricle and prepared for mechanical measurements by wrapping the ends in aluminum foil T-clips for attachment to the mechanical apparatus as previously described [5].

Mechanical Measurements

Experimental physiological Ca^{2+} solutions were calculated as previously described for myofibril [26] and trabeculae [27] mechanics. Details are in the online Supplemental Data. Phosphate (P_i) concentration was reduced in all myofibril solutions to <5 μmol L^{-1} by a P_i scavenging enzyme system (purine-nucleoside-phosphorylase with substrate 7-methyl-guanosine) as previously described [28] except in solutions containing 0.5 mmol L^{-1} added P_i. A low-P_i, pCa 4.0 solution for trabeculae omitted the creatine phosphate and creatine phosphokinase, yielding ~0.1 mmol L^{-1} P_i by NMR analysis. Some trabeculae solutions substituted 5 mmol L^{-1} 2-deoxy-ATP (dATP) for ATP.

For myofibril experiments, mechanical data were collected at 15 °C from small bundles of cardiac myofibrils as previously described [29]. Sarcomere length (SL) was initially set 10 – 20% above slack length and was 2.29 ± 0.02 μm (mean ± S.E.M., n = 63). At this SL, average myofibril initial length (L_o) was 52 ± 3 μm and diameter was 3.88 ± 0.05 μm (4 – 8 myofibrils). Measurement procedures for activation rate (k_{ACT}; with rapid increase in Ca^{2+}), rate of tension redevelopment (k_{TR}; following a rapid release-restretch transient), relaxation rates (slow phase: $k_{REL,slow}$ and fast phase: $k_{REL,fast}$), and slow phase duration are detailed in the Supplementary Material. ANOVA was used to compare between myofibril groups after cTnC exchange and when differences were significant, Student's unpaired *t* tests were used with statistical significance set at $P < 0.05$. All values reported in Table 2 for myofibril tension and kinetics are means ± S.E.M.

For trabeculae experiments, mechanical data were collected at 15 °C from demembranated right ventricular trabeculae mounted on a mechanical apparatus (detailed in the Supplementary Material). SL was initially set to 2.25 μm, L_o was 1.09 ± 0.06 mm, diameter was 107 ± 5 μm (mean ± S.E.M.; n=48) and SL was monitored throughout the experiment. Passive tension was determined at pCa 9.0 and was subtracted from total tension to obtain the active tension values reported. T_{max} measured at pCa 4.0 just prior to cTn exchange was 60.5 ± 4.0 mN mm^{-2} (mean ± S.E.M., n=48). The k_{TR} was determined in trabeculae from

the half-time of tension recovery and tension-pCa and k_{TR}-pCa data were fitted with the Hill Equation to get reported pCa_{50} and n_H values (see Supplementary Material for equations). Reported pCa_{50} and n_H values for tension-pCa are the average of individual fits for each experimental curve ± S.E.M. Reported pCa_{50} values for k_{TR}-pCa represent fits to the average data and are reported ± S.E. of the fits. ANOVA was used to compare between cTn exchange groups after exchange and when differences were significant, Student's unpaired *t* tests were used with statistical significance set at $P < 0.05$.

RESULTS

Ca^{2+} dissociation rate (k_{off}) from cTn

Stopped-flow spectroscopy was used to measure Ca^{2+} dissociation rate (k_{off}) from whole cTn complexes containing recombinant wild type (WT) cTnI, cTnT and either WT cTnC (control), L48Q cTnC, or I61Q cTnC (denoted WT cTn, L48Q cTn, or I61Q cTn, respectively). The k_{off} was determined for each cTn by fitting fluorescence data with exponential curves, as previously described [14]. At 15°C (the temperature used for mechanics measurements) k_{off} was ~30 s^{-1} for WT cTn, in agreement with previous reports [23,30]. The mutations in cTnC altered k_{off}, reducing it by 75% for L48Q cTn (in agreement with a recent report using a modified cTnC with attached fluorescent probe [31]) and increasing it by 2.3-fold for I61Q cTn (Table 1). Reconstitution of thin filaments with WT or mutant whole cTn, Tm, and actin resulted in a 2.2–3.4-fold increase in k_{off} for all cTnC variants versus whole cTn k_{off}. Rates for WT cTn and L48Q cTn in thin filaments agree with recently reported rates [31]. The relative effect of the mutations in cTnC remained the same as for whole cTn, such that k_{off} was reduced by 63% for L48Q cTn and increased by 3.2-fold for I61Q cTn in thin filaments (Table 1).

Maximal activation and relaxation in ventricular myofibrils

WT, I61Q or L48Q cTnC was exchanged into mouse ventricular myofibrils to determine effects on maximal isometric tension development and relaxation kinetics following rapid increase and decrease in bathing $[Ca^{2+}]$. The effectiveness of the cTnC exchange protocol was examined in parallel for each batch of myofibrils using another mutant, D65A cTnC, which does not bind Ca^{2+} at the N-terminus (xcTnC). These myofibrils developed < 3% of the maximal Ca^{2+} activated (pCa 3.5) steady-state tension (T_{max}) obtained for treated controls or those exchanged with WT cTnC (Table 2). Thus, if we assume that cTnC for cTn in myofibrils is relatively similar for the mutants in this study (an assumption based on the fact that the C-terminal portion of the protein is the same), these measurements suggest our protocol was successful in replacing the great majority of native cTnC with mutant cTnC in myofibrils.

Example tension traces of the maximal Ca^{2+} activation-relaxation protocol for three myofibrils exchanged with WT, L48Q or I61Q cTnC are shown in Figure 1. T_{max} and the rates of tension activation (k_{ACT}) and tension redevelopment (k_{TR}) did not differ for L48Q cTnC vs. WT cTnC, and in both of these myofibril groups k_{ACT} did not differ from k_{TR} (Table 2). In contrast, T_{max}, k_{ACT} and k_{TR} were all reduced by ~40–65% with I61Q cTnC, and k_{TR} was significantly faster than k_{act} (Fig. 1C; $P < 0.02$). The slower k_{ACT} vs. k_{TR} suggests that thin filament activation kinetics became rate limiting to tension activation with I61Q cTnC (see Discussion). To further test this idea, we measured k_{ACT} and k_{TR} in the presence of 0.5 mmol L^{-1} inorganic phosphate (P_i), which affects cross-bridge cycling specifically (Fig. 2), without influencing thin filament activation kinetics. Addition of P_i caused a similar decrease in T_{max} (~15%) for control and I61Q cTnC. Interestingly, with I61Q cTnC both k_{ACT} and k_{TR} were increased with P_i, but the increase was greater for k_{TR} ($P < 0.01$) which became similar to the rate for control myofibrils (Fig. 2B, C). In contrast,

0.5 mmol L^{-1} P_i increased k_{TR} and k_{ACT} similarly in control myofibrils. Thus we conclude that I61Q cTnC limits the magnitude (T_{max}) and slows the rate of tension activation (k_{ACT}) via reduction in thin filament activation kinetics. The rates of the slow ($k_{REL,slow}$) and fast ($k_{REL,fast}$) phases of relaxation for did not differ for myofibrils with L48Q cTnC, I61Q cTnC and WT cTnC. However, while the duration of slow phase relaxation was not affected with I61Q cTnC, it was almost doubled with L48Q cTnC ($P < 0.01$; Fig. 1D, Table 2). Thus, L48Q cTnC did not affect the rate of activation or relaxation, but prolonged (slow phase) relaxation by ~40 ms. In contrast, I61Q cTnC had no effect on relaxation, but dramatically slowed activation.

Ca^{2+} dependence of tension development in ventricular trabeculae

To determine the influence of L48Q and I61Q cTnC on cardiac muscle contractile properties at varying Ca^{2+} concentrations, whole cTn containing these cTnC mutants were exchanged into demembranated rat ventricular trabeculae. As with myofibrils, exchange with D65A cTnC (in whole cTn complex) reduced T_{max} to a very low level (~8% of pre-exchanged values), suggesting good protein exchange with this technique (Fig. 3A).

Similar to mouse ventricular myofibrils, T_{max} (Fig. 3A) and $k_{TR,max}$ (Fig. 3C) were not affected by L48Q cTn (vs. WT cTn; Table 3). Example tension traces are shown in Supplementary Figure S1. For I61Q cTn, T_{max} was reduced by ~75%, which was similar to the reduction for mouse ventricular myofibrils (~65%; Table 2). Interestingly, $k_{TR,max}$ (~9 s^{-1}) for I61Q cTn was similar to WT and L48Q cTn (by ANOVA; Table 3). This contrasts results in myofibrils where $k_{TR,max}$ was reduced by ~40% in the absence of P_i (Table 2), but is similar to myofibril results in the presence of 0.5 mmol L^{-1} P_i (Fig. 2). Activation solutions for trabeculae contained 15 mmol L^{-1}creatine phosphate and 5 mmol L^{-1} ATP, which results in a contaminating P_i level of ~0.5 mmol L^{-1} (by NMR analysis; see Supplementary Material) that is likely higher in the center of the preparation due to limited substrate and product diffusion and the coupled ATPase and creatine kinase reactions. This suggests that these results with rat trabeculae are in good agreement with the mouse ventricular myofibril measurements.

When the Ca^{2+} concentration of activation solutions was varied in trabeculae, both Ca^{2+} sensitivity of tension (pCa_{50}) and apparent cooperativity of activation (slope, n_H) were strongly influenced by the cTnC mutants (Fig. 3B, Table 3). L48Q cTn increased pCa_{50} by ~0.4 units compared with WT cTn, and I61Q cTn decreased pCa_{50} by ~0.5 units. These shifts in Ca^{2+} sensitivity were anticipated based on our earlier work with similar skeletal TnC mutants in skeletal fibers [14] and the work of others in trabeculae [17]. However, there was an unexpected large reduction in n_H (~4 units) for both L48Q and I61Q cTn (Table 3), indicating an apparent loss of cooperativity of tension generation with both mutants. This contrasts our previous results in skeletal muscle, where slowing Ca^{2+}-k_{off} of TnC had no effect on n_H [14], and will be discussed in more detail below (see Discussion).

As with steady-state tension, the pCa_{50} of k_{TR} (Fig. 3C) increased with L48Q cTn (5.43 ± 0.06) compared with WT cTn (5.28 ± 0.03). Both lagged behind pCa_{50} of tension [5] by similar amounts (Fig. 3B). Interestingly, the Ca^{2+} dependence of k_{TR} with I61Q cTn was greatly diminished (Fig. 3C). Mono-exponential fits to force traces for I61Q cTn resulted in similar values for k_{TR} (compared with determination from $t_{1/2}$ values), suggesting this loss of Ca^{2+} dependence was not an artifact of measurement techniques at low forces. To determine the influence of cycling cross-bridge number (i.e. steady-state tension level) on k_{TR}, values in Figure 3C were re-plotted as a function of relative tension as Ca^{2+} was varied (Fig. 3D). The data demonstrate k_{TR} did not differ with L48Q cTn vs. WT cTn for similar numbers of cycling cross-bridges (independent of $[Ca^{2+}]$). The situation appears to be quite different with I61Q cTn, where k_{TR} values (8–10 s^{-1}) were equal to or greater than values

obtained for $k_{TR,max}$ with WT (or L48Q) cTn, even though the number of cycling cross-bridges was greatly reduced. These data suggest that while I61Q cTnC greatly limited strong cross-bridge binding, it has no effect or slightly increases attached cross-bridge cycling kinetics.

Effect of strong cross-bridge augmentation on tension in trabeculae

Cardiac thin filament activation is more sensitive to changes in strong cross-bridge binding than skeletal muscle [2,8,32–34], especially during sub-maximal Ca^{2+} activation. To determine how the strong cross-bridge component of thin filament activation might depend on cTnC Ca^{2+} binding properties and whether this (in turn) affects cross-bridge cycling kinetics, we augmented strong cross-bridge binding and cycling (independent of $[Ca^{2+}]$) by replacing ATP with 2 deoxy-ATP (dATP) as the substrate for contraction. dATP increased T_{max} and $k_{TR,max}$ by ~20–35% in trabeculae with WT cTnC (Fig. 4A; $P < 0.05$), as we have reported previously [5,7,8]. Similar increases in T_{max} and $k_{TR,max}$ occurred with L48Q cTnC. Interestingly, with I61Q cTnC, dATP increased T_{max} by 86 ± 9% ($P < 0.01$), to approximately half of T_{max} with WT cTn, while $k_{TR,max}$ was increased by only 19 ± 10% (similar to WT or L48Q cTnC). This much greater effect on T_{max} than on $k_{TR,max}$ suggests I61Q cTnC limits strong cross-bridge binding more than it affects cross-bridge cycling kinetics during isometric contraction.

Measurements of steady-state tension at sub-maximal Ca^{2+} levels are consistent with this idea. Figure 4B shows normalized tension-pCa relationships for paired comparisons between ATP (dashed lines from fit of the data) and dATP (symbols and solid fit lines) in the same trabeculae following cTn exchange. The order of tension measurements (with ATP versus dATP) was varied for individual experiments to reduce the possibility that preparation rundown was a factor in determining values for pCa_{50}. dATP did not affect n_H with WT cTn, L48Q or I61Q cTn, suggesting little or no effect on cooperative thin filament activation. However, while dATP increased pCa_{50} with L48Q cTnC and WT cTnC, it had no effect with I61Q cTnC. For WT cTnC (n=4), pCa_{50} increased from 5.40 ± 0.01 (ATP) to 5.46 ± 0.03 (dATP; $P < 0.05$), in agreement with our previous conclusion that dATP enhances cross-bridge binding at sub-maximal Ca^{2+} [5–7]. The augmentation of cross-bridge binding was similar or greater for L48Q cTn (n=5), where pCa_{50} increased from 5.57 ±0.08 (ATP) to 5.74 ± 0.10 with dATP ($P < 0.01$). In contrast, with I61Q cTn (n=9) the Ca^{2+} sensitivity of tension was not altered by dATP, as pCa_{50} for ATP (4.70 ± 0.05) was not different than for dATP (4.74 ± 0.04; Fig. 4B). Thus, even though dATP almost doubled strong cross bridge binding at all levels of Ca^{2+} activation with I61Q cTn, including T_{max} (Fig. 4A), it could not rescue the dramatic reduction in pCa_{50} or n_H (Fig. 3B; Table 3) caused by the reduced Ca^{2+} binding (to cTnC) component of thin filament activation in cardiac muscle.

Measurements of k_{TR} with dATP at sub-maximal Ca^{2+} levels were consistent with the hypothesis that I61Q cTnC has only minor effects on cross-bridge cycling rates. dATP increases the rates of cross-bridge tension generation and cross-bridge detachment, but appears to affect the former more than the latter, resulting in increased recruitment and rate of cross-bridge cycling at all levels of Ca^{2+} activation in cardiac muscle [5,7]. These effects on crossbrige cycling kinetics were not affected by cTn mutants, as dATP did not affect the k_{TR}-tension relationship with varying Ca^{2+} for trabeculae exchanged with L48Q, I61Q or WT cTn (Fig. 5). The data, with tension normalized to T_{max} for ATP, showed no elevation of k_{TR} at lower tensions but an extension of both tension and k_{TR} at the highest levels of Ca^{2+} activation by dATP for all three cTn exchanges. Back-to-back measurements of T_{max} and $k_{TR,max}$ are marked with an asterisk (*) in each panel of Figure 5 to illustrate increases with dATP during maximal Ca^{2+} activation (summarized in Fig. 4A), and magnitude increases in T_{max} are indicated by a double-headed arrow. Since dATP does not increase k_{TR}

at similar tension levels (compared with ATP), it suggests cross-bridge cycling kinetics were not directly affected by the cTnC mutants used in this study.

DISCUSSION

The purpose of this study was to investigate how altering the Ca^{2+} binding properties of cTn influence cooperative thin filament activation and the kinetics of tension generation and relaxation when exchanged into cardiac muscle. The most significant findings of this work, where cTn complexes with altered k_{off} were exchanged into cardiac myofibrils and demembranated trabeculae, are (1) while the Ca^{2+} sensitivity (pCa_{50}) of tension was decreased by I61Q cTnC (faster k_{off} vs. WT cTn) and increased by L48Q cTnC (slower k_{off} vs. WT cTn) both mutants resulted in an apparent loss of cooperativity of thin filament activation (n_H); (2) I61Q cTnC slowed the rate of thin filament activation, while L48Q cTnC had no effect on activation kinetics but prolonged relaxation (slow phase) duration and (3) cross-bridge binding was modulated by cTn k_{off} but the rate of cross-bridge cycling and tension redevelopment (when Ca^{2+} binding was in near steady-state; k_{TR}) were little affected. As a result, this study suggests that directly modulating the Ca^{2+} binding properties of cTnC using point mutations could alter cardiac contraction, because changes in Ca^{2+} sensitivity are large, with little effect on relaxation kinetics and no effect on myosin kinetics.

cTn Regulation of Ca^{2+} Sensitivity (pCa_{50}) and Cooperative (n_H) Tension Generation

Decreasing (L48Q cTnC) or increasing (I61Q cTnC) cTn Ca^{2+}-k_{off} in whole cTn and reconstituted thin filaments resulted in increased and decreased Ca^{2+} sensitivity of tension (pCa_{50}), respectively. This study correlates Ca^{2+} dissociation rates measured in reconstituted thin filaments with isometric tension in the geometrically constrained lattice structure of cardiac muscle, demonstrating that changes in k_{off} can translate to changes in Ca^{2+} sensitivity of tension for these particular cTnC mutants. A similar result, correlating k_{off} from reconstituted thin filaments with solution myosin ATPase rates, was recently reported [31]. In our study, it was interesting to find that both cTnC mutants (L48Q and I61Q) greatly reduced the slope (n_H) of the tension-pCa relationship (Fig. 3B). The large reduction in n_H with I61Q cTnC was somewhat expected, and this may result from I61Q cTnC disrupting strong-crossbridge-induced increases in Ca^{2+} binding that is normally found in cardiac muscle [2,32–34]. This idea is supported by the tension-pCa curves (Fig. 3B), which show that at ~pCa 5.5, tension begins to rise for WT and I61Q cTnC but the slope of the curve is steep for WT cTnC and more flat for I61Q cTnC, suggesting that some form of cooperativity is lost with I61Q cTnC.

The apparent loss of cooperativity (n_H) with L48Q cTnC likely occurs for a different reason, as pCa_{50} was increased, not decreased (Fig. 3B) and T_{max} is not reduced (Fig. 3A). Tension is initiated at much lower [Ca^{2+}] (i.e. higher pCa) with L48Q cTnC versus WT cTnC (Fig. 3B), and most of the tension increases are observed at low levels of Ca^{2+}-mediated thin filament activation. Although the affinity of L48Q cTnC for the switch peptide of cTnI ($cTnI_{128–180}$) was recently shown to decrease slightly [31], its reduced k_{off} and increased Ca^{2+} binding affinity likely more than compensates for this, making L48Q cTnC a more effective activator of thin filaments at lower [Ca^{2+}] and less dependent on cooperative cross-bridge activation mechanisms. If L48Q cTnC had caused greater strong cross-bridge-induced increases in Ca^{2+} binding, in addition to an increased pCa_{50} we would also expect n_H to also increase, but this did not occur. Therefore, the cooperative coupling between strong-crossbridge binding and increased Ca^{2+} binding to cTnC was likely reduced with L48Q cTnC.

Cardiac vs. Skeletal Muscle Differences in Cooperative Activation

An additional purpose of this study was to compare these results in cardiac muscle with similar measurements in fast skeletal muscle from previous studies. These comparisons can be quite helpful in understanding the molecular mechanisms involved in myofilament regulation of contraction and relaxation. Further, differences in contractile mechanisms may translate to important functional differences between cardiac and skeletal muscle and lead to therapeutics designed specifically for cardiac disease.

The decrease observed in n_H for both L48Q cTnC and I61Q cTnC contrasts results from our similar experiments in fast skeletal muscle[14], suggesting mechanistic differences in cooperative activation of these two striated muscle types. We previously reported that skeletal TnC (sTnC) mutants with decreased (M80Q $sTnC^{F27W}$) or increased (I60Q $sTnC^{F27W}$) sTn Ca^{2+} k_{off} also resulted in increased and decreased (respectively) pCa_{50} of tension in demembranated rabbit psoas fibers [15,19]. However, while these shifts were similar in magnitude to what we report here for cardiac muscle (Table 3), the changes in n_H were minimal. These differences in tension-pCa relationships are illustrated in Figure 6, which shows the fit lines of our data for cardiac and skeletal muscle. Davis *et al.* (2004) also reported that mutant sTnCs reconstituted into skinned skeletal fibers shifted pCa_{50} with little change in n_H, even though these were different point mutations in sTnC than what we had used (see their Fig. 6C [35]). Further, the loss of n_H in cardiac muscle may not be particular to the cTnC mutants selected for this study. Norman *et al.* (2007) reported a similar (but smaller) increase and decrease in pCa_{50} with two different cTnC mutants that also had higher and lower cTnC Ca^{2+} binding affinity (respectively), and these mutants also caused a loss of n_H (see their Fig. 2B [17]). Interestingly, pseudo-phosphorylation of cTnI at serine 23, serine 24, and threonine 144 (which mimics phosphorylation by PKCβ) also increases k_{off}, reduces pCa_{50} and reduces n_H [36]. Thus, cooperative thin filament activation and tension development in cardiac muscle appears to strongly depend on the Ca^{2+} binding properties of cTn, in contrast to fast skeletal muscle. Indeed, cardiac muscle cooperativity appears to depend heavily on strong-binding cross-bridges increasing either Ca^{2+} binding to cTnC *per se* [2,32–34] or interaction strength between cTnC, cTnI, and/or actin. If either are the case, L48Q cTnC and I61Q cTnC may both alter this form of cooperativity and do so within individual functional units of the thin filament (defined as the number of actins made available for myosin binding when Ca^{2+} binds to an individual cTn). In cardiac muscle this functional unit of activation appears to be similar or smaller than a structural regulatory unit (7 actins: 1 Tn: 1 tropomyosin) [8]. This contrasts skeletal muscle, where the functional unit is larger than the structural unit (10–12 actins [9]) and accounts for the majority of cooperative activation (n_H), leaving little role for TnC Ca^{2+} binding kinetics in cooperative activation of skeletal muscle.

cTn Regulation of Tension Development and Relaxation Kinetics

Measurement of tension development and relaxation rates provides insight into the dynamic processes of Ca^{2+}-mediated thin filament activation and cross-bridge cycling. $k_{TR,max}$ was not affected in myofibrils or trabeculae with L48Q cTnC and was slowed with I61Q cTnC only when P_i was ≤ 5 μM. In myofibrils with I61Q cTnC, addition of 0.5 mmol L^{-1} P_i increasd $k_{TR,max}$ to a value similar to those with WT or L48Q cTnC, suggesting I61Q cTnC only affected cross-bridge cycling at sub-physiological [P_i]. Comparisons of k_{ACT} vs. k_{TR} indicate this occurred via thin filament activation dynamics. k_{ACT} reflects the combined rates of Ca^{2+} binding to cTn, thin filament activation, and tension generation, while k_{TR} measures the rate of tension generation when Ca^{2+} binding (to cTn) and thin filament activation are in near steady state [5]. While some thin filament deactivation could occur during shortening length steps [37] with the k_{TR} protocol, Ca^{2+} remains in equilibrium with cTnC (enabling a weak-binding myosin state, i.e. the “closed” thin filament state [3]),

whereas the thin filament is inactivated (i.e. in the "blocked" state) preceding k_{ACT}. Interestingly, the difference in k_{ACT} vs. k_{TR} in myofibrils with I61Q cTnC increased with addition of 0.5 mmol L^{-1} P_i (Fig. 2), which influences cross-bridge cycling specifically. We previously reported a similar difference between k_{ACT} and k_{TR} in trabeculae activated using caged Ca^{2+} in the absence of a phosphate mop, and demonstrated a similar increase in both rates with dATP [5]. Together these studies demonstrate that thin filament activation kinetics can limit the rate tension develops in cardiac muscle, even under saturating Ca^{2+} conditions. This limitation may be even greater at more physiological [P_i], a question that we are investigating.

It has been suggested that k_{TR} during sub-maximal Ca^{2+} activation is slowed by (relatively slow) cooperative events in thin filament activation [38,39], and that loss of these interactions may elevate sub-maximal k_{TR}. Our trabeculae data with I61Q cTnC support this idea (Fig. 3D), but the data with L48Q cTnC do not since pCa_{50} is higher but n_H is lower and k_{TR} is unchanged. In previous studies using sTnC mutants in skeletal muscle we suggested that elevated sub-maximal k_{TR} may result from an effect on cross-bridge attachment and detachment rates (f_{app} and g_{app};[40]) when k_{off} is greatly increased [13,15]. This could occur via reduced TnC-TnI interactions or by indirect effect to increase cross-bridge detachment kinetics, via greater tendency for tropomyosin to move towards an inhibiting position. Because skeletal data and models suggest that crossbridge kinetics are altered when sTnC kinetics are altered [12,13], we test this hypothesis using a four-state mass action kinetic model that incorporates cardiac kinetics and strong-cross-bridge binding feedback (see Appendix and Scheme 1 in Supplementary Material).

Little is known about the influence of thin filament properties on relaxation, especially in cardiac muscle. In fast skeletal muscle, cross-bridge detachment kinetics (g_{app}) are thought to dominate the isometric (slow) phase relaxation rate [4,15,41–43]. The fast phase of relaxation occurs when some sarcomeres lose activation while others rapidly shorten under reduced load, resulting in sarcomere inhomogeneity, deactivation, and exponential tension decline [4,42,43]. In agreement with this, we found no effect of L48Q or I61Q cTnC on the fast ($k_{REL,fast}$) or slow ($k_{REL,slow}$) relaxation phase rates (Table 2). Interestingly, however, the duration of $k_{REL,slow}$ was prolonged with L48Q cTnC, but not I61Q cTnC. This could occur if slower k_{off} allows detaching cross-bridges to re-attach, thus retarding thin filament deactivation without affecting g_{app}.

Conclusion

Regulation of activation and tension development in cardiac muscle must be controlled at the cellular (sarcomere) level because all cardiomyocytes are activated during systole. Our current results suggest the Ca^{2+} binding properties of cTn may allow for optimal cooperativity in activation of contraction, providing rapid tension development that is very sensitive to [Ca^{2+}]. The consequences of a rapid k_{off} of cTnC in cardiac (vs. skeletal) muscle may also be a greater dependence on strong cross-bridge binding for a given level of activation. Thus factors that alter the probability of strong cross-bridge binding, such as myofilament protein phosphorylation or changes in myofilament lattice spacing can have a profound effect in cardiac muscle, steepening the sarcomere length-tension relationship under conditions that affect pre-load and the Frank-Starling relationship. Interestingly, since increasing cTn k_{off} had no effect on relaxation duration or kinetics in this study, it suggests that k_{off} may also be optimized for rapid relaxation, which is advantageous for diastolic function (especially during adrenergic stimulation). Better understanding of these regulatory properties is also important for understanding the genetic basis of familial cardiomyopathies, as disease-linked mutations in several myofilament proteins including cTnC have recently been reported which either increase or decrease the Ca^{2+} sensitivity of steady-state tension in demembranated muscle preparations [44–47] or interfere with TnC-TnI interactions [48].

Ultimately, therapies targeting the Ca^{2+} sensitivity of myofilaments must be specifically designed to account for the kinetics and activation principles of the cardiac system.

Research Highlights

- Cooperative cardiac thin filament activation depends on cTnC Ca^{2+} binding kinetics
- cTnC Ca^{2+} dissociation rate (k_{off}) affects acto-myosin binding but not cycling.
- Altering k_{off} affects Ca^{2+} sensitivity of isometric tension development
- Increasing k_{off} can decrease the rate of contractile activation
- Decreasing k_{off} can increase the duration of isometric relaxation

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We greatly acknowledge Drs. James Sellers (NIH) and Earl Homsher (UCLA) for use of facilities and assistance with the thin filament k_{off} measurements; Dr. Martin Kushmerick for NMR analysis of activation solutions; and Zhaoxiong Luo and An-Yue Tu for construction of cTnC mutants and preparation of cTn complexes. Additional thanks go to Drs. Svetlana Tikunova and Jonathan Davis, who assisted in initial selection of mutations and in methods of analysis for whole cTn k_{off} stopped-flow experiments. We thank Dr. Albert Gordon for comments on the manuscript. This work was supported by USA NIH grants HL61683 and HL65497 to M.R. and Ministero dell Universitá e della Ricera, Italy grants PRIN2006 & 2008 to C.T. and C.P. Financial support by Telethon-Italy (grant # GGP06007) is also gratefully acknowledged. K.L. Kreutziger was a recipient of a Graduate Fellowship in Biomedical Engineering from The Whitaker Foundation. M. Regnier is an Established Investigator of the American Heart Association.

References

1. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. Physiol Rev 2000 Apr;80(2):853–924. [PubMed: 10747208]
2. Hofmann PA, Fuchs F. Evidence for a force-dependent component of calcium binding to cardiac troponin C. Am J Physiol 1987 Oct;253(4 Pt 1):C541–6. [PubMed: 3499084]
3. McKillop DF, Geeves MA. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. Biophys J 1993 Aug;65(2):693–701. [PubMed: 8218897]
4. Poggesi C, Tesi C, Stehle R. Sarcomeric determinants of striated muscle relaxation kinetics. Pflugers Arch 2005 Mar;449(6):505–17. [PubMed: 15750836]
5. Regnier M, Martin H, Barsotti RJ, Rivera AJ, Martyn DA, Clemmens E. Cross-bridge versus thin filament contributions to the level and rate of force development in cardiac muscle. Biophys J 2004 Sep;87(3):1815–24. [PubMed: 15345560]
6. Regnier M, Martyn DA, Chase PB. Calcium regulation of tension redevelopment kinetics with 2-deoxy-ATP or low [ATP] in rabbit skeletal muscle. Biophys J 1998 Apr;74(4):2005–15. [PubMed: 9545059]
7. Regnier M, Rivera AJ, Chen Y, Chase PB. 2-deoxy-ATP enhances contractility of rat cardiac muscle. Circ Res 2000 Jun 23;86(12):1211–7. [PubMed: 10864910]
8. Gillis TE, Martyn DA, Rivera AJ, Regnier M. Investigation of thin filament near-neighbor regulatory unit interactions during skinned rat cardiac muscle force development. J Physiol 2007 Feb 22;580(Pt 2):561–76. [PubMed: 17317743]

9. Regnier M, Rivera AJ, Wang CK, Bates MA, Chase PB, Gordon AM. Thin filament near-neighbour regulatory unit interactions affect rabbit skeletal muscle steady-state force-Ca(2+) relations. J Physiol 2002 Apr 15;540(Pt 2):485–97. [PubMed: 11956338]
10. Li MX, Spyracopoulos L, Sykes BD. Binding of cardiac troponin-I147–163 induces a structural opening in human cardiac troponin-C. Biochemistry 1999 Jun 29;38(26):8289–98. [PubMed: 10387074]
11. Piroddi N, Tesi C, Pellegrino MA, Tobacman LS, Homsher E, Poggesi C. Contractile effects of the exchange of cardiac troponin for fast skeletal troponin in rabbit psoas single myofibrils. J Physiol 2003 Nov 1;552(Pt 3):917–31. [PubMed: 12937281]
12. Moreno-Gonzalez A, Fredlund J, Regnier M. Cardiac troponin C (TnC) and a site I skeletal TnC mutant alter Ca2+ versus crossbridge contribution to force in rabbit skeletal fibres. J Physiol 2005 Feb 1;562(Pt 3):873–84. [PubMed: 15611027]
13. Moreno-Gonzalez A, Gillis TE, Rivera AJ, Chase PB, Martyn DA, Regnier M. Thin-filament regulation of force redevelopment kinetics in rabbit skeletal muscle fibres. J Physiol 2007 Mar 1;579(Pt 2):313–26. [PubMed: 17204497]
14. Kreutziger KL, Gillis TE, Davis JP, Tikunova SB, Regnier M. Influence of enhanced troponin C Ca2+ binding affinity on cooperative thin filament activation in rabbit skeletal muscle. J Physiol 2007 Jun 21;583(1):337–50. [PubMed: 17584846]
15. Kreutziger KL, Piroddi N, Scellini B, Tesi C, Poggesi C, Regnier M. Thin filament Ca2+ binding properties and regulatory unit interactions alter kinetics of tension development and relaxation in rabbit skeletal muscle. J Physiol 2008 Aug 1;586(Pt 15):3683–700. [PubMed: 18535094]
16. Tikunova SB, Davis JP. Designing calcium-sensitizing mutations in the regulatory domain of cardiac troponin C. J Biol Chem 2004 Aug 20;279(34):35341–52. [PubMed: 15205455]
17. Norman C, Rall JA, Tikunova SB, Davis JP. Modulation of the rate of cardiac muscle contraction by troponin C constructs with various calcium binding affinities. Am J Physiol Heart Circ Physiol 2007 Oct;293(4):H2580–7. [PubMed: 17693547]
18. Tikunova SB, Davis JP, Rall JA. Engineering cardiac troponin C (cTnC) Mutants with dramatically altered Ca2+ dissociation rates as molecular tools to study cardiac muscle relaxation. Biophys J 2004;86(1):394a.
19. Kreutziger KL, Piroddi N, Belus A, Poggesi C, Regnier M. Ca2+-binding kinetics of troponin C influence force generation kinetics in cardiac muscle. Biophys J 2007;92:477a.
20. Kreutziger KL, Piroddi N, Belus A, Scellini B, Poggesi C, Regnier M. Effect of TnC with altered Ca2+ binding kinetics on force generation in striated muscle. J Muscle Res Cell Motil 2006;27:501–2.
21. Dong W, Rosenfeld SS, Wang CK, Gordon AM, Cheung HC. Kinetic studies of calcium binding to the regulatory site of troponin C from cardiac muscle. J Biol Chem 1996 Jan 12;271(2):688–94. [PubMed: 8557674]
22. Tikunova SB, Rall JA, Davis JP. Effect of hydrophobic residue substitutions with glutamine on Ca(2+) binding and exchange with the N-domain of troponin C. Biochemistry 2002 May 28;41(21):6697–705. [PubMed: 12022873]
23. Gomes AV, Venkatraman G, Davis JP, Tikunova SB, Engel P, Solaro RJ, et al. Cardiac troponin T isoforms affect the Ca(2+) sensitivity of force development in the presence of slow skeletal troponin I: insights into the role of troponin T isoforms in the fetal heart. J Biol Chem 2004 Nov 26;279(48):49579–87. [PubMed: 15358779]
24. Kohler J, Chen Y, Brenner B, Gordon AM, Kraft T, Martyn DA, et al. Familial hypertrophic cardiomyopathy mutations in troponin I (K183Delta, G203S, K206Q) enhance filament sliding. Physiol Genomics 2003 Jul 7;14(2):117–28. [PubMed: 12759477]
25. Piroddi N, Belus A, Eiras S, Tesi C, van der Velden J, Poggesi C, et al. No direct effect of creatine phosphate on the cross-bridge cycle in cardiac myofibrils. Pflugers Arch 2006 Apr;452(1):3–6. [PubMed: 16395599]
26. Brandt PW, Colomo F, Piroddi N, Poggesi C, Tesi C. Force regulation by Ca2+ in skinned single cardiac myocytes of frog. Biophys J 1998 Apr;74(4):1994–2004. [PubMed: 9545058]

27. Martyn DA, Chase PB, Hannon JD, Huntsman LL, Kushmerick MJ, Gordon AM. Unloaded shortening of skinned muscle fibers from rabbit activated with and without Ca2+ Biophys J 1994 Nov;67(5):1984–93. [PubMed: 7858135]
28. Tesi C, Colomo F, Nencini S, Piroddi N, Poggesi C. The effect of inorganic phosphate on force generation in single myofibrils from rabbit skeletal muscle. Biophys J 2000 Jun;78(6):3081–92. [PubMed: 10827985]
29. Tesi C, Piroddi N, Colomo F, Poggesi C. Relaxation kinetics following sudden Ca(2+) reduction in single myofibrils from skeletal muscle. Biophys J 2002 Oct;83(4):2142–51. [PubMed: 12324431]
30. Davis JP, Norman C, Kobayashi T, Solaro RJ, Swartz DR, Tikunova SB. Effects of thin and thick filament proteins on calcium binding and exchange with cardiac troponin C. Biophys J 2007 May 1;92(9):3195–206. [PubMed: 17293397]
31. Tikunova SB, Liu B, Swindle N, Little SC, Gomes AV, Swartz DR, et al. Effect of calcium-sensitizing mutations on calcium binding and exchange with troponin C in increasingly complex biochemical systems. Biochemistry 2010 Mar 9;49(9):1975–84. [PubMed: 20128626]
32. 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 2001 Jun;80(6):2798–808. [PubMed: 11371454]
33. Martyn DA, Regnier M, Xu D, Gordon AM. Ca2+ - and cross-bridge-dependent changes in N- and C-terminal structure of troponin C in rat cardiac muscle. Biophys J 2001 Jan;80(1):360–70. [PubMed: 11159408]
34. Wang YP, Fuchs F. Length, force, and Ca(2+)-troponin C affinity in cardiac and slow skeletal muscle. Am J Physiol 1994 Apr;266(4 Pt 1):C1077–82. [PubMed: 8178954]
35. Davis JP, Rall JA, Alionte C, Tikunova SB. Mutations of hydrophobic residues in the N-terminal domain of troponin C affect calcium binding and exchange with the troponin C-troponin I96–148 complex and muscle force production. J Biol Chem 2004 Apr 23;279(17):17348–60. [PubMed: 14970231]
36. Lu QW, Hinken AC, Patrick SE, Solaro RJ, Kobayashi T. Phosphorylation of cardiac troponin I at protein kinase C site threonine 144 depresses cooperative activation of thin filaments. J Biol Chem 2010 Apr 16;285(16):11810–7. [PubMed: 20164197]
37. Brenner B, Chalovich JM. Kinetics of thin filament activation probed by fluorescence of N-((2-(Iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1, 3-diazole-labeled troponin I incorporated into skinned fibers of rabbit psoas muscle: implications for regulation of muscle contraction. Biophys J 1999 Nov;77(5):2692–708. [PubMed: 10545369]
38. Fitzsimons DP, Patel JR, Campbell KS, Moss RL. Cooperative mechanisms in the activation dependence of the rate of force development in rabbit skinned skeletal muscle fibers. J Gen Physiol 2001 Feb;117(2):133–48. [PubMed: 11158166]
39. Razumova MV, Bukatina AE, Campbell KB. Different myofilament nearest-neighbor interactions have distinctive effects on contractile behavior. Biophys J 2000 Jun;78(6):3120–37. [PubMed: 10827989]
40. Brenner B. Effect of Ca2+ on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. Proc Natl Acad Sci U S A 1988 May;85(9):3265–9. [PubMed: 2966401]
41. Kress M, Huxley HE, Faruqi AR, Hendrix J. Structural changes during activation of frog muscle studied by time-resolved X-ray diffraction. J Mol Biol 1986 Apr 5;188(3):325–42. [PubMed: 3735425]
42. Huxley AF, Simmons RM. Rapid ‘give’ and the tension ‘shoulder’ in the relaxation of frog muscle fibres. J Physiol 1970 Sep;210(1):32P–3P.
43. Luo Y, Davis JP, Tikunova SB, Smillie LB, Rall JA. Myofibrillar determinants of rate of relaxation in skinned skeletal muscle fibers. Adv Exp Med Biol 2003;538:573–81. discussion 81–2. [PubMed: 15098700]
44. Lim CC, Yang H, Yang M, Wang CK, Shi J, Berg EA, et al. A novel mutant cardiac troponin C disrupts molecular motions critical for calcium binding affinity and cardiomyocyte contractility. Biophys J 2008 May 1;94(9):3577–89. [PubMed: 18212018]

45. Liang B, Chung F, Qu Y, Pavlov D, Gillis TE, Tikunova SB, et al. The familial hypertrophic cardiomyopathy related cardiac troponin C mutation L29Q affects Ca2+ binding and myofilament contractility. Physiol Genomics 2008 Feb 19;33(2):257–66. [PubMed: 18285522]

46. Landstrom AP, Parvatiyar MS, Pinto JR, Marquardt ML, Bos JM, Tester DJ, et al. Molecular and functional characterization of novel hypertrophic cardiomyopathy susceptibility mutations in TNNC1-encoded troponin C. J Mol Cell Cardiol 2008 Aug;45(2):281–8. [PubMed: 18572189]

47. Neulen A, Stehle R, Pfitzer G. The cardiac troponin C mutation Leu29Gln found in a patient with hypertrophic cardiomyopathy does not alter contractile parameters in skinned murine myocardium. Basic Res Cardiol. 2009 Jun 9;10.1007/s00395-009-0038-y

48. Biesiadecki BJ, Kobayashi T, Walker JS, John Solaro R, de Tombe PP. The troponin C G159D mutation blunts myofilament desensitization induced by troponin I Ser23/24 phosphorylation. Circ Res 2007 May 25;100(10):1486–93. [PubMed: 17446435]

Biographies

Kareen L. Kreutziger earned a B.S. in Biomedical Engineering at the University of Rochester (Rochester, NY, USA) in 2001 and her Ph.D. in Bioengineering at the University of Washington (Seattle, WA, USA) in 2007 with Dr. Michael Regnier. She was a Whitaker Foundation Pre-doctoral Fellow in Biomedical Engineering (2001–6). She is currently a Post-doctoral Fellow studying cardiac tissue engineering at the Center for Cardiovascular Biology with Dr. Charles E. Murry in the Department of Pathology at the University of Washington.

Nicoletta Piroddi Born in 1967. B.Sc. in Biology 1993. Ph.D. in Physiology 1998 (University of Florence, Italy) with Professor Corrado Poggesi. Full time Researcher in Physiology at Medical Faculty, University of Florence, since 2004. Teaching Physiology to students at Medical, Science and Pharmacy Faculties, University of Florence, since 2003. Present activity: lab research on molecular mechanisms underlying the force generation process in striated muscle by the use of skeletal and cardiac myofibrils from human and animal muscles.

Chiara Tesi Born 1960. B.Sc. 1985 (University of Florence, Italy). Research Fellowship, Biochemistry Unit Inserm U128 Montpellier-France (1986–1988). Ph.D. 2000 in Physiology (University of Florence). 1992-present: Associated Researcher, Associated Professor and Full Professor of Physiology, Medical Faculty, University of Florence, Italy. Member of the Biomedical PhD School of the University of Florence. Scientific Interests: study of the mechanism of chemomechanical coupling and its regulation in muscle contraction using biochemical and mechanical approaches.

Corrado Poggesi Born 1950. M.D. 1975 (University of Pavia, Italy). Initial academic career, Medical Faculty, University of Pavia. Senior Scientist, Department of Pharmacology, Mayo Clinic, Rochester, MN, USA (1986–87). Since 1994 Professor of Physiology, Medical Faculty, University of Florence, Italy. Visiting Scientist at the Department of Physiology and Biophysics, D. Geffen School of Medicine, UCLA, CA, USA (2003). Chair, Department of Physiological Sciences, University of Florence (2006–08). Present: Head of the Biomedical PhD School of the University of Florence; member of the Academic Senate of the University of Florence.

Michael Regnier is Professor and Vice-Chair of the Department of Bioengineering, and adjunct Professor of Physiology and Biophysics at the University of Washington (Seattle, WA, USA). He is also an Affiliate Investigator of the Benaroya Research Institute at Virginia Mason Hospital (Seattle, WA, USA) and an Established Investigator of the American Heart Association.

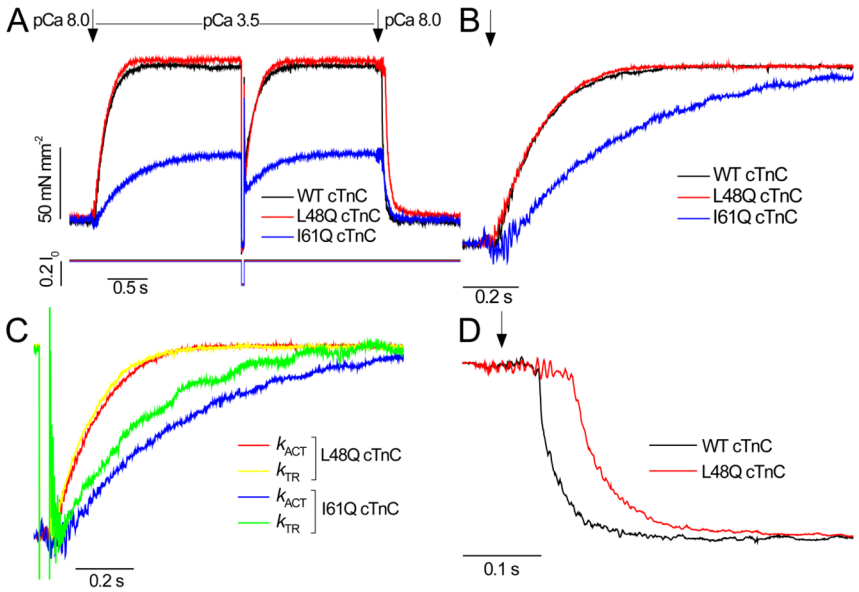

Figure 1.

Example tension traces of activation-relaxation cycle in mouse ventricular myofibrils. A, Tension traces for myofibrils exchanged with WT cTnC (black), L48Q cTnC (red), or I61Q cTnC (blue) are shown for activation from pCa 8 to pCa 3.5 by rapid solution change (first arrow), tension redevelopment resulting from a length release-restretch (see length trace below panel A), and relaxation from pCa 3.5 to pCa 8 (second arrow) at 15 °C and <5 μmol L^{-1} P_i (due to P_i scavenging, see Methods). Passive tension is the difference between initial tension and zero tension during the length release. B, Traces normalized to maximum tension for each condition and displayed with an expanded time scale demonstrate that activation rate (k_{ACT}) was decreased with I61Q cTnC (blue) but not with L48Q cTnC (red). C, Expanded time-scale traces of k_{ACT} and tension re-development rate (k_{TR}) super-imposed to show both rates are the same for L48Q cTnC (red and yellow, respectively) but differ for I61Q cTnC (blue and green, respectively). D, Normalized tension traces demonstrate that during relaxation, slow phase rate ($k_{REL,slow}$) was unchanged but duration is prolonged with L48Q cTnC (red) vs. WT cTnC (black), and that fast phase rate ($k_{REL,fast}$) did not differ.

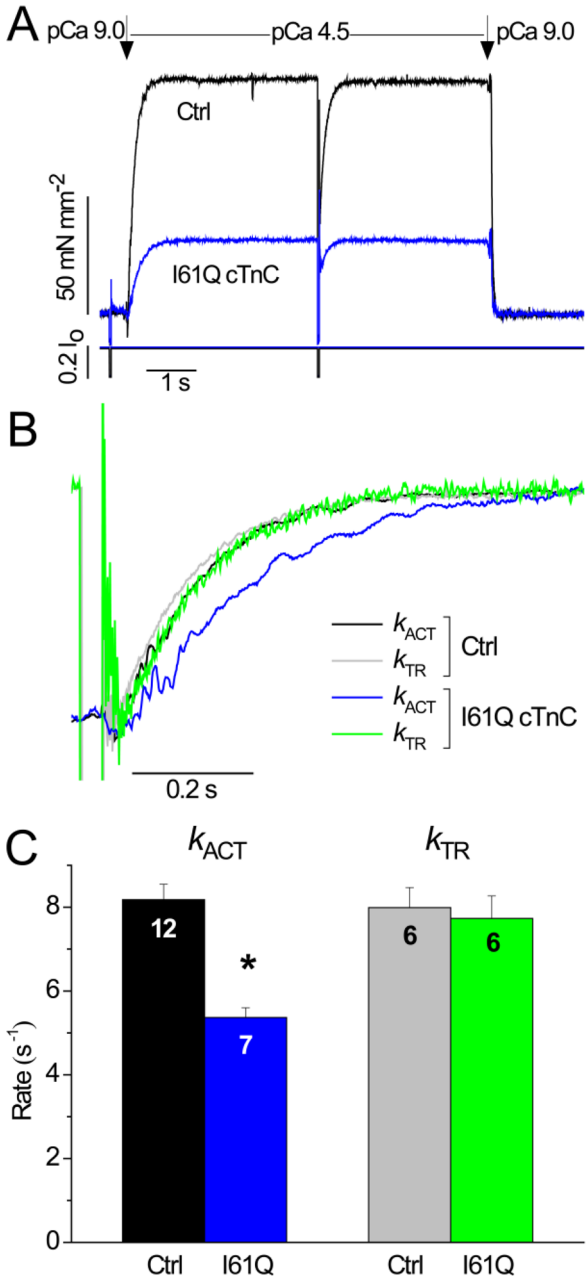

Figure 2.

Activation kinetics in the presence of 0.5 mmol L^{-1} inorganic phosphate (P_i) for control myofibrils vs. myofibrils with I61Q cTnC. A, Example traces of activation and relaxation at pCa 4.5 for WT cTnC (control; black) and I61Q cTnC (blue) with 0.5 mmol L^{-1} P_i. Length trace below tension trace shows transient for measurement of k_{TR}. B, Normalized k_{ACT} and k_{TR} traces demonstrating no difference between these rates for WT cTnC (control; black and grey, respectively), but slower k_{ACT} vs. k_{TR} for I61Q cTnC (blue vs. green, respectively). C, Summary of k_{ACT} and k_{TR} rates for 6–12 myofibrils. * $P < 0.02$.

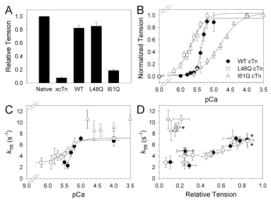

Figure 3.

Tension and k_{TR} as Ca^{2+} was varied for rat trabeculae with WT, L48Q, or I61Q cTn. A, T_{max} for exchange groups is shown relative to pre-exchange native T_{max} (1.0). Control measurements with xcTn (containing D65A cTnC which does not bind Ca^{2+} at site II) reduced T_{max} to <10% of WT cTn, suggesting almost complete exchange of cTn. Exchange with WT or L48Q cTn maintained ~85% T_{max}. With I61Q cTn T_{max} was greatly reduced. B, Tension-pCa curves (normalized to T_{max} for each condition) of summarized data for WT (black circles), L48Q (white squares), and I61Q cTn (white triangles). See Table 3 for fit parameters. C, k_{TR}-pCa data shows increased Ca^{2+} sensitivity of k_{TR} (pCa_{50}) with L48Q cTn vs. WT cTn and a dramatically reduced Ca^{2+} dependence of k_{TR} with I61Q cTn. D, k_{TR} data binned by pCa value and plotted vs. tension to show k_{TR} dependence on cross-bridge number (relative tension). Symbols in panels C and D are the same as panel B, and the black star (D) indicates $k_{TR,max}$ in I61Q cTnC-exchanged trabeculae in a low-P_i pCa 4.0 solution, which was 5.3 ± 0.6 s^{-1} (or ~45% reduced from $k_{TR,max}$ in the traditional activating solutions with ~0.5 mmol L^{-1} contaminating P_i). * Relative T_{max} and $k_{TR,max}$ immediately following cTn exchange (rather than at end of pCa curve).

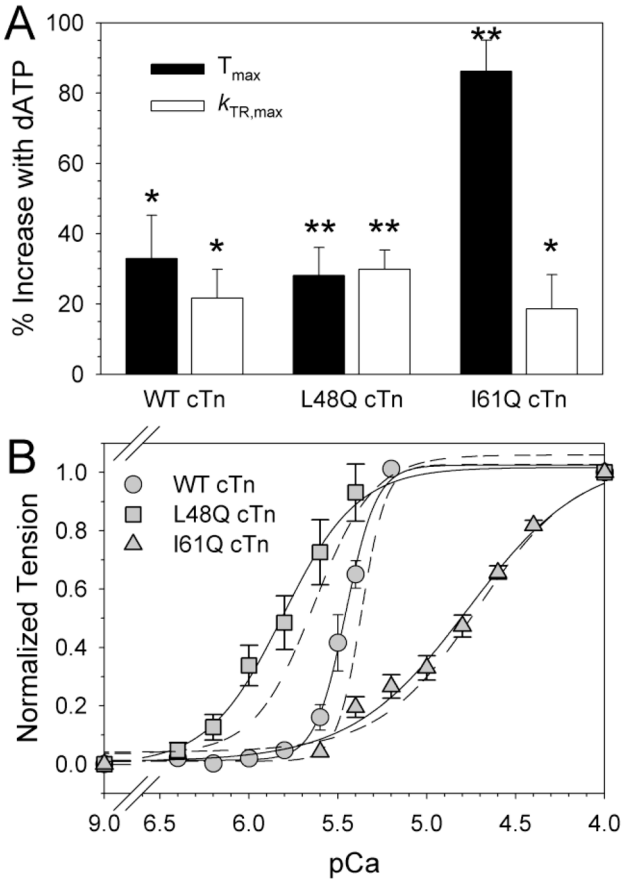

Figure 4.

Effect of 5 mmol L^{-1} dATP on T_{max}, $k_{TR,max}$, and tension-pCa in trabeculae exchanged with WT, L48Q, or I61Q cTn. A, T_{max} and $k_{TR,max}$ increased in the presence of dATP (vs. ATP) in back-to-back maximal activations (pCa 4.0). *P<0.05, **P<0.01 vs. ATP. B, Tension-pCa relationships with dATP for WT (grey circles), L48Q (grey squares), and I61Q cTn (grey triangles). Hill fit curves for ATP are shown as dashed lines for this subset of experiments, where paired comparisons were made with dATP for L48Q (left), WT (middle), and I61Q cTn (right). See text for fit values.

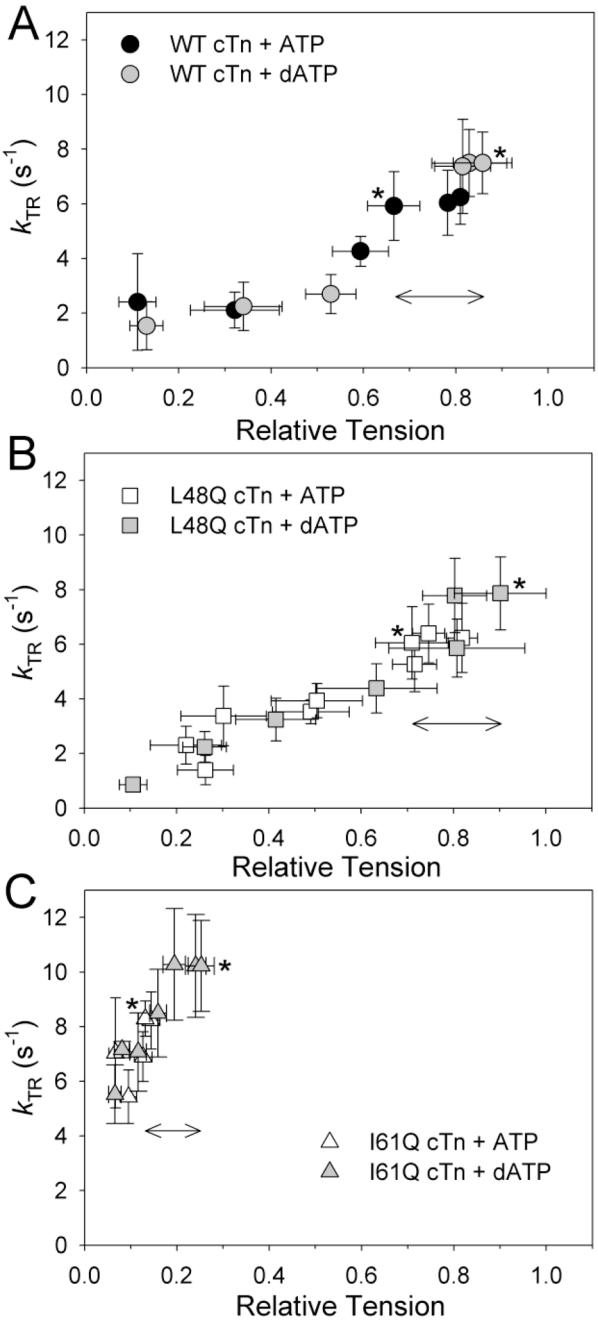

Figure 5.

Effect of 5 mmol L^{-1} dATP on k_{TR}-tension relationship in trabeculae exchanged with WT, L48Q, or I61Q cTn. In a subset of trabeculae exchanged with WT (A), L48Q (B), or I61Q cTn (C), k_{TR}-tension relationships were little affected as pCa was varied in the presence of ATP (A, black symbols; B, C, white symbols) or dATP (grey symbols). * Values from back-to-back maximal activations in ATP and dATP (rather than at end of pCa curve).

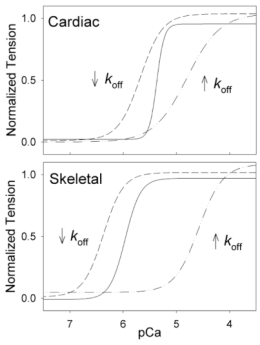

Figure 6.

Hill fits to cardiac data (Fig. 3B) and parallel previous studies with rabbit psoas skeletal fibers with sTnC mutants that decreased (M80Q sTnC^{F27W}; [14]) or increased (I60Q sTnC; [15]) k_{off}. The data, collected under similar solution and temperature conditions, demonstrate that cardiac muscle has a lower pCa_{50}, that altering k_{off} has similar effects on pCa_{50}, but that n_H is greatly reduced in cardiac but not skeletal muscle.

Table 1

Ca^{2+} dissociation rate (k_{off}) from mutant cTnC in whole cTn complex or in reconstituted thin filaments by stopped-flow spectroscopy with Quin-2 fluorescence at 15 °C.

cTn Type	cTn k_{off} (s^{-1}) (n)	TF k_{off} (s^{-1}) (n)
WT cTn	29.7 ± 0.5 (10)	75.4 ± 4.8 (3)
L48Q cTn	7.3 ± 0.1* (9)	28.0 ± 4.1* (3)
I61Q cTn	67.0 ± 9.3* (8)	237.7 ± 30.5* (4)

* P ≤ 0.01 vs. WT. TF, thin filament.

Table 2

Tension generation and relaxation parameters after cTnC exchange in mouse ventricular myofibrils at 15 °C. Values given are mean ± S.E.M.; number in parentheses is number of myofibrils. T_{max}, maximum isometric tension; k_{ACT}, rate constant of tension rise following step-wise pCa decrease (8.0 to 3.5) by fast solution switching; k_{TR}, rate constant of tension redevelopment following release-restretch of maximally activated myofibrils; k_{REL}, rate constant of tension relaxation during slow or fast phase.

MYOFIBRIL BATCHES	TENSION GENERATION			RELAXATION		
				Slow Phase		Fast Phase
	T_{max} (mN mm^{-2})	k_{ACT} (s^{-1})	k_{TR} (s^{-1})	Duration (ms)	k_{REL} (s^{-1})	k_{REL} (s^{-1})
Sham Treated Control	111 ± 10 (10)	7.26 ± 0.36 (11)	7.31 ± 0.48 (11)	59 ± 4 (10)	1.68 ± 0.21 (9)	21.8 ± 3.0 (10)
xcTnC	3 ± 0.7 (7)*°	-	-	-	-	-
WT cTnC	112 ± 12 (10)	7.71 ± 0.32 (10)	7.84 ± 0.28 (10)	47 ± 5 (10)	1.62 ± 0.17 (10)	24.1 ± 2.8 (10)
L48Q cTnC	97 ± 9 (9)	7.97 ± 0.30 (11)	8.01 ± 0.37 (11)	85 ± 6 (10)*	1.75 ± 0.34 (10)	14.5 ± 1.4 (10)
I61Q cTnC	40 ± 4 (20)*°	3.40±0.29 (20)*°	4.55± 0.35 (20)*°	48 ± 2 (19)$	1.71 ± 0.16 (18)	22.6 ± 2.4 (20)

° P < 0.01 vs. Control

* P < 0.01 vs. WT

$ P < 0.02 vs. Control

Table 3

Steady-state tension parameters after whole cTn exchange in demembranated rat trabeculae at 15 °C.

cTn Type	Relative T_{max}	pCa_{50}	n_H	$k_{TR,max}$ (s^{-1})
WT cTn (8)	0.83 ± 0.05	5.25 ± 0.08	6.5 ± 1.3	7.6 ± 0.8
L48Q cTn (8)	0.85 ± 0.06	5.63 ± 0.08*	2.6 ± 0.4*	7.2 ± 1.4
I61Q cTn (14)	0.19 ± 0.02*	4.77 ± 0.05*	2.2 ± 0.4*	9.2 ± 0.8

* P ≤ 0.01 vs. WT