Effect of Ca\(^{2+}\) binding properties of troponin C on rate of skeletal muscle force redevelopment

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SKELETAL MUSCLE CONTRACTION is regulated by Ca\(^{2+}\)-dependent interactions between the thick and thin filaments. The thin filaments are organized into repeating structural units each containing one tropomyosin (Tm) dimer and one troponin (Tn) complex attached per seven helically arranged actins (for review, see Refs. 8, 31). When the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) increases, Ca\(^{2+}\) binds to TnC (the Ca\(^{2+}\) binding subunit of the heterotrimeric Tn complex), which results in a series of conformational changes that shift Tm across actin to expose strong cross-bridge binding sites. This process is believed to involve three states of actin-Tm: 1) a blocked state, where Tm blocks strong binding of cross bridges to actin; 2) a closed state, in which cross bridges are able to weakly attach to actin; and 3) an open state, in which cross bridges are able to strongly attach to actin and produce force (12, 17). According to this model, the blocked, closed, and open states exist in equilibrium, with the blocked state predominating in the absence of Ca\(^{2+}\). Once Ca\(^{2+}\) binds to TnC, this shifts the thin filament equilibrium from the blocked to the closed state, which also appears to be the preferred state of Tm in the absence of Tn (16). Strongly bound cross bridges then further shift the thin filament equilibrium from the closed to the open state. This hypothesis implies that both Ca\(^{2+}\) binding to the thin filament and strongly bound cross bridges are required for activation of the thin filament and maximal force production. However, it is unclear to what degree each of these processes is involved in regulating the rate of contraction.

The rate of contraction can be approximated by a rapid shortening-restretch maneuver, developed by Brenner, who interpreted the results as a simple two-state system (1, 3). The rate of force redevelopment (k\textsubscript{ur}) was considered the sum of the rate of cross bridges transitioning from a non-force-producing state to a force-producing state (f\textsubscript{app}) and the rate of cross bridges detaching from a force-producing state to a non-force-producing state (g\textsubscript{app}), i.e., k\textsubscript{ur} = f\textsubscript{app} + g\textsubscript{app}. It is well established that k\textsubscript{ur} in striated muscle becomes faster with increasing [Ca\(^{2+}\)]\textsubscript{i}, which has been explained as a Ca\(^{2+}\)-dependent effect on f\textsubscript{app} (2, 4, 5, 10, 33).

The mechanism by which Ca\(^{2+}\) modulates the rate of contraction and k\textsubscript{ur} is still under debate; however, evidence suggests that it is unlikely to be a direct effect of Ca\(^{2+}\) on the intrinsic kinetics of the cross-bridge cycle (9, 11, 13, 19, 20, 25, 32). It has been suggested that cooperative interactions within the myofilaments influence the rate of contraction (4). For instance, reducing the cooperativity via partial TnC extraction and addition of N-ethylmaleimide-modified myosin subfragment-1 (NEM-S1), a strong-binding cross bridge analog, elevated k\textsubscript{ur} at low Ca\(^{2+}\) concentration. This results suggest that the TnC mutants predominantly affected k\textsubscript{ur} through modulating the level of thin filament activation and not by altering intrinsic cross-bridge cycling properties. To corroborate this, NEM-S1, a non-force-generating cross-bridge analog that activates the thin filament, fully recovered maximal k\textsubscript{ur} for I60QTnC at low Ca\(^{2+}\) concentration. Thus TnC mutants with altered Ca\(^{2+}\) binding properties can control the rate of contraction by modulating thin filament activation without directly affecting intrinsic cross-bridge cycling rates.

rate of force redevelopment; mutation; N-ethylmaleimide-modified myosin subfragment-1; troponin C extraction/reconstitution
have attempted to address this issue by incorporating either thin filament activation or cooperativity; however, a model incorporating both has not yet been developed, and the relative importance of their contributions to $k_{tr}$ is still under debate (4, 22).

Level of thin filament activation, cooperativity, and cross-bridge kinetics are at least three mechanisms that affect $k_{tr}$. To help distinguish which of these mechanisms TnC affects, we have engineered TnC mutants with altered Ca$^{2+}$ binding properties and incorporated them into single-skinned psoas fibers from rabbits. We have determined the effects of the TnC mutants on the Ca$^{2+}$ sensitivity of force production, $k_{tr}$, and the cooperativity of these processes. We also utilized partial TnC extraction and NEM-S1 to elucidate the mechanism by which altering the Ca$^{2+}$ binding properties of the thin filament might affect $k_{tr}$. We propose that altering the Ca$^{2+}$ binding properties of TnC will affect both the level of thin filament activation, as indicated by the level of steady-state force produced, and cooperative interactions to modulate $k_{tr}$.

MATERIALS AND METHODS

Materials. Phenyl-Sepharose CL-4B and EGTA were purchased from Sigma Chemical (St. Louis, MO). Quin-2 was purchased from Calbiochem (La Jolla, CA). IAANS was purchased from Molecular Probes (Eugene, OR).

Protein mutagenesis and purification. The pET-24 plasmid encoding rabbit skeletal TnC was a generous gift from Dr. Michael Regnier (University of Washington, Seattle, WA). TnC mutants were constructed, expressed, and purified from the TnC plasmid as previously described (30). Tnl, Tn1, and myosin subfragment-1 (S1) were purified from rabbit skeletal muscle as previously reported (28). Myosin S1 was further modified with NEM as previously described (28).

Labeling of TnC and reconstitution of Tn complexes. TnC and its mutants were labeled with the environmentally sensitive probe IAANS for 3–5 h at 22°C with constant shaking in labeling buffer (in mM: 50 Tris, 90 KCl, and 1 EGTA, with 6 M urea, pH 7.5). The labeling reaction was stopped by addition of 2 mM DTT, and the labeled protein was exhaustively dialyzed against labeling buffer at 4°C to remove unreacted label. Tn complexes were reconstituted as previously described (6).

Determination of Ca$^{2+}$ dependence of conformational changes in IAANS-labeled Tn complexes ($k_{Ca}$). All steady-state fluorescence measurements were performed with a Perkin-Elmer LS55 spectrophotometer at 15°C. IAANS fluorescence was excited at 330 nm and monitored at 450 nm as microliter amounts of CaCl$_2$ were added to 2 ml of each labeled Tn complex (0.15 μM) in (in mM) 200 MOPS (to prevent pH changes on addition of Ca$^{2+}$), 150 KCl, 2 EGTA, 1 EDTA, and 3 MgCl$_2$, with 0.02% Tween 20, pH 7.0 at 15°C. The free [Ca$^{2+}$] was calculated with the computer program EGCA02 developed by Robertson and Potter (26). The Ca$^{2+}$ sensitivities of conformational changes were reported as a dissociation constant, $k_{Ca}$ (μM), representing a mean ± SE of three or four separate titrations. The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described (30).

Determination of Ca$^{2+}$ dissociation kinetics. All kinetic measurements were performed with the use of an Applied Photophysics (Leatherhead, UK) model SX.18 MV stopped-flow instrument with a dead time of ~1.4 ms at 15°C. The Ca$^{2+}$ dissociation rates ($k_{Ca}$) from unlabeled Tn complexes were directly measured with the fluorescent Ca$^{2+}$ chelator quin-2. Quin-2 was excited with a 150-W xenon arc source at 330 nm. Quin-2 emission was monitored through a 510-nm broad-band interference filter from Oriel (Stratford, CT). The rates of conformational changes due to Ca$^{2+}$ removal by EGTA from labeled Tn complexes were measured by following IAANS fluorescence decay. The samples were excited at 330 nm. The IAANS emission was monitored through a 420- to 470-nm band-pass interference filter from Oriel. The buffer used in the stopped-flow experiments was (in mM) 10 MOPS, 150 KCl, 1 EDTA, and 3 MgCl$_2$, with 0.02% Tween 20, pH 7.0. The data were fit with a program (by P. J. King, Applied Photophysics) that utilizes the nonlinear Levenberg-Marquardt algorithm. Each data set represents an average of at least three separate experiments, each averaging at least five traces fit with a single-exponential equation.

Standard solutions for muscle experiments. The solutions for skinned fiber experiments were prepared as previously described (23). Large batches of pCa 9.0 and pCa 4.0 solutions were divided into aliquots and stored at ~80°C. These aliquots were thawed and mixed to make intermediate-pCa solutions, which were stored at 4°C and used within a week.

Preparation of rabbit psoas fibers. All protocols were approved by the Institutional Animal Care and Use Committee. Rabbit psoas muscle was harvested, cut into small bundles (~2 mm long), and stored in 50% relaxing/50% glycerol solution overnight at 4°C. Fibers were then stored at ~20°C for up to 3 wk. Single fibers were isolated from these bundles, attached on both ends with aluminum T clips, fixed at the clamped areas with 25% glutaraldehyde, and then skinned in relaxing solution with 1% Triton X for 5 min. The skinned fibers were then mounted between the arms of a high-speed length controller (model 322C, Aurora Scientific) and an isometric force transducer (model 403A, Aurora Scientific) in a chamber filled with pCa 9.0 solution. The resting sarcomere length was set at ~2.5 μm as determined by the first-order diffraction pattern from a HeNe laser directed through the fiber. Cross-sectional area was calculated from width measurements assuming a circular circumference. Passive force was measured by rapidly shortening the fiber (1 ms, 10% of total length) in a pCa 9.0 solution. This resulted in a drop of force below the resting force baseline, which was measured as the passive force. Contractile force was measured by washing the fiber for 1 min in hexamethylenediamine-N,N,N′,N′-tetraacetate (HDTA) solution containing (in mM) 6.6 HDTA, 0.4 EGTA, 1.0 Mg free, 14.5 phosphocreatine, 4.39 MgATP, and 20.0 imidazole, pH 7.0. After isometric force was allowed to develop and plateau, the total force developed was measured as the difference between the plateau and the baseline upon shortening. Active force generated by the fiber in various pCa solutions was calculated as the total force minus the resting force. The force per cross-sectional area (F/CSA) was calculated from the second of two maximal activations at the beginning of the experiment to determine maximum endogenous force. The mean F/CSA of 34 fibers used in this study was 86 ± 6 mm²/mg². All experiments were performed at 15°C.

Protocol for measuring rate of force redevelopment. A rabbit psoas fiber was contracted in a solution ranging from pCa 4.0 to pCa 7.5 until the force reached a plateau. It was then rapidly shortened (1 ms, 10% of total length), held at this position for 10 ms, and then rapidly restretched (1 ms) to its original length. An in-house Lab View program was utilized to control the movement of the high-speed length controller. Force redevelopment to similar initial levels, and $k_{tr}$ was measured by assuming a single-exponential process as $k_{tr} = \ln(2)/t_{1/2}$, where $t_{1/2}$ is the time to 50% of force redevelopment. To account for rundown over the course of the experiment, activations in pCa 4.0 were repeated at the middle and end of the experiments.

Extraction and reconstitution of TnC. After maximal force and $k_{tr}$ were determined for the unextracted fibers, fibers were soaked for 2 min in TnC extraction buffer containing (in mM) 10 HEPES, 5 EDTA, and 0.5 trifluoperazine (TFP) at pH 7.0. The fibers were then washed three times for 1 min in a pCa 9.0 solution to remove residual TFP and then maximally activated by a pCa 4.0 solution. Force routinely fell to 5 ± 2% of maximal force ($F_{max}$). Subsequently the fibers were reconstituted for 2 min in 400 μl of pCa 9.0 solution with 16 μM TnC and then maximally activated twice to measure percent force recovery. The fibers were then contracted in randomly selected...
pCa solutions to measure force and $k_o$. After each pCa measurement, the fiber was fully relaxed in pCa 9.0. Fibers that showed rundown $>10\%$ F$_{max}$ over the course of the experiment were excluded. For partial extraction of TnC fibers were prepared as above, but after maximal endogenous force was measured they were only soaked in TnC extraction solution for 7–10 s. Force and maximal endogenous force was measured they were only soaked in 10% F$_{max}$ over the course of the experiment were excluded. For

Results

Following Ca$^{2+}$ binding and exchange with TnC in Tn complex. We measured the Ca$^{2+}$ sensitivity and $k_{off}$ of the engineered TnCs incorporated into the Tn complex by labeling the native Cys$^{90}$ residue of TnC with the environmentally sensitive probe IAANS. The whole Tn complex was used since we believe it provides a reasonable approximation for the Ca$^{2+}$ binding properties of skeletal TnC in muscle (6). Accordingly, we could monitor the Ca$^{2+}$-induced changes in fluorescence that occur when Ca$^{2+}$ bound to the regulatory domain of the wild-type TnIAANS-Tn-TnT complex (here designated here as WTTnIAANS), as well as the TnC mutants V43QTnIAANS, I60QTnIAANS, and T70DTnIAANS. As seen in Fig. 1, the fluorescence intensity for all the TnIAANS complexes decreased with the addition of Ca$^{2+}$. Compared with WTTnIAANS (pCa$\approx$ 7.25 ± 0.01), the V43QTnIAANS mutation increased the Ca$^{2+}$ sensitivity of the TnIAANS complex ~1.9-fold (pCa$\approx$ 7.53 ± 0.02) while the T70DTnIAANS (pCa$\approx$ 5.53 ± 0.03) and I60QTnIAANS (pCa$\approx$ 5.53 ± 0.07) mutations decreased the Ca$^{2+}$ sensitivity of the TnIAANS complex ~5.0- and 52-fold, respectively.

Effect of TnC mutations on rates of Ca$^{2+}$ dissociation from unlabeled and labeled Tn complexes. Figure 2A shows the EGTA-induced rates of structural change ($k_{offIAANS}$) reported by the IAANS probe as Ca$^{2+}$ dissociated from the labeled TnIAANS complexes in a stopped-flow apparatus. Compared with WTTnIAANS (6.8 ± 0.2 s$^{-1}$), $k_{offIAANS}$ for V43QTnIAANS (1.9 ± 0.1 s$^{-1}$) decreased ~3.6-fold, while $k_{offIAANS}$ for T70DTnIAANS (38.9 ± 0.5 s$^{-1}$) and I60QTnIAANS (145 ± 4 s$^{-1}$) increased ~5.7-fold and ~21-fold, respectively. These rates can be compared with the actual Ca$^{2+}$ dissociation rates ($k_{offQuin-2}$) measured from unlabeled Tn complexes by following changes in the fluorescence of the Ca$^{2+}$ chelator quin-2 as shown in Fig. 2B and summarized in Table 1. Compared with WTTn (6.8 ± 0.2 s$^{-1}$), $k_{offQuin-2}$ for V43QTn (1.73 ± 0.01 s$^{-1}$) decreased ~3.9-fold, while $k_{offQuin-2}$ for T70DTn (37.2 ± 0.2 s$^{-1}$) and I60QTn (103 ± 3 s$^{-1}$) increased ~5.5-fold and ~15-fold, respectively. These results suggest that the conformational change in the regulatory domain of the Tn complex occurred concomitantly with Ca$^{2+}$ dissociation. However, there is a slight discrepancy for I60QTn, as the rate of IAANS-reported conformational change was ~1.4-fold faster than the rate of Ca$^{2+}$ dissociation measured with quin-2. Assuming $K_d = k_{off}/k_{on}$ we calculated the rate of Ca$^{2+}$ association ($k_{onCa^{2+}}$) for the TnIAANS complexes as seen in Table 1. $k_{onCa^{2+}}$ values were

![Fig. 1. Effect of troponin (Tn)C mutations on Ca$^{2+}$ binding to the Tn complex. Ca$^{2+}$-dependent changes in IAANS fluorescence are shown for wild type (WT), V43Q, T70D, and I60Q as a function of pCa: 100% IAANS fluorescence corresponds to the highest fluorescence value, whereas 0% fluorescence corresponds to the lowest fluorescence value for each individual TnIAANS complex. Each data point represents the mean ± SE of 3 or 4 titrations fit with a logistic sigmoid curve.](http://ajpcell.physiology.org/)

![Fig. 2. Effect of TnC mutations on rate of Ca$^{2+}$ dissociation from the Tn complex. A: time course of increase (Δ) in IAANS fluorescence as Ca$^{2+}$ was removed by EGTA from the regulatory Ca$^{2+}$ binding sites of WTTnIAANS, V43QTnIAANS, T70DTnIAANS, and I60QTnIAANS. Each Tn complex (1 μM) in (mM) 10 MOPS, 150 KCl, 1 DTT, and 3 MgCl, with 0.02% Tween 20, pH 7.0, + 100 μM Ca$^{2+}$ was rapidly mixed with an equal volume of the same buffer + 10 mM EGTA at 15°C. B: time course of increase in quin-2 fluorescence as Ca$^{2+}$ was removed by quin-2 from the regulatory Ca$^{2+}$ binding sites of WTTn, V43QTn, T70DTn, and I60QTn. Each Tn complex (3 μM) in (mM) 10 MOPS, 150 KCl, 1 DTT, and 3 MgCl, with 0.02% Tween 20, pH 7.0 was rapidly mixed with an equal volume of the same buffer + 150 μM quin-2 at 15°C.](http://ajpcell.physiology.org/)
similar for WTTbAANS (1.2 ± 0.1 × 10^8 M^{-1}s^{-1}) and T70DTbAANS (1.3 ± 0.1 × 10^8 M^{-1}s^{-1}), while \( k_{\text{offCa}^{2+}} \) for V43QTbAANS (0.64 ± 0.03 × 10^8 M^{-1}s^{-1}) and I60QTbAANS (0.49 ± 0.07 × 10^8 M^{-1}s^{-1}) decreased ~1.9-fold and ~2.4-fold, respectively. Thus mutations in TnC can alter both the \( Ca^{2+} \) association and dissociation rates.

**Effect of TnC mutations on \( Ca^{2+} \) sensitivity of force development.** The unlabeled mutant TnCs were incorporated into rabbit skinned psoas fibers via an extraction/reconstitution protocol. We first compared the effects of the unlabeled WTTnC to the results obtained for endogenous TnC to ensure that the extraction and reconstitution process did not have any significant effect (see Table 2). Similar to the endogenous TnC, reconstitution of fibers with WTTnC yielded 101 ± 3% force recovery with a \( pC_{50} \) of 5.98 ± 0.02 and a Hill coefficient of 2.5 ± 0.3 (Fig. 3, Table 2). The \( Ca^{2+} \) sensitivity values of force development by the TnC mutants were then compared with the WTTnC values. V43QTnC returned maximal force (103 ± 2%) and sensitized force development to \( Ca^{2+} \) ~7.9-fold (\( pC_{50} \) of 6.88 ± 0.08), consistent with it having a higher \( Ca^{2+} \) sensitivity. On the other hand, T70DTnC reduced maximal recovered force (90 ± 3%) and desensitized force production to \( Ca^{2+} \) ~1.7-fold (\( pC_{50} \) 5.74 ± 0.03). I60QTnC produced results similar to T70DTnC but to a greater extent, with 76 ± 4% maximal force recovery and ~4.0-fold (\( pC_{50} \) 5.35 ± 0.06) lower \( Ca^{2+} \) sensitivity of force production. In addition, both V43QTnC (Hill coefficient of 1.1 ± 0.2) and I60QTnC (Hill coefficient of 1.7 ± 0.1) caused significant reductions in cooperativity of force development, but not T70DTnC (Hill coefficient 2.7 ± 0.2). Partial extraction of TnC to force levels similar to that recovered by I60QTnC had a significant effect on neither the \( Ca^{2+} \) sensitivity of force nor the cooperativity of force development (see Table 2). These results demonstrate that the \( Ca^{2+} \)-desensitizing effects and decrease in cooperativity caused by I60QTnC are not due to incomplete incorporation of TnC.

**Effect of TnC mutations on \( Ca^{2+} \) sensitivity of \( k_{\text{tr}} \).** After determining that these TnC mutants drastically affected the \( Ca^{2+} \) sensitivity of force development, we examined their effects on \( k_{\text{tr}} \). Representative force traces demonstrating the force redevelopment protocol from fibers reconstituted with WTTnC, V43QTnC, I60QTnC, or T70DTnC are shown in Fig. 4 at \( pC_{a} \) 4.0, 5.6, and 6.0. \( k_{\text{tr}} \) was calculated by assuming a single-exponential process. Clearly, altering the \( Ca^{2+} \) binding properties of TnC can have a drastic effect on \( k_{\text{tr}} \) at matched \( pC_{a} \).

As seen in Table 3, the WTTnC did not have a significant effect on the \( Ca^{2+} \) sensitivity (\( pC_{50} \) 5.72 ± 0.03) of \( k_{\text{tr}} \), cooperativity of the increase in \( k_{\text{tr}} \) (Hill coefficient of 1.7 ± 0.2), or minimal (i.e., the lowest measurable) \( k_{\text{tr}} \) (22 ± 1%) compared with the endogenous TnC. However, there was a slight reduction in the recovery of maximal \( k_{\text{tr}} \) for WTTnC (93 ± 3%) compared with endogenous TnC (Table 3), where maximal \( k_{\text{tr}} \) for endogenous TnC was 11.6 ± 1.1 s^{-1} (\( n = 4 \)). The \( Ca^{2+} \) sensitivities of \( k_{\text{tr}} \) for mutant TnCs were compared with those for WTTnC. As seen in Fig. 5 and summarized in Table 3, V43QTnC recovered maximal \( k_{\text{tr}} \) (98 ± 4%) and sensitized \( k_{\text{tr}} \) to \( Ca^{2+} \) ~1.8-fold (\( pC_{50} \) 6.08 ± 0.1). Maximal \( k_{\text{tr}} \) recovery was reduced for both T70DTnC (87 ± 6%) and

### Table 1. Comparison of \( Ca^{2+} \) binding properties of TnC mutants

<table>
<thead>
<tr>
<th>TnC</th>
<th>( pC_{50} )</th>
<th>( n )</th>
<th>( k_{\text{onCa}^{2+}} ), s^{-1}</th>
<th>( k_{\text{offCa}^{2+}} ), s^{-1}</th>
<th>Calculated ( k_{\text{offCa}^{2+}} ), from ( k_{\text{onCa}^{2+}} ), × 10^8 M^{-1}s^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.25 ± 0.01</td>
<td>1.34 ± 0.03</td>
<td>( 6.8 ± 0.2 )</td>
<td>( 6.8 ± 0.2 )</td>
<td>( 1.2 ± 0.1 )</td>
</tr>
<tr>
<td>V43Q</td>
<td>7.53 ± 0.02*</td>
<td>1.35 ± 0.07</td>
<td>( 1.9 ± 0.1* )</td>
<td>( 1.73 ± 0.01* )</td>
<td>( 0.64 ± 0.03* )</td>
</tr>
<tr>
<td>T70D</td>
<td>6.55 ± 0.03*</td>
<td>0.86 ± 0.05*</td>
<td>( 3.89 ± 0.5* )</td>
<td>( 37.2 ± 0.2* )</td>
<td>( 1.3 ± 0.1 )</td>
</tr>
<tr>
<td>I60Q</td>
<td>5.53 ± 0.07*</td>
<td>0.70 ± 0.07*</td>
<td>( 145 ± 4* )</td>
<td>( 103 ± 3* )</td>
<td>( 0.49 ± 0.07* )</td>
</tr>
</tbody>
</table>

Values are means ± SE. TnC, troponin C; \( n \), Hill coefficient; \( k_{\text{offCa}^{2+}} \), dissociation rate; \( k_{\text{onCa}^{2+}} \), association rate; WT, wild type. *Significantly different from WT as determined by a Student’s \( t \)-test.

### Table 2. Comparison of effects of TnC mutants on \( Ca^{2+} \) sensitivity of force development

<table>
<thead>
<tr>
<th>TnC</th>
<th>( n )</th>
<th>( pC_{50} )</th>
<th>( nH )</th>
<th>%F Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>7</td>
<td>5.91 ± 0.03</td>
<td>2.9 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>WT</td>
<td>4</td>
<td>5.98 ± 0.02</td>
<td>2.5 ± 0.3</td>
<td>101 ± 3</td>
</tr>
<tr>
<td>WT NEM-S1</td>
<td>6</td>
<td>6.02 ± 0.02</td>
<td>1.9 ± 0.2*</td>
<td>88 ± 3*</td>
</tr>
<tr>
<td>V43Q</td>
<td>5</td>
<td>6.88 ± 0.08*</td>
<td>1.1 ± 0.2*</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>T70D</td>
<td>4</td>
<td>5.74 ± 0.03*</td>
<td>2.7 ± 0.2</td>
<td>90 ± 3*</td>
</tr>
<tr>
<td>I60Q</td>
<td>4</td>
<td>5.35 ± 0.06*</td>
<td>1.7 ± 0.1*</td>
<td>76 ± 4*</td>
</tr>
<tr>
<td>I60Q NEM-S1</td>
<td>4</td>
<td>5.43 ± 0.03*</td>
<td>1.4 ± 0.1*</td>
<td>71 ± 5*</td>
</tr>
<tr>
<td>Partial extraction</td>
<td>4</td>
<td>5.9 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>75 ± 1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. TnC, troponin C; NEM-S1, N-ethylmaleimide-modified myosin subfragment-1; NA, not applicable. *Significantly different from WT as determined by a Student’s \( t \)-test.

**Fig. 3. Effect of TnC mutations on \( Ca^{2+} \) dependence of skeletal muscle force generation.** \( Ca^{2+} \) dependence of isometric force generation in single-skinned psoas fibers reconstituted with WTTnC, V43QTnC, T70DTnC, and I60QTnC is shown as a function of \( pC_{a} \). Experimental conditions are described under MATERIALS AND METHODS. Each data point represents the mean ± SE from at least 4 separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
I60QTnC (48 ± 4%). T70DTnC and I60QTnC also decreased the Ca\(^{2+}\) sensitivity of \(k_{tr}\) ~2.5-fold (p\(Ca_{50}\) 5.45 ± 0.4) and ~6.9-fold (p\(Ca_{50}\) 5.0 ± 0.1), respectively. However, the three mutants had varying effects on the cooperativity of \(k_{tr}\). Significant changes were only seen for V43QTnC (Hill coefficient of 0.8 ± 0.1), which decreased cooperativity, and T70DTnC (Hill coefficient of 2.4 ± 0.4), which increased cooperativity. Partial extraction of TnC to maximal force recovery levels similar to those for I60QTnC did not affect the Ca\(^{2+}\) sensitivity or cooperativity of \(k_{tr}\) or maximal or minimal \(k_{tr}\) (Table 3).

**Relationship between \(k_{tr}\) and relative force.** To examine whether the TnC mutants altered \(k_{tr}\) by modulating the availability of myosin binding sites on actin or by altering the apparent rate of cross-bridge cycling, we plotted the force vs. \(k_{tr}\) relationship for the different TnCs (Fig. 6). At matched relative forces ~30%, all the TnCs displayed a similar force vs. \(k_{tr}\) relationship. However, at relative forces ~30%, there was a ~1.5-fold increase in \(k_{tr}\) for both V43QTnC and I60QTnC compared with WTTnC (Table 3). Thus for relative forces >30%, it would appear that the mutant TnCs do not alter the apparent rate of cross-bridge cycling. However, since \(k_{tr}\) for I60QTnC could only be measured up to ~70% relative force, it was unclear whether this relationship still held at relative forces >70%.

**Effect of NEM-S1 on force and \(k_{tr}\) for WTTnC and I60QTnC.** To determine whether I60QTnC affected the intrinsic cross-bridge cycling rate, we utilized NEM-S1, a strong-binding cross bridge analog that can trap the thin filament into an open conformation (28). Incorporation of 6 \(\mu\)M NEM-S1 with WTTnC fibers resulted in a ~12% reduction of force, no significant effect on the Ca\(^{2+}\) sensitivity of force (p\(Ca_{50}\) of 6.02 ± 0.2), and a decrease in the cooperativity of force production (Hill coefficient of 1.9 ± 0.2) (Fig. 7A; Table 2). These results agree well with previously reported effects of NEM-S1 on fibers containing endogenous TnC (9). On the other hand, adding NEM-S1 to I60QTnC did not have a

### Table 3. Comparison of effects of TnC mutants on Ca\(^{2+}\) sensitivity of \(k_{tr}\) development

| TnC          | \(n\) | p\(Ca_{50}\) | Hill coefficient | Maximum \(k_{tr}\), s\(^{-1}\) | \(k_{tr}\) Recovery at p\(Ca\) 4.0 | % Minimum \(k_{tr}\)<br> | % Minimum \(k_{tr}\)<br>
<table>
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</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>7</td>
<td>5.59 ± 0.09</td>
<td>1.89 ± 0.03</td>
<td>11.6 ± 1.1</td>
<td>NA</td>
<td>22 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>WT</td>
<td>4</td>
<td>5.72 ± 0.03</td>
<td>1.7 ± 0.2</td>
<td>10.8 ± 1.3</td>
<td>93 ± 3</td>
<td>22 ± 1</td>
<td>32 ± 1*</td>
</tr>
<tr>
<td>WT NEM-S1</td>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>11.7 ± 1.2</td>
<td>101 ± 3</td>
<td>NA</td>
<td>32 ± 1*</td>
</tr>
<tr>
<td>V43Q</td>
<td>5</td>
<td>6.08 ± 0.10*</td>
<td>0.8 ± 0.1*</td>
<td>12.0 ± 0.9</td>
<td>98 ± 4</td>
<td>32 ± 1*</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>T70D</td>
<td>4</td>
<td>5.45 ± 0.4*</td>
<td>2.4 ± 0.4*</td>
<td>10.3 ± 1.1</td>
<td>87 ± 6</td>
<td>NA</td>
<td>32 ± 1*</td>
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<tr>
<td>I60Q</td>
<td>4</td>
<td>5.0 ± 0.1*</td>
<td>1.4 ± 0.4</td>
<td>5.7 ± 1.7*</td>
<td>48 ± 4*</td>
<td>NA</td>
<td>32 ± 1*</td>
</tr>
<tr>
<td>I60Q NEM-S1</td>
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<td>NA</td>
<td>NA</td>
<td>12.6 ± 2.7</td>
<td>70 ± 2*</td>
<td>NA</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Partial extraction</td>
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<td>5.8 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>10.7 ± 1.2</td>
<td>92 ± 4</td>
<td>17 ± 3</td>
<td></td>
</tr>
</tbody>
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Values are means ± SE. \(k_{tr}\), Rate of force redevelopment. *Significantly different from WT as determined by a Student’s \(t\)-test. NA represents data not collected and thus not available.
significant effect on maximal recovered force, Ca$^{2+}$ sensitivity of force development, or cooperativity of force development (Tables 2 and 3). However, at low relative forces in the presence of NEM-S1, $k_r$ was at maximal preextraction values for both WTTnC and I60QTnC fibers (Fig. 7B). At intermediate levels of relative force $k_r$ decreased for both WTTnC and I60QTnC fibers, whereas at higher forces only with WTTnC was maximal preextraction $k_r$ reached. Thus I60QTnC can exhibit maximal preextraction $k_r$ if the thin filament is activated by NEM-S1.

**DISCUSSION**

Previous work has shown that $k_r$ can be affected by the level of thin filament activation (15, 24), changes in cross-bridge kinetics (1), and cooperative interactions (9) (for further review see Ref. 27). The level of thin filament activation and cross-bridge kinetics appear to have the most significant influences on $k_r$. However, whereas cross-bridge kinetics have a direct effect on $k_r$, thin filament activation modulates $k_r$ by limiting the number of cross bridges that can bind to the thin filament. Our goal in this study was to examine the effects that Ca$^{2+}$ binding properties of TnC have on $k_r$ and how they do so in relation to these three factors that modulate $k_r$.

Previous studies that have addressed the role of TnC in modulating $k_r$ in skeletal muscle utilized TnC extraction, TnC isoforms, and the Ca$^{2+}$-sensitizing compound calmidazolium (5, 15, 18, 24). Simple extraction of TnC or incorporation of a TnC that cannot bind Ca$^{2+}$ into muscle decreases maximal force without altering maximal $k_r$ (5, 18). On the other hand I60QTnC$^{527W}$, a Ca$^{2+}$-desensitizing mutant, decreases $k_{off}$ and decreased maximal force and $k_r$ (15), similar to our findings with I60QTnC. Incorporation of cardiac TnC or M80QTnC (a Ca$^{2+}$-sensitizing mutation that slows $k_{off}$) into skeletal muscle caused an elevation of $k_r$ at submaximal [Ca$^{2+}$] (5, 15), whereas calmidazolium, which sensitizes skeletal muscle to Ca$^{2+}$ and slows $k_{off}$, elevates $k_r$ only at intermediate [Ca$^{2+}$] (24). All these studies clearly indicate that modulation of TnC Ca$^{2+}$ binding properties affects $k_r$. However, it is unclear whether it does so by influencing the degree of thin filament activation or by modulating the cooperative interactions during contraction.

Our studies suggest that TnC Ca$^{2+}$ binding properties modulate the state of thin filament activation to regulate $k_r$. Our results further suggest this can be accomplished without altering intrinsic cross-bridge kinetics, in agreement with previous results (22). As seen in Fig. 6, at matched forces $k_r$ was unchanged for all the TnC mutants at >30% $F_{max}$ even with their varied effects on $k_d$, $k_{on}$, and $k_{off}$, similar to what we previously observed in cardiac muscle (23). The observation that $k_r$ was similar for the various TnC mutations at matched force levels suggests that the intrinsic cross-bridge kinetics were not affected. If the TnC mutants were affecting the cross-bridge kinetics, we would have expected the force vs. $k_r$ relationship to have shifted either up or down compared with WTTnC, representing an increased or decreased rate of cross-bridge cycling at matched states of thin filament activation (i.e., force). However, neither I60QTnC nor T70DTnC recovered maximal force or $k_r$. Partial extraction of TnC resulted in loss of force without a decrease in maximal $k_r$; thus the reductions in both $F_{max}$ and maximal $k_r$ for T70DTnC and I60QTnC were not due to incomplete reconstitution. Although both I60QTnC and T70DTnC have faster $k_{off}Ca^{2+}$ and decreased $F_{max}$, our previous work showed (7) that there is no direct correlation between the two. In addition, I60QTnC and V43QTnC both share depressed $k_{on}$, yet only I60QTnC reduces maximal $k_r$. Thus it is apparent that when examining the effects of TnC constructs on $k_r$ and force, we cannot reintegrate these properties to the isolated aspects of either $k_{off}$ and/or $k_{on}$.

The significance of thin filament activation and how it might regulate force and $k_r$ can be simulated by a recent model of muscle activation (34). This model incorporates interactions

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**Fig. 5.** Effect of TnC mutations on Ca$^{2+}$ dependence of $k_r$. Ca$^{2+}$ dependence of $k_r$ in single-skinned psoas fibers reconstituted with WTTnC, V43QTnC, T70DTnC, and I60QTnC is shown as a function of pCa. Each data point represents the mean ± SE from at least 4 separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.

**Fig. 6.** Dependence of relative $k_r$ on relative force for single-skinned psoas fibers reconstituted with TnC mutants. Average relative $k_r$ values at each pCa shown in Fig. 5 are plotted vs. the average levels of relative force generated at each pCa. For each fiber $k_r$ was normalized to the pCa 4.0 $k_r$ of the fiber before TnC extraction and reconstitution. Data from WTTnC, V43QTnC-, T70DTnC-, and I60QTnC-reconstituted fibers are shown on the same plot. Each data point represents the mean ± SE from at least 4 separate fibers.
between the Tn complex, tropomyosin, actin, and myosin to predict the position of tropomyosin on actin and thus the level of thin filament activation. As seen in Fig. 8A, the shape of the simulated relative apparent $k_{tr}$ vs. fractional thin filament activation is similar to that of our experimental $k_{tr}$ vs. force relationship. With this model, the experimental force vs. pCa data were iteratively simulated with the $Ca^{2+}$ binding properties of the mutant Tn complexes (Fig. 8, B–D). As described in

![Fig. 7. Effect of N-ethylmaleimide-modified myosin subfragment-1 (NEM-S1) on $Ca^{2+}$ dependence of force generation by WTTnC and I60QTnC. A: $Ca^{2+}$ dependence of isometric force generation in single-skinned psoas fibers reconstituted with WTTnC or I60QTnC in the absence (filled symbols) or presence (open symbols) of NEM-S1 (6 μM) as a function of pCa. Each data point represents the mean ± SE from at least 4 separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation. B–D: various simulations (denoted by the traces) of the mutant TnCs compared with the WTTnC. Experimental data are shown by black traces for WTTnC in B–D. B: V43QTnC data (gray traces). Dotted line simulates the effects of only increasing the $Ca^{2+}$ binding affinity of Tn, similar to that observed for the V43Q Tn complex. Broadly spaced dashed line simulates the effect of increasing the $Ca^{2+}$ binding affinity and decreasing the actin affinity of apo Tn (2-fold) (which can be interpreted as the intrinsic ability of Tn to keep the thin filament in the blocked state independent of $Ca^{2+}$). Dashed line simulates having 1 $Ca^{2+}$ binding site with high affinity while the other site maintains the WT $Ca^{2+}$ binding affinity, in addition to a 2-fold lower actin affinity of apo Tn. Solid trace simulates the effects of decreasing the $Ca^{2+}$ affinity and the ability of $Ca^{2+}$ to promote the transition from the blocked to closed state by 18%. C: T70DTnC (gray traces) and its various simulated fits. Dotted line simulates the effects of only decreasing the $Ca^{2+}$ affinity of Tn, similar to that observed for the T70D Tn complex. Dashed line simulates the effects of decreasing the Tn affinity and the ability of $Ca^{2+}$ to promote the transition from blocked to closed state by 53%. D: I60QTnC (gray traces) and its various simulated fits. Dashed line simulates the effects of only decreasing the $Ca^{2+}$ affinity of Tn, similar to that observed for the I60Q Tn complex. Dotted line simulates the effects of decreasing the $Ca^{2+}$ affinity of Tn and decreasing the actin affinity of apo Tn. Solid trace represents both of the above effects and also decreasing the ability of $Ca^{2+}$ to promote the transition from blocked to closed state by −53%.

![Fig. 8. Simulated data based on a thin filament model of activation compared with experimental data. A: the model’s simulated relative apparent $k_{tr}$ ($k_{app}$) vs. fractional thin filament activation (M), which is comparable in shape to the experimental plot of force vs. $k_{tr}$. B–D: various simulations (denoted by the traces) of the mutant TnCs compared with the WTTnC. Experimental data are shown by black traces for WTTnC in B–D. B: V43QTnC data (gray traces). Dotted line simulates the effects of only increasing the $Ca^{2+}$ binding affinity of Tn, similar to that observed for the V43Q Tn complex. Broadly spaced dashed line simulates the effect of increasing the $Ca^{2+}$ binding affinity and decreasing the actin affinity of apo Tn (2-fold) (which can be interpreted as the intrinsic ability of Tn to keep the thin filament in the blocked state independent of $Ca^{2+}$). Dashed line simulates having 1 $Ca^{2+}$ binding site with high affinity while the other site maintains the WT $Ca^{2+}$ binding affinity, in addition to a 2-fold lower actin affinity of apo Tn. Solid trace simulates the effects of decreasing the $Ca^{2+}$ affinity and the ability of $Ca^{2+}$ to promote the transition from the blocked to closed state by 18%. C: T70DTnC (gray traces) and its various simulated fits. Dotted line simulates the effects of only decreasing the $Ca^{2+}$ affinity of Tn, similar to that observed for the T70D Tn complex. Dashed line simulates the effects of decreasing the Tn affinity and the ability of $Ca^{2+}$ to promote the transition from blocked to closed state by 53%. D: I60QTnC (gray traces) and its various simulated fits. Dashed line simulates the effects of only decreasing the $Ca^{2+}$ affinity of Tn, similar to that observed for the I60Q Tn complex. Dotted line simulates the effects of decreasing the $Ca^{2+}$ affinity of Tn and decreasing the actin affinity of apo Tn. Solid trace represents both of the above effects and also decreasing the ability of $Ca^{2+}$ to promote the transition from blocked to closed state by −53%.]
Fig. 8, the model suggests that thin filament activation can be modulated by at least the Ca\(^{2+}\) sensitivity of the two regulatory Ca\(^{2+}\) binding sites of TnC, the affinity of TnI for actin and/or TnC, and the ability of Ca\(^{2+}\) to promote the transition from the blocked to closed state. Clearly more work is required to experimentally verify this model and simulate the effects of the mutant TnCs on \(k_{ir}\). Regardless, there appear to be multiple mechanisms that can affect the cooperativity and level of thin filament activation.

Since I60QTnC recovered only \(~75\%\) of maximum force and \(~48\%\) of maximum \(k_{ir}\), we decided to further test whether I60QTnC decreased maximal \(k_{ir}\) through alteration of cross-bridge kinetics or solely by a decrease in thin filament activation. Thus we utilized NEM-S1, a strong-binding cross bridge analog that activates the thin filament but does not produce force (28). Addition of NEM-S1 to reconstituted I60QTnC fibers recovered maximal \(k_{ir}\) at low Ca\(^{2+}\), providing evidence that the cross bridges could cycle at their maximal intrinsic rate. The decreases in \(k_{ir}\) at intermediate and maximal [Ca\(^{2+}\)] observed with I60QTnC in the presence of NEM-S1 were most likely due to incomplete spread of NEM-S1. At low [Ca\(^{2+}\)], regulatory units of the thin filament with NEM-S1 are preferentially activated by Ca\(^{2+}\) and display maximal activation. However, at higher [Ca\(^{2+}\)], regulatory units without NEM-S1 began to contract at the rate limited by I60QTnC’s decreased ability to activate the thin filament. Unlike I60QTnC, fibers reconstituted with WTTnC can contract at maximal preactivation rates at high [Ca\(^{2+}\)] even after addition of NEM-S1, consistent with full activation of the thin filament.

At force levels <30% of \(F_{max}\), \(k_{on}\) was slightly elevated for both V43QTnC and I60QTnC but not for T70DTnC compared with WTTnC. These results are surprising considering that V43QTnC and I60QTnC have opposite effects on the Ca\(^{2+}\) sensitivity of force and \(k_{ir}\) development as well as on Ca\(^{2+}\)-\(K_d\) and \(k_{off}\). Two traits V43QTnC and I60QTnC shared, though, were decreases in Ca\(^{2+}\)-\(k_{on}\) and cooperativity of force development as demonstrated by lowered Hill coefficient. Consistent with cooperative interactions being the cause of the elevation in submaximal \(k_{ir}\), Campbell’s model (4) of \(k_{ir}\) suggests that decreasing cooperative interactions serves to enhance \(k_{ir}\). This effect is most pronounced at low [Ca\(^{2+}\)], where there is a large number of noncycling cross bridges for cooperative recruitment to cycling cross bridges. As more cross bridges are cooperatively recruited into the cycling population, they progressively elevate the final steady-state force without affecting cross-bridge cycling rates, effectively slowing down the observed \(k_{ir}\). However, our results do not rule out the possibility that Ca\(^{2+}\)-\(k_{on}\) and/or \(k_{off}\) might also play a role in elevating \(k_{ir}\) at low [Ca\(^{2+}\)]. In this case, \(k_{on}\) may be controlled by the kinetics at which the thin filament equilibrates between the open and closed states, regulating the availability of strong cross-bridge binding sites. At higher [Ca\(^{2+}\)], though, this effect would be overridden by the predominance of strongly bound myosin forcing the thin filament into the open state, causing \(k_{off}\) to be similar for the different TnC mutants at matched forces. Interestingly, the behavior of I60QTnC was similar to that observed with extraction and reconstitution of cardiac TnC in psoas fibers except that \(k_{ir}\) was elevated over a greater range of force levels (22). Clearly the mechanisms behind the effects of cardiac TnC and I60QTnC are different.

Since the Hill coefficient serves as an aggregate measurement for many cooperative interactions possible during contraction, the mechanisms by which V43QTnC and I60QTnC decrease cooperativity are likely to be different. Although cooperativity via longitudinal spread of activation between regulatory units has a significant effect on steady-state force (14, 21), nearest-neighbor regulatory unit cooperativity does not significantly affect \(k_{ir}\) (14, 15, 22). Thus cooperative interactions that affect \(k_{ir}\) must occur within the regulatory unit of seven actins per Tm dimer and Tn complex (8, 31). These processes include, but are not limited to, feedback interactions between cross bridges, cross bridges and Tm, and Ca\(^{2+}\) binding to TnC. Since V43QTnC is already sensitized to Ca\(^{2+}\), any cooperative mechanisms that normally increase Ca\(^{2+}\) binding to TnC (such as the ability of TnC to interact with TnI) might be attenuated. In addition, V43QTnC may cause the thin filament to favor an activated state, reducing the role of cross bridge activation of the thin filament to recruit more cross bridges. On the other hand, I60QTnC may reduce the cooperativity of thin filament activation by strongly favoring the inactivated state and ablating cooperative effects.

In conclusion, the Ca\(^{2+}\) binding properties of TnC can affect the rates of force redevelopment primarily via modulation of the level of thin filament activation, but also through cooperative interactions at low forces as evidenced by V43QTnC and I60QTnC. Our results highlight the importance of incorporating both thin filament activation and cooperativity in accurately modeling \(k_{ir}\); however, combining these two properties into a single model is beyond the scope of this article. In addition, the modulation of \(k_{ir}\) by cooperative effects is minimal compared with that caused by the level of thin filament activation.

**REFERENCES**


