Effect of the Ca\(^{2+}\) Binding Properties of Troponin C
On Skeletal and Cardiac Muscle Force Development

Thesis

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Abstract

Many factors play a role in regulating striated muscle contraction; however, the relative importance of the thin filament in this process is still under poorly understood. In this work, the role of troponin C (TnC) on the rate of skeletal muscle contraction and as a regulator of the Ca$^{2+}$ sensitivity of cardiac force production were studied. To investigate how TnC might affect the rate of contraction, skeletal TnC constructs with altered Ca$^{2+}$ binding properties were reconstituted into single skinned psoas fibers from rabbits to assess the Ca$^{2+}$ dependence of force development and the rate of force redevelopment ($k_{tr}$) at 15°C. This procedure resulted in a sensitization of both force and $k_{tr}$ to Ca$^{2+}$ for V43QTnC, whereas T70DTnC and I60QTnC desensitized force and $k_{tr}$ to Ca$^{2+}$, with I60QTnC causing a greater desensitization. In addition, T70DTnC and I60QTnC depressed both maximal force ($F_{max}$) and maximal $k_{tr}$. Even though V43QTnC and I60QTnC had drastically different effects on the Ca$^{2+}$ binding properties of TnC, they both exhibited decreases in the cooperativity of force production and elevated $k_{tr}$ at force levels less than 30% $F_{max}$ compared to wild-type TnC. However, at matched force levels greater than 30% $F_{max}$, $k_{tr}$ was similar for all the TnC constructs. These results suggest that the TnC mutants primarily affected $k_{tr}$ through modulating the level of thin filament activation and not by altering intrinsic cross-bridge cycling properties. To corroborate these results, NEMS-1, a non force...
generating cross-bridge analogue that activates the thin filament, fully recovered maximal $k_n$ for the I60QTnC at low [Ca$^{2+}$].

Additionally, a protocol was developed to passively exchange whole human Tn into rat skinned cardiac trabeculae to study how TnC works in conjunction with other thin filament proteins to regulate the Ca$^{2+}$ sensitivity of force production. To this end, the disease related abnormal Ca$^{2+}$ sensitivities resulting from a truncation of TnI (residues 1-192) and a single residue ΔK210 TnT deletion on the force-pCa relationship were first characterized. TnCs were then specifically engineered and combined with these disease related mutations in hopes of correcting their effects on the Ca$^{2+}$ sensitivity of force. Our results demonstrate that TnC is able to attenuate and even completely restore dysfunctional Ca$^{2+}$ sensitivity of force generation due to TnI or TnT modifications. Thus, it has been shown that TnC can control the rate of contraction by modulating the level of thin filament activation and is capable of correcting the aberrant Ca$^{2+}$ binding properties due to modifications in other thin filament proteins.
Dedicated to my parents
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Chapter 1: Introduction

A: General striated muscle physiology

Muscle contraction is regulated by the Ca\(^{2+}\) dependent interactions between the thick (myosin) and thin (actin) filaments (28, 29). When an action potential is sent to initiate a contraction, it travels along the plasma membrane of the muscle fiber and down tubular structures known as T-tubules to release the cell’s intracellular Ca\(^{2+}\) stores. As the free [Ca\(^{2+}\)] in the cell increases, it binds to the protein troponin C (TnC), the Ca\(^{2+}\) regulatory unit of the heterotrimeric troponin complex (Tn) on the thin filament.

Thin filaments are organized into repeating structural units each containing one tropomyosin dimer (Tm) and one troponin complex attached per seven helically arranged actins (for review, see (9, 34)). The troponin complex as a whole plays a vital role in regulating thin filament activation, and in addition to TnC, the troponin complex also consists of troponin T (TnT), which anchors Tn to Tm, and troponin I (TnI), the inhibitory subunit which binds to actin and anchors Tm to block myosin binding sites in the absence of Ca\(^{2+}\).

As determined by X-ray crystallography and NMR imaging, the structure of TnC consists mainly of two globular heads connected by a helical linker (Figure 1.1). These globular heads are distinguished as an N-domain and a C-domain and each contains a pair of Ca\(^{2+}\) binding sites numbered I-IV. Sites I and II of the N-domain are more Ca\(^{2+}\)
specific, however, cardiac TnC only contains one active site while both function in skeletal TnC. Meanwhile sites III and IV of the C-domain have a higher affinity for Mg$^{2+}$, and since the C-domain primarily serves to bind TnC to the N-terminus domain of TnI, it is also known as the structural domain. Removal of Mg$^{2+}$ and Ca$^{2+}$ from the C-domain via a chelating compound, such as EDTA, will actually cause TnC to dissociate from the Tn complex (20). On the other hand, the N-domain of TnC is known as the regulatory domain because it binds Ca$^{2+}$ and initiates muscle contraction by interacting with the C-domain of TnI.

**Figure 1.1: Variations in skeletal and cardiac TnC structure.** Metal ion binding sites are represented by gray spheres. As can be seen, skeletal TnC contains two regulatory domains while cardiac TnC only contains one.

The molecular mechanism by which TnC interacts with TnI during a contraction varies between the cardiac and skeletal isoforms. In skeletal muscle, when Ca$^{2+}$ binds to the N-terminus of TnC, it causes a conformational change to expose a hydrophobic pocket which can then bind to a switch region of TnI (Figure 1.2). This shifts the inhibitory region of TnI away from actin to allow Tm movement and exposure of myosin.
binding sites. In the absence of Ca\(^{2+}\), this hydrophobic pocket is buried within TnC and unavailable for TnI binding. In cardiac muscle though, Ca\(^{2+}\) binding to TnC does not open up the hydrophobic pocket (7). The TnI switch region must also interact with TnC-Ca\(^{2+}\) to induce a conformational change and open the hydrophobic pocket (Figure 1.3). Ultimately this has the same effect as in skeletal muscle, exposing myosin binding sites on actin.

![Skeletal Troponin C Opens with Ca\(^{2+}\)](image)

**Figure 1.2: Representation of the conformational changes that occur in skeletal TnC during calcium binding.** The hydrophobic pocket in TnC is represented in yellow while the rest is in red. In the apo state, the pocket is enclosed, however, upon calcium binding the pocket is exposed. TnI, as seen in purple, can then bind to it.
Cardiac Troponin C Opens with Ca\textsuperscript{2+} and Troponin I

Figure 1.3: Representation of the conformational changes that occur in cardiac TnC during calcium binding. The hydrophobic pocket in TnC is represented in yellow while the rest is in red. In both the apo and calcium saturated state, the hydrophobic pocket remains buried inside cTnC. Once TnI, represented in blue, binds to it though, the pocket opens up.

When Ca\textsuperscript{2+} binds to TnC, the series of conformational changes that occur are believed to shift Tm across actin to expose strong cross-bridge myosin binding sites. This process is believed to involve three states of actin-Tm: a) a blocked state, where Tm blocks strong binding of cross-bridges to actin; b) a closed state, in which cross-bridges are able to weakly attach to actin, and c) an open state, in which cross-bridges are able to strongly attach to actin and produce force (Figure 1.4) (14, 19). According to this model, the blocked and closed states exist in equilibrium, with the blocked state predominating in the absence of Ca\textsuperscript{2+}. Upon addition of Ca\textsuperscript{2+}, TnC will bind with Ca\textsuperscript{2+} and then interact with TnI to remove Tm inhibition of myosin binding sites on actin. This results in a shift of the thin filament from the blocked to the closed state. Strongly bound cross-bridges then further shift the thin filament from the closed to the open state. This hypothesis
implies that both Ca\textsuperscript{2+} binding to the thin filament and strongly bound cross-bridges are required for complete activation of the thin filament and maximal force production.

**Both Ca\textsuperscript{2+} Binding to TnC and Cross-Bridge Binding to Actin Are Required for Full Muscle Activation**

**Figure 1.4: Representation of the three state model of thin filament activation.**

Actin is represented in light grey while tropomysin is depicted in dark grey. Strong myosin binding sites are in red while weak binding sites are in light blue. In the Blocked state (no calcium), tropomysin overlaps the strong myosin binding sites. Upon addition of calcium, the thin filament enters the Closed state where tropomysin shifts its position to partially expose the strong myosin binding sites. Finally, upon myosin cross-bridge binding the thin filament enters the Open state where the strong myosin binding sites are fully exposed (Reproduced from (14)).
B: Studying the role of TnC through mutagenesis in skeletal muscle

Given that TnC plays a crucial role in regulating muscle contraction, one of the major goals in our lab has been to study how altering TnC’s Ca\(^{2+}\) binding properties might affect the rates of muscle contraction and force generation. Thus to characterize TnC, the native Cys\(^{99}\) residue of skeletal TnC was labeled with the environmentally sensitive probe IAANS and a novel fluorescent cardiac TnC\(^{TE3C}_{IAANS}\) was developed to study the Ca\(^{2+}\) binding properties of TnC in biochemical systems ranging from the Tn complex to the reconstituted thin filaments (Manuscript in preparation). Labeling of TnC with IAANS had minimal effects on TnC’s biochemical and physiological properties and allowed us to monitor the Ca\(^{2+}\) induced changes in fluorescence that occur when Ca\(^{2+}\) bound to the regulatory domain of the TnC\(_{IAANS}\)-TnI-TnT complex (herein designated as Tn\(_{IAANS}\)). Utilizing these probes, we could design TnC mutants with faster and slower Ca\(^{2+}\) dissociation rates and greater and lower Ca\(^{2+}\) binding affinities. These mutated constructs serve as tools to better understand how TnC affects muscle function.

Our lab has engineered numerous TnCs with altered Ca\(^{2+}\) binding properties. To study how TnC might affect the rate of skeletal muscle contraction, three rabbit skeletal TnC mutants with different Ca\(^{2+}\) binding properties, V43QTnC, T70DTnC, and I60QTnC were developed and characterized in the Tn\(_{IAANS}\) complex. As seen in Fig. 1.5 and summarized in Table 1, we first studied the Ca\(^{2+}\) sensitivity of the Tn\(_{IAANS}\) complex fluorescence intensity for all the Tn\(_{IAANS}\) complexes. Compared to the WildTypeTn\(_{IAANS}\) the V43QTn\(_{IAANS}\) mutation increased the Ca\(^{2+}\) sensitivity of the Tn\(_{IAANS}\) complex while the T70DTn\(_{IAANS}\) and I60QTn\(_{IAANS}\) mutations decreased the Ca\(^{2+}\) sensitivity of the
TnIAANS, with I60QTnIAANS causing a greater decrease in sensitivity. Furthermore, the Ca\(^{2+}\) dissociation rates (k\(_{off}\)IAANS) were measured via the EGTA (a Ca\(^{2+}\) chelating compound) induced rates of structural change from the TnIAANS complex for the three mutants (Fig. 1.6, Table 1). Compared to WTTnC, the V43QTnC decreased k\(_{off}\)IAANS, while T70DTnC and I60QTnC increased the k\(_{off}\)IAANS, with I60QTnC increasing it the most. These rates were confirmed by measuring the true Ca\(^{2+}\) dissociation rates by utilizing Quin-2. Similar to EGTA, Quin-2 is a Ca\(^{2+}\) chelating compound, but it also fluoresces when it binds Ca\(^{2+}\). We could then use Quin-2 fluorescence to directly measure the rate at which Ca\(^{2+}\) dissociates from these TnCs and confirm that the conformational change in the regulatory domain of the TnIAANS complex occurred concomitantly with Ca\(^{2+}\) dissociation. Since K\(_d\) = k\(_{off}\)k\(_{on}\), we calculated the k\(_{on}\)Ca\(^{2+}\) for the TnIAANS complexes and found the T70DTnC to have a similar k\(_{on}\)Ca\(^{2+}\) as WTTnC while V43QTnC and I60QTnC decreased k\(_{on}\)Ca\(^{2+}\). These three TnCs with drastically different Ca\(^{2+}\) binding properties were then incorporated into rabbit skinned psoas fibers to study TnC’s effects on the rate of contraction.
Figure 1.5: Effect of TnC mutations on Ca$^{2+}$ binding to the rabbit skeletal Tn complex.

The Ca$^{2+}$ dependent changes in IAANS fluorescence are shown for 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 Tn$\text{IAANS}$ complex. Each data point represents the mean ± SE of 3 or 4 titrations fit with a logistic sigmoid curve. (Manuscript in preparation)
Figure 1.6. Effect of TnC mutations on the rate of Ca\(^{2+}\) dissociation from the rabbit skeletal Tn complex.

Panel A shows the time course of increase in IAANS fluorescence as Ca\(^{2+}\) was removed by EGTA from the regulatory Ca\(^{2+}\) binding sites of WTTn\(_{IAANS}\), V43QTn\(_{IAANS}\), T70DTn\(_{IAANS}\) and I60QTn\(_{IAANS}\). Panel B shows the 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, I60QTn. (Manuscript in preparation)
<table>
<thead>
<tr>
<th>TnC</th>
<th>pCa50</th>
<th>nH</th>
<th>$k_{\text{off\ IANNS}}$ (1/s)</th>
<th>$k_{\text{off\ Quin-2}}$ (1/s)</th>
<th>Calculated $k_{\text{On\ IANNS}}$ from $k_{\text{off\ IANNS}}$ ($\times 10^8$ 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>
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<tr>
<td>T70D</td>
<td>*6.55 ± 0.03</td>
<td>*0.86 ± 0.05</td>
<td>*38.9 ± 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>

**Table 1. Comparison of the Ca$^{2+}$ binding properties of the rabbit skeletal TnC mutants.**

Values significantly different from WT as determined by a Student’s T-Test are denoted by *. (Manuscript in preparation)

*C: Studying skeletal muscle contraction in skinned fibers*

Ultimately, our goal was to study how our TnC constructs might affect muscle function, thus we incorporated them into rabbit skinned psoas fibers. The skinned muscle preparation is used as a surrogate for *in vivo* preparations because it allows us to simulate different environments in the cell that we would otherwise be unable to control (31). Skinning the muscle with a detergent, such as Triton-X, removes the sarcolemma, leaving only the basic contractile machinery, ie. the thin and thick filaments with their associated proteins. We can then expose the muscle to specific concentrations of Ca$^{2+}$ in the presence of ATP to induce contraction. Furthermore, removal of the plasma membrane allows us to incorporate our mutant TnCs into the muscle via an extraction/reconstitution process and study their effects on the rate of contraction (6).
Due to Ca$^{2+}$ diffusing into the fiber limiting the rate of contraction, we cannot measure the true rate of contraction in this skinned fiber preparation. However, it can be approximated by a rapid shortening-restretch maneuver where the fiber is first activated at a fixed [Ca$^{2+}$] until the force reaches a plateau. The fiber is then rapidly shortened to mechanically shear the attached cross-bridges, dropping force to zero, then the fiber is rapidly stretched back to its original length (Fig 1.7). The rate at which the cross-bridges reattach and recover force to the original level is known as the rate of force redevelopment (k$_{tr}$) and this method was developed by Brenner, who interpreted the results as a simple two state cross-bridge system (1, 3). The rate of force redevelopment is then the sum of the rate of cross-bridges transitioning from a non-force producing state to a force producing state (f$_{app}$) and the rate of cross-bridges detaching from a force-producing state to a non-force producing state (g$_{app}$), ie. k$_{tr}$ = f$_{app}$ +g$_{app}$. It is well established that k$_{tr}$ in striated muscle becomes faster with increasing [Ca$^{2+}$], which has been explained as a Ca$^{2+}$ dependent effect on f$_{app}$ (2, 4, 5, 12, 36).
**Figure 1.7: Example of the measurement of $k_{tr}$.** On the left we see the calcium activation of the muscle and subsequent generation of force. Once force reaches a steady state plateau, the muscle is rapidly shortened and restretched to its original length. This corresponds to a drop in force and the subsequent rate at which force returns to its original levels is $k_{tr}$. The differences between $k_a$ and $k_{tr}$ can be seen on the right which is an actual trace of a $k_{tr}$ measurement. $k_a$, as seen in the top panel is limited by $Ca^{2+}$ diffusion.

**D: Mechanism of $Ca^{2+}$ modulation of contraction kinetics in skeletal muscle**

The mechanism by which $Ca^{2+}$ modulates the rate of contraction ($k_{tr}$) is still under debate. Evidence suggests that it is unlikely to be a direct effect of $Ca^{2+}$ on the intrinsic kinetics of the cross-bridge cycle (10, 13, 15, 21, 22, 26, 35). It is clear though that the simple two state model is insufficient in accurately describing $k_{tr}$. For
example, the effect of the different states of thin filament activation have been shown to affect $k_{fr}$ and have been incorporated into a simple four state model (12). This model suggested that altering the $Ca^{2+}$ binding properties of TnC can affect the level of thin filament activation (i.e. exposure of strong binding cross-bridge sites) and/or directly modulate the rate of cross-bridge attachment and detachment (17).

It has also been suggested that cooperative interactions within the myofilaments influence the rate of contraction, especially at low $[Ca^{2+}]$ (4). Cooperativity itself is the concept wherein activation involving interactive multiple units occurs more easily as compared to single independent units. In the myofilament, cooperative interactions would include cooperative cross-bridge binding to the thin filament; strongly bound cross-bridges increasing TnC’s affinity for TnI; strongly bound cross-bridges shifting Tm movement; and $Ca^{2+}$ binding to TnC influencing $Ca^{2+}$ binding to adjacent TnCs. According to this model, cooperativity decreases $k_{fr}$ by progressively recruiting non-cycling crossbridges into the cycling population to elevate the final steady state force without increasing the rate of contraction. Thus the apparent $k_{fr}$ is depressed. For instance, reducing the cooperativity via partial TnC extraction and addition of NEMS-1, a strong binding but non-force producing cross-bridge analogue, would inhibit cooperative activation of near neighbor regulatory units and cross-bridge binding, resulting in elevated $k_{fr}$ at low $Ca^{2+}$ (10). Recent models have attempted to address the shortcomings of the original interpretation of $k_{fr}$ by incorporating either thin filament activation or cooperativity, however, a model incorporating both has not yet been developed and the relative importance of their contribution to $k_{fr}$ is still under debate.
Thus the effects of TnC mutants in skeletal muscle on the Ca\textsuperscript{2+} sensitivity of force production, rate of force redevelopment, and the cooperativity of these processes were studied. Partial TnC extraction and NEMS-1 were also used to elucidate the mechanism by which altering the Ca\textsuperscript{2+} binding properties of the thin filament might affect k\textsubscript{fr}. Our hypothesis is that altering the Ca\textsuperscript{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\textsubscript{fr}.

\textit{E: TnC’s role in the Tn complex to regulate the Ca\textsuperscript{2+} sensitivity of force production in cardiac muscle}

Although our goal is to understand how TnC regulates the contractile properties of muscle, ultimately we want to determine if it might be a viable therapeutic target in treating dysfunctional states. For example, aberrant myofilament Ca\textsuperscript{2+} responses are routinely observed in familial cardiomyopathies. One fairly common subtype of familial cardiomyopathies, dilated cardiomyopathy (DCM), is characterized by ventricular dilation and systolic dysfunction and its effects are at least partially due to decreased myofilament Ca\textsuperscript{2+} sensitivity. In addition, aberrant myofilament Ca\textsuperscript{2+} sensitivities can be caused by non-inherited cardiac dysfunctions such as after an ischemia reperfusion injury. In this case, myofilament protein proteolysis, a TnI 1-192 truncation, has been suggested as the cause for the observed change in myofilament Ca\textsuperscript{2+} sensitivity (33). In both cases, evidence has shown that correcting the dysfunctional Ca\textsuperscript{2+} sensitivity ameliorates the diseased effects and improves cardiac function.
For example, a TnT deletion mutation (ΔK210) results in the symptoms associated with DCM in a transgenic mouse model (8). Treatment with the myofilament Ca\textsuperscript{2+} sensitizing drug pimobendan ameliorated the diseased effects seen in the mice. However, due to the non-specificity of pimobendan and other drugs, their effectiveness as treatments is limited. Given our interest in TnC’s role as the central regulatory switch for thin filament activation, we wanted to determine if TnC could be used to correct abnormal behavior due to mutations in the other thin filament proteins, TnI and TnT. If so, then TnC could be a possible therapeutic target for treating these dysfunctional states without the side effects due to drug therapies. Thus we decided to utilize the cardiac TnI 1-192 truncation and TnT ΔK210 deletion, due to their opposite and significant effects on cardiac muscle function, to test TnC’s ability to correct their aberrant Ca\textsuperscript{2+} sensitivity of cardiac muscle force generation.

TnI 1-192 truncation and TnT ΔK210 were first characterized in reconstituted thin filaments (Figure 1.8, Table 2). The TnI 1-192 increased TnC’s Ca\textsuperscript{2+} binding affinity significantly compared to the control. On the other hand, the TnT ΔK210 decreased TnC’s Ca\textsuperscript{2+} binding affinity compared to control. Cardiac TnCs were then specifically engineered with opposite Ca\textsuperscript{2+} binding properties, a desensitizing S69D, D73N TnC for the TnI 1-192 and a sensitizing M45Q, S69D TnC for the TnT ΔK210 to hopefully correct the diseased properties. As seen in Figure 1.8, the designer TnCs were successful in correcting the altered Ca\textsuperscript{2+} sensitivities of both the TnI 1-192 truncation and TnT ΔK210 back to control levels. These biochemical studies served as a predictor for how these mutant proteins might behave in vivo, however, testing them in more physiologically relevant systems was crucial in ascertaining their true
role. Thus the next logical step was to incorporate them into the skinned muscle system and test the hypothesis that engineered TnCs can correct the abnormal Ca\(^{2+}\) sensitivity of force generation due to disease related thin filament mutations.

Unfortunately, the simple extraction/reconstitution method as used in skeletal muscle was untenable for our studies in cardiac muscle. Extraction of skeletal TnC results in extraction of approximately 98% of the native TnC. However, extraction of cardiac TnC is less efficient, only extracting about 70% of the endogenous TnC. In addition, we can only extract TnC, not TnI or TnT.

Thus to incorporate our mutant proteins into rat skinned cardiac trabeculae, a protocol was developed to passively exchange our engineered Tn complexes with the endogenous Tn (See Methods section). Previous studies have utilized Tn exchange into skinned muscle, but they either had poor exchange or did not allow for an internal control with the endogenous Tn. Via rigorous testing and experiment under various conditions, our protocol achieves a high level of exchange while maintaining trabeculae integrity. This then allowed us to confirm our hypothesis that TnC plays a vital role in regulating the contractile properties of muscle and may be a potential therapeutic target for treatment of cardiomyopathies.
Figure 1.8. Effect of disease related protein modifications and their associated correcting TnCs on the Ca\(^{2+}\) binding properties of reconstituted cardiac thin filaments.

Panel A shows the Ca\(^{2+}\) dependent changes in IAANS fluorescence for control Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (□), TnI (1-192)Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (△), S69D,D73NTnC Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (◇), and TnI (1-192)-S69D,D73NTnC Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (▲) in reconstituted thin filaments as a function of pCa. Panel B shows the Ca\(^{2+}\) dependent changes in IAANS fluorescence for control Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (□), TnT ΔK210 Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (▽), M45Q,S69DTnC Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (♦), and TnT ΔK210-M45Q,S69DNTnC Tn\(^{\text{T53C}}\)\(_{\text{IAANS}}\) (▼) in reconstituted thin filaments as a function of pCa. The data sets were normalized individually for each combination. For the disease related protein modifications, each data point represents the mean ± S.E. of three titrations fit with a logistic sigmoid function while for control, each data point represents the mean ± S.E. of five titrations fit with a logistic sigmoid function. The IAANS fluorescence was excited at 330 nm and monitored at 450 nm. (Manuscript in preparation)
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<th>Protein</th>
<th>pCa50</th>
<th>Ca2+ Kd (uM)</th>
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<td>1.28±0.06</td>
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<td>0.71±0.05 *</td>
<td>1.14±0.09</td>
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</tr>
<tr>
<td>TnT ΔK210</td>
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<td>15.43±1.18 *</td>
<td>1.19±0.06</td>
<td>↓3.2</td>
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<tr>
<td>S69D,D73N</td>
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<td>17.05±2.45 *</td>
<td>1.12±0.05</td>
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<tr>
<td>M45Q,S69D</td>
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<td>1.60±0.16 *</td>
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</tr>
<tr>
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<tr>
<td>ΔK210-M45Q,S69D</td>
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<td>4.32±0.80</td>
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Table 2. Comparison of the Ca²⁺ binding affinity of the various combinations of mutations in reconstituted cardiac thin filaments.

Values significantly different from control as determined by a Student’s T-Test are denoted by *. (Manuscript in preparation)
Chapter 2: Materials and Methods

Standard Solutions for Muscle Experiments.

The solutions for skinned fiber experiments were prepared as previously described (24). 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 (~ 2mm long), and stored in 50 % relaxing / 50 % glycerol solution overnight at 4°C. Fibers were then stored at -20°C for up to 3 weeks. Single fibers were isolated from these bundles, attached on both ends with aluminum T-clips, fixed at the clipped areas with 25 % gluteraldehyde, 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 1st 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 (1ms, 10 % of the 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. Contraction in various pCa solutions was preceded by washing the fiber for 1 minute in HDTA solution containing 6.6 mM HDTA, 0.4 mM EGTA, 1.0 mM Mg free, 14.5 mM Phosphocreatine, 4.39 mM MgATP, and 20.0 mM Imidazole, pH 7.0. After allowing isometric force 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 mN/mm². All experiments were performed at 15°C.

Protocol for Measuring the Rate of Force Redevelopment (k_tr).

A rabbit psoas fiber was contracted in a pCa solution (ranging from pCa 4.0 to pCa 7.5) until the force reached a plateau. It was then rapidly shortened (1ms, 10 % of the total length), held at this position for 10ms, and then rapidly restretched (1ms) to its original length. An in-house Lab View program was utilized to control the movement of the high-speed length controller. Force redeveloped to similar initial levels and the rate of force redevelopment (k_tr) was measured by assuming a single exponential process as: \( k_{tr} = \frac{\ln(2)}{t_{1/2}} \), where \( t_{1/2} \) is the time to 50 % of force
redevelopment. In order 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 in Rabbit Skeletal Fibers**

After determining maximal force and $k_{fr}$ for the un-extracted fibers, fibers were soaked for 2 minutes in TnC extraction buffer containing 10 mM Hepes, 5 mM EDTA, and 0.5 mM TFP (Trifluoperazine) at pH 7.0. The fibers were then washed three times for one minute in a pCa 9.0 solution to remove residual TFP, and then maximally activated by a pCa 4.0 solution. Force routinely fell to 5.31±1.67% of $F_{max}$. Subsequently the fibers were reconstituted for two minutes in 400 µL of pCa 9.0 solution with 16 µM TnC and then maximally activated twice to measure % force recovery. The fibers were then contracted in randomly selected pCa solutions to measure force and $k_{fr}$. After each pCa measurement, the fiber was fully relaxed in pCa 9.0. Fibers that showed rundown greater than 10% $F_{max}$ over the course of the experiment were excluded. For partial extraction of TnC, fibers were prepared as above, but after measuring maximal endogenous force, they were only soaked in TnC extraction solution for 7-10 seconds. Force and $k_{fr}$ were then measured at varying pCa levels as described above.

**NEMS-1 Muscle Experiments.**

Fibers were prepared, extracted and reconstituted as described above. Upon maximally activating the fiber once after reconstitution with TnC, the fiber was
soaked for 15 minutes in pCa 9.0 solution with 6 µM of NEMS-1, and returned to pCa 9.0 before continuing with the ktr protocol. In addition, 6 µM NEMS-1 (generously provided by Dr. Darl Swartz) was added to the pre-activation HDTA wash.

**Preparation of Rat Cardiac Trabeculae**

Male LBN-F1 rats weighing 150-200g were anesthetized with 1ml of sodium pentobarbital via intraperitoneal injection. Upon opening the thoracic cavity, 0.1ml of heparin was injected into the apex of the heart and then the heart was rapidly excised and placed into relaxing solution at 4°C. Unbranched trabeculae were harvested from the right ventricle, attached on both ends with aluminum t-clips, and skinned overnight in relaxing solution with 1% Triton-X at 4°C. The skinned trabeculae were then used within 2 days. The trabeculae were mounted as above for psoas fibers at 15°C, but the sarcomere length was set to 2.2µm. Passive and active tension were measured as above, but the trabeculae was rapidly shortened by 20% of the resting length, held at that position for 3 seconds, and then returned to its original length over 5 seconds. No pre-contraction HDTA solution was used before a contraction in a pCa solution. F/CSA was calculated assuming an elliptical circumference from the second maximal contraction. The mean F/CSA of 23-trabeculae used in this study was 48 ± 3 mN/mm².

**Human Troponin Exchange in Rat Cardiac Trabeculae**
After maximal force was measured in the pre-exchanged trabeculae, trabeculae were shortened by 20% of the resting length and soaked in a Rigor Buffer consisting of 10mM MOPS, 150mM KCl, 20mM BDM (a drug which prevents cross-bridges from strongly binding to actin), 0.01% NaN₃, 0.5mM DTT, and 3mM MgCl₂ for 30 min. The temperature was then elevated to 25°C and the trabeculae was soaked in an exchange buffer consisting of Rigor Buffer with 7-15μM human Tn, and 500μM Ca²⁺ for 2.5 hours. In the case of the mock Tn exchange, no Tn was added to the exchange buffer. Every 15 minutes the exchange buffer in the chamber was briefly mixed. After exchange, the trabeculae was stretched back to its original length, transferred to a pCa 9.0 solution with 20mM BDM and the passive tension was measured at 15°C. Then the trabeculae was washed 3 times in pCa 9.0 solution for 5 minutes each to remove residual BDM. Afterwards, maximal tension at pCa 4.0 was measured twice to determine the % of maximal force recovery. The trabeculae was then randomly contracted in solutions of varying [Ca²⁺] with a maximal contraction performed in the middle and end to determine rundown. Muscles that exhibited greater than 20% rundown in the maximal force over the course of contracting in the various pCa solutions were excluded.

Development of the Protocol for Tn Exchange

The protocol for exchange of whole human Tn into rat skinned cardiac trabeculae was adapted from several previous studies using both trabeculae and single myofibrils (11, 18, 27, 33). After extensive testing, we found each of these previous methods to be inadequate for our study. Overnight exchange precluded measurement
of the initial maximal endogenous force levels but it also typically resulted in a reduction of the F/CSA by ~20mN/mm^2. The 2 hour exchange protocols did not reduce the F/CSA as much as the overnight exchange, but they also did not exchange the Tn efficiently, resulting in at most ~40% exchange of the endogenous Tn. Our protocol has addressed most of these problems. Preliminary quantification of our Tn exchanged trabeculae via western blot analysis has shown that we have exchanged from ~60-70% of the endogenous Tn with our mutant Tns (Thanks to Brandon Biesiadecki) (Fig 2.1). From our observations, the temperature at which the exchange is conducted and the concentration of Tn in the exchange buffer are the most crucial aspects in efficient exchange. However, higher temperatures also tended to result in a lower % force recovery upon exchange.
Figure 2.1 Western Blot Analysis of Tn Exchange into Rat Cardiac Trabeculae.

A Western blot analysis was performed as a preliminary means of quantifying the amount of Tn exchanged into the rat cardiac trabeculae. A TnI specific antibody was utilized to image the Tn and the gel was overexposed to improve the clarity of the bands. Pure Tn was run alongside single exchanged trabeculae as a means of comparison. Gel courtesy of Brandon Biesiadecki.
Crucial in maintaining trabeculae integrity over the course of the exchange and improving the % force recovery were sodium azide and BDM. Without sodium azide, a bacteriostatic, maximal force in mock exchanged trabeculae deteriorated significantly and the calcium sensitivity of force increased to levels similar to the TnI 1-192 truncation. We believe this may have been due to bacterial growth in the exchange chamber which resulted in protein degradation in the trabeculae, however we did not exhaustively test this possibility. BDM also helped maintain trabeculae integrity as well, since lack of BDM resulted in increased passive tension and further reductions in % force recovery. This may be due to the extended rigor period the trabeculae undergoes, which might result in “dead” myosin heads becoming permanently attached to the thin filament and unable to cycle. BDM may help prevent this by inhibiting strongly bond cross-bridges to form.

Clearly, there is room for improvement in the exchange protocol to yield a better % force recovery. One method might be decreasing the time of exchange, since skinned fibers tend to deteriorate at elevated temperatures. However, trabeculae would need to be tested thoroughly to ensure an optimal exchange time and maximize Tn exchange. Another option might be to increase the concentration of BDM in both the rigor and exchange buffers. Although, both solutions already contain 20mM of BDM, rigor force is still approximately 15-30% of maximal force during the exchange. Increasing the BDM concentration may reduce this rigor force, thus lessening strain on the fiber and preserving structural integrity during the exchange process.
Chapter 3: Results

Effect of TnC Mutations on the Ca$^{2+}$ Sensitivity of Force Development in Rabbit Skeletal Muscle.

The unlabeled mutant TnCs were incorporated into rabbit skinned psoas fibers via an extraction/reconstitution protocol. We first compared the effects of the unlabelled WTTnC to the results obtained for endogenous TnC to ensure that the extraction and reconstitution process did not have any significant effect (see Table 3). Similar to the endogenous TnC, reconstitution of fibers with WTTnC yielded 101 ± 3% force recovery with a pCa$_{50}$ of 5.98 ± 0.02, and a Hill coefficient of 2.5 ± 0.3 (Figure 3.1, Table 3). The Ca$^{2+}$ sensitivity values of force development by the TnC mutants were then compared to the WTTnC values. V43QTnC returned maximal force (103 ± 2%) and sensitized the force development to Ca$^{2+}$ ~7.9 fold (pCa$_{50}$ 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 (pCa$_{50}$ 5.74 ± 0.03). I60QTnC produced similar results as T70DTnC but to a greater extent, with 76 ± 4% maximal force recovery and ~4.0 fold (pCa$_{50}$ 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 coefficients 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 neither a significant effect on the
Ca$^{2+}$ sensitivity of force nor cooperativity of force development (see Table 3). These results demonstrate that the Ca$^{2+}$ desensitizing effects and decrease in cooperativity caused by I60QTnC are not due to incomplete incorporation of TnC.
Figure 3.1. Effect of TnC mutations on the Ca\(^{2+}\) dependence of rabbit skeletal muscle force generation. Shown are the Ca\(^{2+}\) dependence of isometric force generation in single skinned psoas fibers reconstituted with WTTnC (■), V43QTnC (●), T70DTnC (▲), and I60QTnC (▼) as a function of pCa. The experimental conditions are described under “Materials and Methods.” Each data point represents the mean ± S.E. from at least four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Table 3. Comparison of the effects of TnC mutants on the Ca\(^{2+}\) sensitivity of force development. Values significantly different from WT as determined by a Student’s T-test are denoted by *. NA represents data not collected and thus not available. One way analysis of variance (ANOVA) was used to determine the influence of the factor TnC on the dependents pCa50, Hill Coefficient, and % Force Recovery. TnC was found to be significant for all dependents (p<0.0001).

Effect of TnC Mutations on Ca\(^{2+}\) Sensitivity of k_{tr} in Rabbit Skeletal Muscle.

After determining that these TnC mutants drastically affected the Ca\(^{2+}\) sensitivity of force development, we examined their effects on k_{tr}. Representative force traces demonstrating the force redevelopment protocol from fibers reconstituted with WTTnC, V43QTNc, I60QTNc or T70DTnC are shown in Figure 3.2A-D at pCa 4.0, 5.6, and 6.0. k_{tr} was calculated assuming a single exponential process. Clearly, altering the Ca\(^{2+}\) binding properties of TnC can have a drastic effect on k_{tr} at matched pCa.

As seen in Table 4, the WTTnC did not have a significant effect on the Ca\(^{2+}\) sensitivity (pCa\(_{50}\) 5.72 ± 0.03) of k_{tr}, cooperativity of the increase in k_{tr} (Hill coefficient
of 1.7 ± 0.2), or minimal (i.e. the lowest measurable) $k_{fr}$ (22 ± 1%) compared to the endogenous TnC. However, there was a slight reduction in the recovery of maximal $k_{fr}$ for WTTnC (93 ± 3%) compared to endogenous TnC (Table 4), where maximal $k_{fr}$ for endogenous TnC was 11.6 ± 1.1/s (N=4). The $Ca^{2+}$ sensitivity of $k_{fr}$ for mutant TnCs were compared to those for WTTnC. As seen in Fig. 3.3 and summarized in Table 4, V43QTnC recovered maximal $k_{fr}$ (98 ± 4%) and sensitized $k_{fr}$ to $Ca^{2+}$ ~1.8-fold (pCa$_{50}$ 6.08 ± 0.1). Maximal $k_{fr}$ recovery was reduced for both T70DTnC (87 ± 6%) and I60QTnC (48 ± 4%). T70DTnC and I60QTnC also decreased the $Ca^{2+}$ sensitivity of $k_{fr}$ ~2.5-fold (pCa$_{50}$ 5.45 ± 0.4) and ~6.9-fold (pCa$_{50}$ 5.0 ± 0.1), respectively. However, the three mutants had varying effects on the cooperativity of $k_{fr}$. 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 similar maximal force recovery levels as I60QTnC did not affect the $Ca^{2+}$ sensitivity, cooperativity of $k_{fr}$, nor maximal or minimal $k_{fr}$ (Table 4). Clearly, TnC mutants that affect the $Ca^{2+}$ sensitivity of force development, likewise affect the $Ca^{2+}$ sensitivity of $k_{fr}$. 
Figure 3.2. Effect of TnC mutations on $k_r$ in rabbit skeletal muscle. Panels A-D show representative traces of the rapid shortening-restretch maneuver from single skinned psoas fibers reconstituted with WTTnC, V43QTnC, T70DTnC and I60QTnC at pCa 4.0, 5.6 and 6.0.
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<th>%kₜr Recovery at pCa 4.0</th>
<th>% Minimum kₜr</th>
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<td>V43Q (N=5)</td>
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<td>10.7 ± 1.2</td>
<td>92 ± 4</td>
<td>17 ± 3</td>
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</table>

Table 4. Comparison of the effects of TnC mutants on the Ca²⁺ sensitivity of kₜr development. Values significantly different from WT as determined by a Student’s T-Test are denoted by *. NA represents data not collected and thus not available. One way analysis of variance (ANOVA) was used to determine the influence of the factor TnC on the dependents pCa50, Hill Coefficient, and kₜr. TnC was found to be significant for both all dependents (p<0.0001).
Figure 3.3. Effect of TnC mutations on the Ca\textsuperscript{2+} dependence of k_{tr} in rabbit skeletal muscle. Shown are the Ca\textsuperscript{2+} dependence of k_{tr} in single skinned psoas fibers reconstituted with WTTnC (■), V43QTNc (●), T70DTnC (▲), and I60QTNc (▲) as a function of pCa. Each data point represents the mean ± S.E. from at least four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Relationship Between $k_tr$ and Relative Force in Skeletal Muscle.

To examine whether the TnC mutants altered $k_tr$ by modulating the availability of myosin binding sites on actin or by altering the rate of cross-bridge cycling, we plotted the force vs. $k_tr$ relationship for the different TnCs (Fig 3.4). At matched relative forces greater than 30%, all the TnCs displayed a similar force vs. $k_tr$ relationship. However, at relative forces less than 30%, there was a ~1.5-fold increase in $k_tr$ for both V43QTnC and I60QTnC compared to WTTnC (Table 4). Thus for relative forces greater than 30%, it would appear that the mutant TnCs do not alter the 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 greater than 70%.
Figure 3.4. The dependence of relative $k_{tr}$ on relative force for single skinned psoas fibers reconstituted with TnC mutants. Average relative $k_{tr}$ values at each pCa shown in Fig. 7 are plotted vs. the average levels of relative force generated at the corresponding pCa. For each fiber $k_{tr}$ was normalized to the pCa 4.0 $k_{tr}$ of the fiber prior to 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 ± S.E. from at least four separate fibers.
Effect of NEM-S1 on Force and $k_{tr}$ for WTTnC and I60QTnC.

In order to determine if I60QTnC affected the intrinsic cross-bridge cycling rate, we utilized NEMS-1, a strong binding cross-bridge analogue that can trap the thin filament into an open conformation (32). Incorporation of 6µM NEMS-1 with WTTnC fibers resulted in a ~12% reduction of force, no significant effect on the Ca$^{2+}$ sensitivity of force (pCa$_{50}$ of 6.02 ± 0.2), and a decrease in the cooperativity of force production (Hill coefficient of 1.9 ± 0.2) (Figure 3.5a; Table 3). These results agree well with previously reported effects of NEMS-1 on fibers containing endogenous TnC (10). On the other hand, adding NEMS-1 to I60QTnC did not have a significant effect on maximal recovered force, Ca$^{2+}$ sensitivity of force development, or cooperativity of force development (Table 3, Table 4). However, at low relative forces in the presence of NEMS-1, $k_{tr}$ was at maximal pre-extraction values for both WTTnC and I60QTnC fibers (Figure 3.5b). At intermediate levels of relative force, $k_{tr}$ decreased for both WTTnC and I60QTnC fibers, whereas at higher forces, only with WTTnC was maximal pre-extraction $k_{tr}$ reached. Thus I60QTnC can exhibit maximal pre-extraction $k_{tr}$ if the thin filament is activated by NEMS-1.
Figure 3.5. Effect of NEM-S1 on the Ca\(^{2+}\) dependence of force generation by WTTnC and I60QTnC in skeletal muscle. Panel A shows the 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 (6µM) as a function of pCa. Each data point represents the mean ± S.E. from at least four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation. Panel B shows the average relative k\(_{tr}\) values at each pCa shown in Panel A plotted vs. the average levels of relative force generated at the corresponding pCa. For each fiber k\(_{tr}\) was normalized to the pCa 4.0 k\(_{tr}\) of the fiber prior to TnC extraction and reconstitution. Each data point represents the mean ± S.E. from at least four separate fibers for WTTnC (■) and I60QTnC (●) in the absence (filled symbols) or presence (open symbols) of NEM-S1 (6µM).
Effect of Human Tn Exchange into Rat Cardiac Trabeculae

IAANS labeled human Tn complexes were passively exchanged into rat skinned cardiac trabeculae. We first compared the effects of mock Tn exchange with that of control Tn and found that incorporation of human Tn did not significantly affect the Ca\(^{2+}\) sensitivity of force production (pCa\(_{50}\) of 5.7±0.1), the cooperativity of force (nH of 2.0±0.2) production, nor force recovery upon exchange (55 ± 7%) (Fig 3.6, Table 5). The mutant Tn complexes were then compared to the control Tn. The TnI 1-192 truncation recovered higher maximal force (86 ± 8%) than the control Tn and sensitized the force development to Ca\(^{2+}\) ~11.2 fold (pCa\(_{50}\) of 6.76±0.11) (Fig 3.7). TnI 1-192 truncation was the only Tn to recover force that was significantly different from control in addition to producing significant levels of Ca\(^{2+}\) independent force at pCa 9. On the other hand, the TnT K210 deletion recovered 52 ± 4% force and desensitized force production to Ca\(^{2+}\) ~2.2 fold (pCa\(_{50}\) 5.24±0.1) (Fig 3.8). Incorporation of the correcting S69D D73NTnC with the mutant TnI 1-192 Tn recovered 70 ± 6% of maximal force and restored the Ca\(^{2+}\) sensitivity of force back to control levels (pCa\(_{50}\) 5.75±0.1). Upon exchange of the correcting M45Q S69DTnC TnT ΔK210 Tn into the trabeculae, it recovered 52± 3% of maximal force and corrected the decreased Ca\(^{2+}\) sensitivity of force from the ΔK210 TnT back to control levels (pCa\(_{50}\) 5.61±0.04). These results demonstrate that combining TnCs specifically engineered to counteract the altered Ca\(^{2+}\) sensitivity of force production due to TnI and TnT mutations can correct the diseased effects.
Figure 3.6. Effect of the Tn exchange protocol on the Ca$^{2+}$ dependence of cardiac muscle force generation. Shown is the Ca$^{2+}$ dependence of isometric force generation in skinned trabeculae exchanged with Control Tn (■) and the mock exchange (♦) as a function of pCa. Panel A shows the data normalized to the starting maximal endogenous levels while Panel B normalizes the data to the maximal force upon exchange. Each data point represents the mean ± S.E. from four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Figure 3.6
Figure 3.7. Effect of TnI 1-192 and S69D,D73N TnC on the Ca$^{2+}$ dependence of cardiac muscle force generation. Shown is the Ca$^{2+}$ dependence of isometric force generation in skinned trabeculae exchanged with control Tn (■), TnI (1-192) Tn (○), and the combined TnI (1-192) S69D,D73N Tn (▲) as a function of pCa. Panel A shows the data normalized to the starting maximal endogenous levels while Panel B normalizes the data to the maximal force upon exchange. Each data point represents the mean ± S.E. from four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Figure 3.7
Figure 3.8. Effect of TnT ΔK210 Tn and M45Q,S69D TnC on the Ca$^{2+}$ dependence of cardiac muscle force generation. Shown is the Ca$^{2+}$ dependence of isometric force generation in skinned trabeculae exchanged with control Tn (■), TnT ΔK210 Tn (♦), and the combined ΔK210 Tn M45Q,S69D TnC Tn (★) as a function of pCa. Panel A shows the data normalized to the starting maximal endogenous levels while Panel B normalizes the data to the maximal force upon exchange. Each data point represents the mean ± S.E. from four separate fibers individually fit with a logistic sigmoid equation mathematically equivalent to the Hill equation.
Table 5. Comparison of the effects of Tn mutants on the Ca\(^{2+}\) sensitivity of force development in cardiac trabeculae. Values significantly different from WT as determined by a Student’s T-Test are denoted by * . One way analysis of variance (ANOVA) was used to determine the influence of the factor TnC on the dependents pCa50, Hill Coefficient, and % Force Recovery. TnC was found to be significant for both all dependents (p<0.0001).

<table>
<thead>
<tr>
<th>Force vs pCa</th>
<th>Normalized to Endogenous</th>
<th>Normalized to Exchanged Tn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCa50</td>
<td>nH</td>
</tr>
<tr>
<td>mock exchange</td>
<td>5.39±0.2</td>
<td>*1.1±0.3</td>
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<tr>
<td>cntrl IAANS</td>
<td>5.7±0.1</td>
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<td>TnI1-192</td>
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<tr>
<td>K210</td>
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<td>2.4±1.0</td>
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<tr>
<td>M45Q S69D K210</td>
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<td>1.5±0.2</td>
</tr>
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</table>
Chapter 4: Discussion

Skeletal Muscle

Previous work has shown that $k_{tr}$ can be affected by the level of thin filament activation (17, 25), changes in cross-bridge kinetics (1), and cooperative interactions at low forces (10) (for further review see (30)). The level of thin filament activation and cross-bridge kinetics appear to have the most significant influences on $k_{tr}$, however whereas cross-bridge kinetics have a direct effect on $k_{tr}$, thin filament activation modulates $k_{tr}$ by limiting the number of cross-bridges that can bind to the thin filament. Our goal in studying skeletal muscle was to examine the effects that Ca$^{2+}$ binding properties of TnC have on $k_{tr}$ and how it does so in relation to these three factors that modulate $k_{tr}$.

Previous studies that have addressed the role of TnC in modulating $k_{tr}$ in skeletal muscle utilized TnC extraction, TnC isoforms, mutations, and the Ca$^{2+}$ sensitizing compound calmidazolium (5, 17, 20, 25). Simple extraction of TnC or incorporation of a TnC that cannot bind Ca$^{2+}$ into muscle, decreases maximal force without altering maximal $k_{tr}$ (5, 20). On the other hand I60QTnC$^{F27W}$, a Ca$^{2+}$ desensitizing mutant that increases $k_{off}$, decreased maximal force and $k_{tr}$ (17), 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 $k_{tr}$ at
submaximal \([\text{Ca}^{2+}]\) (5, 17), whereas calmidazolium, which sensitizes skeletal muscle to \(\text{Ca}^{2+}\) and slows \(\text{k}_{\text{off}}\), elevates \(\text{k}_{\text{tr}}\) only at intermediate \([\text{Ca}^{2+}]\) (25). All these studies clearly indicate that modulation of TnC \(\text{Ca}^{2+}\) binding properties affect \(\text{k}_{\text{tr}}\). However, it is unclear what the mechanism(s) are behind the modulation of \(\text{k}_{\text{tr}}\) through TnC \(\text{Ca}^{2+}\) binding properties.

Our studies suggest that TnC \(\text{Ca}^{2+}\) binding properties modulate the state of thin filament activation to regulate \(\text{k}_{\text{tr}}\). Our results further suggest that this can be accomplished without altering cross-bridge kinetics. As seen in Fig. 3.4, at matched forces, \(\text{k}_{\text{tr}}\) is unchanged for all the TnC mutants at greater than 30% \(F_{\text{max}}\) even with their varied effects on \(K_d\), \(k_{\text{on}}\), and \(k_{\text{off}}\). The observation that \(\text{k}_{\text{tr}}\) was similar for the various TnC mutations at matched force levels, suggests that cross-bridge kinetics were not affected. If the TnC mutants were affecting the cross-bridge kinetics, we would have expected the force vs \(\text{k}_{\text{tr}}\) relationship to have shifted either up or down compared to WTTnC, representing an increased or decreased rate of cross-bridge cycling at matched states of thin filament activation (ie. force). However, neither I60QTnC nor T70DTnC recovered maximal force or \(\text{k}_{\text{tr}}\). Partial extraction of TnC resulted in loss of force without a decrease in maximal \(\text{k}_{\text{tr}}\). Thus, the reductions in both \(F_{\text{max}}\) and maximal \(\text{k}_{\text{tr}}\) for T70DTnC and I60QTnC were not due to incomplete reconstitution. Although both I60QTnC and T70DTnC have faster \(\text{k}_{\text{off}}\) and decreased \(F_{\text{max}}\), our previous work has shown that there is no direct correlation between the two (6). Thus, it is apparent that \(\text{k}_{\text{off}}\) cannot be the only consideration when examining the effects of TnC constructs on \(\text{k}_{\text{tr}}\) and force.
Since I60QTnC recovered only ~75% of maximum force and ~48% of maximum $k_{tr}$, we decided to further test whether I60QTnC decreased maximal $k_{tr}$ through alteration of cross-bridge kinetics or solely by a decrease in thin filament activation. Thus we utilized NEMS-1, a strong binding cross-bridge analogue that activates the thin filament but does not produce force (32). Addition of NEMS-1 to reconstituted I60QTnC fibers recovered maximal $k_{tr}$ at low Ca$^{2+}$, providing evidence that the cross-bridges could cycle at their maximal intrinsic rate. The decreases in $k_{tr}$ at intermediate and maximal [Ca$^{2+}$] observed with I60QTnC in the presence of NEMS-1 were most likely due to incomplete spread of NEMS-1. At low [Ca$^{2+}$], regulatory units of the thin filament with NEMS-1 are preferentially activated by Ca$^{2+}$ and display maximal activation. However, at higher [Ca$^{2+}$], regulatory units without NEMS-1 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 pre-extraction rates at high [Ca$^{2+}$] even after addition of NEMS-1, consistent with full activation of the thin filament.

At force levels less than 30% of $F_{max}$, $k_{tr}$ was slightly elevated for both V43QTnC and I60QTnC but not for T70DTnC when compared to WTTnC. These results are surprising considering that V43QTnC and I60QTnC have opposite effects on the Ca$^{2+}$ sensitivity of force and $k_{tr}$ development as well as on their Ca$^{2+}$ $K_d$ and $k_{off}$. Two traits V43QTnC and I60QTnC shared though, were decreases in their Ca$^{2+}$ $k_{on}$ and cooperativity of force development. Consistent with cooperative interactions being the cause of the elevation in $k_{tr}$, Campbell’s model of $k_{tr}$ suggests that decreasing cooperative interactions serves to enhance $k_{tr}$ (4). This effect is most
pronounced at low [Ca\textsuperscript{2+}] where there is a large number of non-cycling 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\textsubscript{tr}. However, our results do not rule out the possibility that Ca\textsuperscript{2+} k\textsubscript{on} might also play a role in elevating k\textsubscript{tr} at low [Ca\textsuperscript{2+}].

The mechanisms by which V43QTnC and I60QTnC decrease cooperativity might be different. It has been shown that altered nearest neighbor regulatory unit cooperativity does not significantly affect k\textsubscript{tr} (16, 17, 23), so cooperative interactions that affect k\textsubscript{tr} must occur within the regulatory unit of seven actin per Tm dimer and Tn complex (9, 34). These processes include, but are not limited to, feedback interactions between cross-bridges, cross-bridges and TnC, cross-bridges and Tm, and Ca\textsuperscript{2+} binding to TnC. Since V43QTnC is already sensitized to Ca\textsuperscript{2+}, any cooperative mechanisms that normally increase Ca\textsuperscript{2+} binding to TnC 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\textsuperscript{2+} binding properties of skeletal 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\textsubscript{tr}. 
Cardiac Muscle

The exchange of whole human Tn into rat skinned cardiac trabeculae is a promising step in utilizing engineered TnCs to correct the aberrant physiological properties due to cardiomyopathy related Tn mutations. Although the exchange protocol is fairly harsh on maintenance of trabeculae integrity, since even the mock exchange only recovered 68±5% of maximal force, the altered Ca$^{2+}$ sensitivities of force for both the TnI 1-192 truncation and the ΔK210 TnT were successfully corrected back to control values with our TnCs. Interestingly, the TnI 1-192 displayed a significant increase in active force at pCa 9.0, suggesting that this TnI truncation partially removes thin filament inhibition of cross-bridge cycling. We know that this is active force and not an increase in passive tension since addition of BDM to inhibit cross-bridge cycling resulted in passive forces similar to endogenous levels in pCa 9.0.

These results demonstrate TnCs importance in regulating muscle contraction and hint upon its role as a central hub for modulating interactions in the thin filament. More work is necessary in improving the exchange protocol, such as improving the force recovery upon exchange, and in further characterizing TnC’s ability to correct diseased behavior due to other thin filament mutations. More mutations need to be studied and how they affect the rates of contraction and relaxation will need to be determined. If TnC is able to correct all of these contractile parameters, then it will indeed be an ideal treatment target and the next step would be to study it in intact muscle with the eventual goal of characterizing it in human tissue. In conclusion, the
results from rat cardiac trabeculae demonstrate that TnC is a viable treatment target for correcting the aberrant Ca^{2+} sensitivity of force production due to diseased states.
REFERENCES


