INFLUENCE OF THE THIN FILAMENT CALCIUM ACTIVATION
ON MUSCLE FORCE PRODUCTION AND RATE OF CONTRACTION IN
CARDIAC MUSCLE

DISSertation

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* * * * *

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ABSTRACT

Cardiac muscle contraction is initiated by Ca\textsuperscript{2+} binding to troponin C (TnC), which triggers conformational changes on the thin filament, allowing the myosin heads (or crossbridges) to attach to actin and the thick filaments to slide along the thin filaments. This study investigated the influence of the thin filament Ca\textsuperscript{2+} binding affinity on modulating the rate of contraction at various levels of Ca\textsuperscript{2+} activation in rat skinned cardiac trabeculae at 15°C. The rate of contraction was assessed as the rate of force (tension) redevelopment, or k_{tr}.

Our novel approach was to directly change the level of thin filament Ca\textsuperscript{2+} activation by incorporating into trabeculae cardiac TnC mutants with various Ca\textsuperscript{2+} binding affinities. The TnC mutants V44QTnC\textsuperscript{F27W} and F20QTnC\textsuperscript{F27W}, when reconstituted into trabeculae, increased and decreased, respectively, the Ca\textsuperscript{2+} sensitivity of force production, both in conditions of normal or accelerated crossbridge cycling induced by the presence of added inorganic phosphate (Pi). The rates of contraction at submaximal levels of Ca\textsuperscript{2+} activation were increased or decreased, respectively, when the muscle was sensitized or desensitized to Ca\textsuperscript{2+}, but the rates of contraction at saturating Ca\textsuperscript{2+} activation were similar. When crossbridges cycle faster in the presence of Pi, enhancing or reducing the thin filament Ca\textsuperscript{2+} activation can still increase or decrease the rate of contraction at
submaximal levels of force production, but the rates of contraction at maximal levels of Ca\(^{2+}\) activation were similar for trabeculae reconstituted with TnC mutants. This study indicates that the rate of cardiac muscle contraction is modulated, at submaximal levels of Ca\(^{2+}\) activation, by the Ca\(^{2+}\) binding properties of TnC, and at maximal levels of Ca\(^{2+}\) activation, by the kinetics of crossbridge cycling.

These results have physiological relevance and possible clinical applications considering that, on a beat-to-beat basis, the heart contracts at submaximum Ca\(^{2+}\) activation. In heart failure, Ca\(^{2+}\) sensitizing TnC mutants would increase the rate of contraction and improve contractility, whereas in hypertrophic cardiomyopathy, Ca\(^{2+}\) desensitizing TnC mutants would correct for the cardiac sensitization and/or the hypercontractile state.
Dedicated to my parents
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CHAPTER 1

INTRODUCTION

*Structural and biochemical basis of cardiac muscle contraction.* In striated muscle, cyclic interactions between thin (actin) filaments and thick (myosin) filaments result in filament sliding, muscle shortening and force production. The organization of the contractile proteins represents a sarcomere, or the functional unit of the striated muscle. Within a sarcomere, the actin and myosin filaments interdigitate, such as in cross-sectional representation, they form a hexagonal lattice, with six actin filaments surrounding one myosin filament. The light microscopy and electron microscopy images of muscle fibers revealed a striated pattern, resulting from alternating light bands (I bands, mostly actin) and dark bands (A bands, mostly myosin). A sarcomere spans between two Z-lines and it contains the A band in the middle, neighbored by half of the I band on each side. Furthermore, X-ray diffraction studies revealed the detailed structure of the contractile machinery, particularly the myosin heads or crossbridges extending towards actin. When Ca$^{2+}$ binds to the thin filament, myosin crossbridges are able to reach to actin and slide the thin filaments past the thick filaments. This mechanism of contraction, known as the
sliding filament theory of contraction, was first proposed by A.F. Huxley (Huxley, 1957a). The “coupler” between biochemical and mechanical processes is ATP, which initiates contraction of muscle by providing the energy required for myosin to attach to actin and slide the thin filaments towards the middle of the sarcomere. After the release of the hydrolysis products, Pi and ADP, ATP binding to myosin is also required for the detachment of myosin from actin and the muscle relaxes.

In both skeletal and cardiac muscle, a vast amount of information about the processes involved in muscle contraction has resulted from skinned muscle preparations. In these preparations, removal of the sarcolemma gives access to the contractile machinery inside the cell, allowing exposure of the cell to different Ca\(^{2+}\) activation solutions. In this manner, both steady-state Ca\(^{2+}\) binding properties of the muscle and the kinetics of relaxation and activation can be investigated. Overall, the skinned preparation can simulate different environments that the cells \textit{in vivo} can be exposed to and provides valuable information regarding the mechanism of contraction and relaxation.

Along the thin filament, one troponin complex is bound to every 7-actin monomers. The troponin complex is comprised of three proteins arranged in a 1:1:1 stoichiometric ratio, known as troponin C (TnC or the Ca\(^{2+}\) binding subunit), troponin I (TnI, which inhibits actin–myosin interaction in the absence of Ca\(^{2+}\)), and troponin T (TnT), which binds the troponin complex to tropomyosin. Initiation of contraction occurs when Ca\(^{2+}\) binds to troponin C in the troponin complex on
the thin filament. The actin filaments are organized in a double-helical structure by polymerization of actin monomers. The helical polymers are composed of repetitive 13 actin monomers on each helical turn. Fiber diffraction patterns, calculated from oriented gels of filamentous actin (Holmes, et al. 1990), showed that actin has mainly two domains: one involved in actin-actin interactions and one forming the nucleotide-binding pocket. The domains move closer in the filamentous form, closing the nucleotide-binding pocket. Tropomyosin (Tm) is a double–stranded protein, which binds end-to-end along the thin filament. Each tropomyosin covers 7 actin monomers. Tropomyosin can rotate on the surface of the actin filament, movement influenced by both Ca^{2+} binding to TnC and crossbridge binding to actin. In the absence of Ca^{2+}, Tm blocks the myosin binding sites on actin. When Ca^{2+} binds to TnC, a series of conformational changes propagate along the troponin complex to tropomyosin, which moves in a position closer to the groove of the thin filament, exposing the myosin binding sites and allowing myosin to attach to actin.

Troponin C is the Ca^{2+} “sensor” in both cardiac and skeletal muscle. Troponin complex was first identified biochemically by Katz (Katz, 1966) and biochemically purified by Ebashi (Ebashi, et al. 1967). Later on, Greaser and Gergely (1971) defined the three distinct proteins that comprise the troponin complex. Troponin C structure, as described by NMR imaging and X-ray crystallography, consists of two globular heads (N-domain and C-domain) connected through a helical central linker. This structure is similar to a dumbbell
shaped molecule, each of the heads containing two common motifs, EF-hand or a helix-loop-helix, which can bind different cations. Each globular head contains a pair of Ca$^{2+}$-binding sites, numbered sites I through IV from the N-domain. Sites I and II of the N-domain are referred to as Ca$^{2+}$-specific sites, whereas sites III and IV in the C-domain can bind Ca$^{2+}$ or Mg$^{2+}$. The cation binding sites are positioned spatially similar to a pentagonal bipyramid, due to orientation of the amino acids in the binding motif. The EF-hands in the C-domain are high affinity Ca$^{2+}$ and Mg$^{2+}$ binding sites, primarily anchoring the TnC to the N-terminus domain of TnI. Therefore, the C-domain is also known as the structural domain. The N-domain of TnC contains only one functionally active EF-hand in cardiac muscle, in comparison with skeletal muscle, which has two active Ca$^{2+}$ binding sites in the N-domain. In cardiac muscle, the first EF-hand is inactive because of amino acid substitutions at three coordinating positions in the Ca$^{2+}$ binding site. The N-domain favors the binding of Ca$^{2+}$ over Mg$^{2+}$ and it contains the physiological Ca$^{2+}$ binding sites, triggering the initiation of force production. For this reason, the N-domain is also known as the regulatory domain, as it is able to exchange Ca$^{2+}$ fast enough to regulate diastolic and systolic processes. The Ca$^{2+}$ binding constants of each site, measured in ATP-free solutions, are $K_{Ca} = 7.4 \times 10^7$ M$^{-1}$, $K_{Mg} = 0.9 \times 10^3$ M$^{-1}$ for sites III and IV and $K_{Ca} = 1.2 \times 10^6$ M$^{-1}$, $K_{Mg} = 1.1 \times 10^2$ M$^{-1}$ for site II (Kobayashi & Solaro, 2005). In the presence of MgATP, there are no changes in the affinities of sites III and IV, but the Ca$^{2+}$ affinity of site II decreases to $K_{Ca} = 3.0 \times 10^5$ M$^{-1}$ (Pan & Solaro. 1987). In cardiac muscle, the
strong crossbridge interaction with actin and/or crossbridge dissociation can influence the Ca\(^{2+}\) binding to the regulatory site of TnC (as a feedback mechanism) just as Ca\(^{2+}\) binding to TnC triggers conformational changes propagated along the troponin complex and tropomyosin to actin, facilitating myosin attachment (see review, Gordon et al, 2000). This mechanism might play an important role in the regulation of cardiac muscle contraction, as will be discussed later. Kinetic studies indicated that the regulatory domain of TnC is important for the modulation of the Ca\(^{2+}\) binding properties of the thin filament and it is important for the regulation of contraction (Johnson, et al. 1979, Putkey, et al. 1989, Robertson, et al. 1981).

Troponin I is the subunit of the troponin complex that establishes multiple interactions with TnC, TnT and actin, many of these interactions influenced by Ca\(^{2+}\) binding to TnC. Troponin I binds to the N-terminal region of actin and inhibits the binding of myosin, even though not directly, because TnI is present in a 1:7 ratio on actin. It is implied that TnI can help anchor the troponin complex on actin in the absence of Ca\(^{2+}\). The cardiac isoform has an N-terminal extension, which is not present in the skeletal muscle isoforms. There are six identified functional regions of TnI: 1) a N-terminal extension, cardiac specific, which contains the PKA–dependent phosphorylation sites Ser-23 and Ser-24, 2) a region that binds to the C-domain of TnC, 3) a region that binds to the C-domain of TnT, 4) an inhibitory region which binds to actin, 5) a switch region, and 6) a second actin-binding region. The most critical is the inhibitory region, which is
highly conserved among muscle types, even though in the cardiac isoform, a Pro has been replaced with Thr at position 143. This Thr is a major phosphorylation site for PKC in cardiac muscle. TnI-TnC interaction is a major step in the Ca\(^{2+}\)-mediated interaction between the regulatory proteins. Most of the information about the TnC-TnI interaction has been provided by diffraction studies, circular dichroism, NMR and fluorescence studies of the complex, (Kobayashi & Solaro, 2005) but the definitive, complete structure, as crystallization of the full complex, has not been achieved yet. Troponin I is oriented in an anti-parallel fashion as related to TnC orientation, such that the C-domain of TnC binds the N-domain of TnI. The molecular mechanisms by which Ca\(^{2+}\) binds to the N-domain of TnC and triggers contraction are different in cardiac muscle, compared with skeletal muscle. In skeletal TnC, Ca\(^{2+}\) binding to the N-domain induces a conformational change known as the "open" state, which exposes a hydrophobic patch to which TnI can bind. In the resting state, this patch is deeply “buried” in the N-domain and unavailable for TnI. The inhibitory peptide of TnI is bound to actin at the low levels of Ca\(^{2+}\) and inhibits crossbridge binding. Upon [Ca\(^{2+}\)] rising, the switch peptide of TnI binds to the exposed hydrophobic patch of TnC, which pulls the TnI inhibitory peptide away from actin, relieving the actin inhibition and facilitating myosin binding to actin (Li et al, 2004).

In cardiac muscle, Ca\(^{2+}\) binding to the regulatory domain cannot open the hydrophobic patch on TnC. Opening of the hydrophobic patch requires the interaction with the switch site of TnI and ultimately, this interaction induces a
structural re-arrangement among the troponin complex, which results in movement of tropomyosin further in the actin groove, exposing the myosin-binding sites on actin. Recently, more information about the intricacies of interaction among the proteins in the troponin complex has been provided by crystal structure of the core domain of TnC, TnI and TnT in the presence of Ca\(^{2+}\) (Takeda, et al. 2003).

Phosphorylation of cardiac proteins is a key component of the modulation of contractile function of the heart during β-adrenergic stimulation. On β-adrenergic stimulation in the heart, there is rapid formation of the intracellular second messenger cAMP, which causes a direct activation of protein kinase A (PKA) within the cardiac cell. The activated catalytic subunit of PKA is known to have multiple intracellular targets in cardiac muscle: the sarcoplasmic reticulum (SERCA pump), phospholamban of the sarcoplasmic reticulum, and two key myofilament proteins, TnI of the thin filament and myosin binding protein C (MyBP-C) of the thick filament. The adrenergic stimulation not only increases the cardiac output to match the metabolic demands on a beat-to-beat basis, but also accelerates relaxation. It seems that PKA phosphorylation of Ser 23 and Ser 24 on TnI is most important for the regulation of the sensitivity of myofilaments to Ca\(^{2+}\) and for speeding the crossbridge cycling kinetics, whereas PKC phosphorylation of both TnI and TnT appears to decrease the crossbridge cycling rate. Many studies suggest that various kinases can phosphorylate multiple substrates and that there is a substantial cross talk among kinases in the heart.
Nevertheless, the molecular interactions in the troponin complex and the mechanisms of regulation of the Ca\textsuperscript{2+} dependent activation of the thin filament are not completely elucidated yet.

Troponin T is the biggest component of the troponin complex and it establishes extensive interactions with proteins within the troponin complex and with tropomyosin and actin. TnT is an elongated protein, with a globular part, the C-terminal region, which is part of the head of the troponin complex, and an N-terminal region forming the tail of the protein. The tail binds to the region of tropomyosin overlap and it is thought to be involved in the cooperative binding of myosin S1 to actin. As seen in crystal structure, the C-terminal region binds to TnC and TnI (Kobayashi & Solaro. 2005). The cardiac isoforms arise from alternative splicing of TnT transcripts. There are four isoforms of cardiac TnT, which are expressed in different conditions, during development and in pathological conditions, such as heart failure (Gomes, et al. 2002).

Tropomyosin is a double stranded helical protein arranged as a coiled coil, which forms filaments sitting in the groove of the actin double helix. Stability of the coiled coil is maintained by hydrophobic interactions between the nonpolar side chains of the amino acids in each chain. Each chain spans 7 actins in the thin filament. Neighboring tropomyosins overlap in a head–to-tail configuration along the thin filament. X-ray images of tropomyosin decorating F-actin (Lorenz, et al. 1995) showed that tropomysin binds at the periphery at the actin filament
through electrostatic interactions, which may be responsible for the degree of flexibility of tropomyosin on actin. Cardiac tropomyosin seems to be more flexible than the skeletal isoform, as suggested by the fluorescence measurements reported by a probe attached to cysteine 190 (Chandy, et al. 1999). It was demonstrated that the head–to-tail overlap provides the stability of tropomyosin on actin, as deleting the overlap region greatly reduced the affinity of binding to actin (Mak & Smillie. 1981). The troponin complex binds close to the overlap region, one–third of the way from the C-terminal of Tm and toward the N-terminal region. Myosin binding increases the binding of Tm to actin and decreases its flexibility, and also the troponin complex further anchors Tm to actin through sites greatly influenced by Ca$^{2+}$. The movement of Tm over actin filaments, induced by both Ca$^{2+}$ binding to troponin and myosin heads binding on actin, is an important part of the regulation of contraction (Szczesna & Fajer. 1995, Szczesna, et al. 1996). The constitutive proteins of the troponin complex are arranged in a 1:1:1 stoichiometric ratio and are distributed along the actin filament, with 1 troponin complex bound to every 7 actin monomers.

The thick filament is composed of the motor protein myosin II, which is comprised of two heavy chains and four light chains. The heavy chains form a parallel two-chain coil-coil structure arranged as rods, which form the backbone of the thick filament. The large N-terminal extension forms a globular region, known as a myosin head (subfragment 1, S1, also as a crossbridge (XB). The two heads of each myosin molecule project outward from the thick filament and
are helically arranged around it with a repeat of about 14.3 nm, as indicated by X-ray diffraction studies. Subfragment S1 is responsible for the enzymatic activity of myosin. It has three sub-domains: an upper, middle and lower sub-domain. The lower sub-domain contains the actin-binding site; the ATP-binding site is located in between the upper and the middle sub-domains, separated from the actin-binding site by only 4 nm. The myosin head is linked to the rod portion of the myosin molecule through a short part, known as the “myosin neck”. The connecting structure between the myosin head and the myosin neck is called the “converter region”. One pair of the light chains binds to the myosin neck. The main function of the neck is to behave as a lever arm, to amplify the movements transmitted to the converter domain during ATP hydrolysis. The structural relationship of actin and myosin molecules, as shown by X-ray diffraction images (Rayment, et al. 1993a, Rayment, et al. 1993b) and electron micrographic reconstruction (Milligan & Flicker. 1987, Milligan, et al. 1990), suggested that myosin and actin establish two different chemical interactions during contraction. The first interaction is an ionic interaction, which involves the negative charges on the surface of the actin monomers and positively charged Lys residues on the myosin head. After the strong binding of myosin, at the end of the power stroke, a second set of hydrophobic interactions occur (Milligan. 1996). Biochemical studies measuring the kinetics of ATP binding and by-product release provided valuable insight into the coupling of chemical and mechanical energy of muscle force production. ATP binding to the myosin head triggers a conformational
change, causing a rapid dissociation of the myosin head from actin (Holmes, et al. 2003, Kawai, et al. 1993). Following detachment from actin, ATP is rapidly hydrolyzed to ADP and Pi, both of which remain very tightly bound to the myosin head. The free energy of ATP hydrolysis is not released but remains within the structure of the M.ADP.Pi complex. In this state, the myosin head can bind to actin via weak ionic interactions. After Ca$^{2+}$ binding to the thin filament and subsequent tropomyosin movement on actin, myosin-binding sites on actin are exposed. The affinity of M.ADP.Pi for actin is significantly higher than that of M.ATP. If an actin site is within reach of the myosin head, it will bind rapidly and reversibly to the actin site. This allows stronger hydrophobic interactions to occur between myosin and actin (Zhao & Kawai. 1994). It was considered that this stronger interaction triggers a major conformational change, known as isomerization, which is accompanied by the dissociation of Pi and the power stroke (Tyska & Warshaw. 2002).

Crystal structures suggest that the power stroke consists of a reorientation of part of the myosin head distal to the actin-binding site which results in the relative sliding of actin and myosin filaments by a distance of up to 10 nm. The internal strain resulting from the conformational change in the myosin head is conveyed to the “lever arm” region, part of the S2 structure (Tyska & Warshaw. 2002). This strain triggers lever arm bending, mostly as a torque motion directed longitudinally rather than transversely. Under isometric conditions, the estimated longitudinal force per interaction is ~2 pN/crossbridge (Cooke. 1997). After the
force-generating step, ADP is released, followed rapidly by ATP rebinding and myosin head detachment from actin. After completing the cycle, ATP is again hydrolyzed and the entire process starts over again. This sequence of steps in the crossbridge cycle reflects mostly conformational changes of the myosin molecule associated with chemical reactions, as actin is thought to undergo very small conformational changes, which are less understood.

The mechanism of contraction will be dictated by the sequence of reactions among ATP, ADP, Pi, myosin and actin and the affinities of binding, which will drive the reaction forward (as the free energy of binding is positive) or backward (by decreasing the free energy). The crossbridge cycle has been described from structural studies (crossbridge visualization in electron micrograph reconstructions of decorated actin, (Huxley et al, 1969), combined with biochemical studies (ATP-ase measurements, Lymn at Taylor, 1971). It was remarkably difficult to actually visualize a crossbridge during the swing motion. New development of X-ray sources and resolved X-ray diagrams from contracting muscle provided evidence for the movement of the crossbridge during its cycling interaction with actin (Huxley at al, 1981, Irving at al, 1992). A schematic representation of the crossbridge cycle (Gordon, et al. 2000, Cooke. 2004), with a detailed explanation follows below. For unbound myosin, the sequence of reaction is indicated by the steps 1’, 3’ and 5’. The hydrolysis of ATP in step 3’ is relatively rapid (~125 s⁻¹), whereas 5’ is very slow in the absence of actin.
After ATP binds to actomyosin (step 1), myosin rapidly dissociates from actin (step 2), followed by a fast, reversible cleavage of ATP in the myosin head. During step 4, there is a rapid re-association of actin with myosin, forming the weakly bound A-M•ADP•Pi. The isomerization process takes place during step 5, which is thought to be mediated by Ca\(^{2+}\) (Ma & Taylor. 1994). As a result of the isomerization, the strongly bound state AM•ADP•Pi is formed. This form is associated with force production and lever arm swinging (step 6), which is followed by Pi release in step 7. The force generation and the Pi release steps are represented as different events, even though it was considered that they might occur at the same time (Gordon, et al. 2000). There is general consensus though that the isomerization process and the transition from weak to strong binding involves storage of energy in the myosin head and represents the transition associated with movement of the lever arm and force generation (power stroke). The rate of Pi release is followed by another isomerization during
which ADP is released. The rate constant for these reactions has been estimated from solution studies and skinned fiber studies (for review, see Gordon et al., 2000). These studies showed that the rate of Pi release in isometrically contracting fibers is ~ 30 s\(^{-1}\) (Millar & Homsher, 1990), much faster than the overall muscle ATPase rate (Chock, et al. 1979). This implies that Pi release is not the rate limiting-step of an isometric contraction. Therefore, the rate-limiting step might occur after Pi release, possibly when the ADP, the second product of hydrolysis, is released.

**Ca\(^{2+}\) activation of force production.** In vivo control systems that regulate cardiac muscle contractility are complex and organized at multiple levels. Contractility is under the influence of the autonomic nervous system (sympathetic and parasympathetic innervations of the heart) to match the cardiac performance in accordance with the demands, but intrinsic mechanisms of the myocardium contribute to these control systems. As an example, the increase in venous return to the heart results in an increase in the stoke volume, a classical observation described as the Frank-Starling effect. Nevertheless, the structural and functional mechanisms of the Frank-Starling effect have not been completely elucidated. At the level of myofilaments, the activation of contraction is initiated by Ca\(^{2+}\) binding to the thin filament, which is considered to behave like a “switch” mechanism, which “turns on” the thick filament and allows myosin to bind to actin, resulting in filament sliding and force production. Even though Ca\(^{2+}\) binding is the critical step in initiation of contraction, this event by itself cannot explain the
variations in the contractile properties of the heart on a beat-to-beat basis. If Ca\(^{2+}\) activation behaves only as a simple “regulatory switch”, this would not explain the variations in the contractile parameters in physiological conditions under the sympathetic tone or the more complex changes the heart undergoes in pathological conditions. In recent years, a large amount of work has shown that the regulation of cardiac muscle contraction is modulated by many factors at many levels of interaction. Thus, the phosphorylation of many myofibrillar proteins, the cooperative interactions along the thin filament, the “cross-talk” between the level of activation of the thin and thick filament and the progressive activation of the thick filaments can contribute to regulating the speed and force of contraction.

The analysis of the isometric force as a function of Ca\(^{2+}\) is described by the Hill equation. The force-pCa relationship is sigmoidal and well fit with the Hill equation: 

\[
\frac{F}{F_0} = \frac{1}{1 + 10^n (pCa - pCa_{50})}
\]

where \(F_0\) is the maximal force, \(F\) is the force produced at any particular Ca\(^{2+}\) activation, \(pCa_{50}\) is the Ca\(^{2+}\) activation solution required to generate half-activation and \(n\) is the Hill coefficient. It is considered that \(n\) is an estimation of the minimum number of binding sites involved in the regulation of contraction. In cardiac muscle, \(n\) has been reported to be higher than 3, also the range is quite broad, many studies have reported a Hill coefficient as low as ~1.5 and as high as ~9 (Gordon et al., 2000). Nevertheless, the apparent discrepancy between the Hill coefficient and the presence of just one regulatory binding site on cardiac TnC means that there are cooperative
interactions involved in the process of activation. The force produced at any 
\( \text{Ca}^{2+} \) activation is therefore greater than it would have been predicted based on 
\( \text{Ca}^{2+} \) binding to a single binding site on TnC. Thus, a complete description of the 
activation of contraction includes cooperative interactions at various levels: along 
the thin filament, along the thick filament and also the feedback of the thick 
filament interaction with actin on the activation level of the thin filament. In a 
conceptual context, cooperativity of activation means that events in the sequence 
of activation will facilitate the occurrence of subsequent events of the sequence. 
(Campbell. 1997). A thin filament functional group was estimated to be 
comprised of ~10-14 actin monomers. Along this functional unit, \( \text{Ca}^{2+} \) activation 
will facilitate the myosin binding by exposing the myosin binding sites on actin. 
The force produced by the muscle will depend on this interaction, in terms of 
number of attached crossbridges, the force developed by each crossbridge and 
the availability of binding sites on actin. The maximum number of attached 
crossbridges during contraction was estimated mostly from structural studies and 
this number can be highly variable. It was suggested that, per seven-actin unit, 
no more than four myosin S1 heads could attach. During an isometric 
contraction, only 20-50% of the available crossbridges can attach (Brenner. 
1986), which means that ~1-2 myosin heads can attach per 7 actins-1 
tropomyosin-1 troponin complex (known as a regulatory unit or RU).
Cooperativity of cardiac muscle activation. Many models of activation currently imply that there are four mechanisms of cooperative activation, which have been supported by experimental data. The initiation of activation proceeds when Ca\(^{2+}\) binds to a RU, resulting in crossbridge binding to myosin binding sites on actin. This sequence of events can be referred to as RU→XB interaction. By sharing a 7-actin strand and Tm, the RU and XB can interact in the following ways: RU→RU, XB→RU and XB→XB interaction.

The RU→RU interaction is a source of cooperativity between Ca\(^{2+}\) binding sites on the thin filament, such as Ca\(^{2+}\) binding to one site enhances Ca\(^{2+}\) binding to the adjacent sites, a coupling which occurs most likely through head-to-tail interactions between neighboring tropomyosin molecules. (Grabarek, et al. 1983; Mehegan & Tobacman. 1991). Fluorescence probes on TnC which monitor changes in Ca\(^{2+}\) binding to TnC in the presence of TnI - TnT or actin, with or without myosin, suggested that there is cooperative binding to the Ca\(^{2+}\)-specific sites of troponin C in regulated actin in skeletal muscle (Grabarek, et al. 1983). Binding studies of TnC or TnC - TnI to actin-tropomyosin in cardiac thin filaments suggest that, even in the absence of myosin, long range allosteric interactions occur between troponin molecules. Studies in skeletal muscle using either partial extraction of TnC (Moss, et al. 1985) or replacement of native cardiac TnC in thin filaments with a cardiac mutant, which cannot bind Ca\(^{2+}\) (Butters, et al. 1997), showed that, at maximal Ca\(^{2+}\) activation, either ATP-ase activity or force is correlated with the fraction of
functional TnC units. Moreover, partial extraction of TnC decreased the Hill coefficient (Brandt, et al. 1982) and this implied that the cooperative interaction among all troponin complexes on the thin filament is perturbed. As the tension the muscle develops correlates with the Ca$^{2+}$ binding to TnC, a study reported that maximum Ca$^{2+}$ tension occurred when one third of the TnC along the thin filament was extracted (Moss, et al. 1985). This observation suggested that the size of the activating unit is about three A$_T$mTn units long. Overall, various studies imply that adjacent TnCs must bind Ca$^{2+}$ and facilitate S1 binding and force generation, a process which seems to be more important for complete activation in cardiac muscle compared with skeletal muscle.

The XB$\rightarrow$RU interaction implies that, when crossbridges strongly attach to actin, this tight interaction between myosin and actin influences, presumably through tropomyosin and troponin complex interactions, the Ca$^{2+}$ binding properties of TnC, such as more Ca$^{2+}$ can bind to the thin filament. There is evidence for increased activation of the thin filament in the presence of rigor crossbridges both in the skeletal and cardiac muscle. It was suggested, based on conformational changes in TnC in response to strong crossbridge binding to the thin filaments, that the cycling crossbridges could increase the Ca$^{2+}$ bound to the thin filament, (Hofmann & Fuchs. 1987, Pan & Solaro. 1987), a process more important in cardiac muscle than in skeletal muscle. More support for the importance of this cooperative event in cardiac muscle has been provided indirectly by shortening-deactivation experiments, where intracellular Ca$^{2+}$ was
measured with fluorescent indicators. Allowing a muscle to shorten reduced the amount of subsequent force production by detaching the crossbridges, and this event initiated an extra Ca$^{2+}$ transient. This transient was interpreted as resulting from Ca$^{2+}$ coming off TnC when the influence of strongly bound crossbridges to increase TnC Ca$^{2+}$ binding affinity was lost (Allen & Kentish, 1988, Gordon & Ridgway, 1987). Other investigators (Hofmann & Fuchs, 1987) showed that this phenomenon does not involve length changes that can occur as a result of crossbridge detachment. They showed that decreasing muscle force production in the presence of vanadate, reduced the Ca$^{2+}$ binding properties of the thin filament independent of length changes. These studies showed that indeed the strong crossbridge binding increases the Ca$^{2+}$ binding affinity of the thin filament. This mechanism seems to be more important in cardiac muscle, which manifests a greater length dependence of force (Frank – Starling phenomenon). The physiological significance of this cooperative process still remains undefined, because it is not yet understood if the TnC increase in affinity is simply a consequence of increased number of attached crossbridges or it is required to promote more crossbridge binding to further increase the level of force production.

The XB→XB interaction was first shown in biochemical experiments, where myosin binding to regulated actin was facilitated by the presence of rigor crossbridges (Bremel & Weber, 1972). This mechanism implies that strongly attached crossbridges to a RU can enhance the binding of crossbridges to the
neighboring RU in an allosteric or graded manner. More studies investigated the same effect by using fluorescence imaging of myofibrils in the presence of rhodamine-labeled S1 (Swartz, et al. 1990), or by manipulating the concentrations of ADP or Pi (Dantzig, et al. 1991, Dantzig, et al. 1992) to alter the distribution of crossbridges between strong and weak binding states. Direct quantitative information has been provided using the substrate NEM-S1 (Swartz & Moss. 1992), which binds strongly to the thin filament but does not produce force. Both biochemical and skinned cardiac muscle experiments measuring the ATP-ase activity and force in the presence of Ca\(^{2+}\), respectively, suggested that the increase in the ATP-ase activity or sensitivity of force production was a result of increased crossbridge binding facilitated by NEM-S1.

The mechanism of this cooperation seems to be through the allosteric effects on the regulatory strand on actin, which results in the spread of activation between the neighboring regulatory units, exposing more myosin-binding sites. This mechanism was suggested by the lack of effect of NEM-S1 at maximal activation (when the thin filament is saturated with Ca\(^{2+}\)) and by activation of force at pCa 9.0, when virtually no Ca\(^{2+}\) was bound to TnC. The latter observation implies that, in the absence of Ca\(^{2+}\), the thin filament is not completely “turned off”. This implication was used by Geeves to propose more activation states of the thin filament (see below).
Structural models of Ca\textsuperscript{2+} regulation of contraction. H.E. Huxley and his collaborators proposed, based on three-dimensional image reconstruction of electron micrograph and X-ray diffraction studies, the steric blocking model of regulation (Huxley. 1957b). This model stated that, in the absence of Ca\textsuperscript{2+}, tropomyosin is blocking the myosin-binding site on actin and, upon Ca\textsuperscript{2+} binding to TnC, the tropomyosin moves and allows a myosin head to interact with the site previously blocked. Therefore, this model described the role of the thin filament as a “switch”, being in either an “off” conformation (blocking myosin binding) or an “on” conformation (allowing myosin binding). There was no cooperativity implied between neighboring regulatory units and the cooperative thin filament unit was composed of 7 actins, 1 troponin complex and 1 tropomyosin. This simple model of regulation was re-evaluated by later studies, which suggested that the attached crossbridges might activate the thin filament even in the absence of Ca\textsuperscript{2+}, thus implying that there are three conformational states of the thin filament instead of two, suggesting a more complex mechanism of activation.

Geeves and collaborators described the tropomyosin position on actin as blocked, closed and open (Geeves & Lehrer. 1994). In the blocked state, there is no myosin binding, in the closed state, there is only weak myosin binding and in the open state, the myosin can enter a strong binding, force-generating state. Ca\textsuperscript{2+} binding to the troponin complex initiates the transition from a blocked to a closed state. This model takes into account the cooperative binding of myosin to
actin, therefore the size of the cooperative unit was increased in comparison to previous models to 14 actins, to suggest that tropomyosin overlap is important for propagating the activation to the neighboring actin regulatory units. This model was supported by structural studies showing the three thin filament activation states (McKillop & Geeves. 1993). The model suggests that Ca\textsuperscript{2+}, by itself, does not completely activate the thin filament and it requires strongly attached crossbridges to further move tropomyosin in the groove on actin and favor stronger, hydrophobic interactions between myosin and actin. The implication of this model was that, regarding the states of myosin binding to actin, there are only two possible situations: weak binding or strong binding to actin (Maytum, et al. 1999).

A modified version of the model described by Geeves has been proposed by Lehrer, (Lehrer & Geeves. 1998) which suggested that the thin filament is not fully blocked. Different from Geeves model, Lehrer implied that the blocked state is not completely due to tropomyosin, but also due to TnI binding to actin. TnI will move away from actin in the presence of Ca\textsuperscript{2+} to bind to TnC, thus allowing weak binding of myosin. Furthermore, the weak binding will promote strong binding and transition to the M state. This alternative was less likely as TnI does not span seven actins as does tropomyosin and the influence of a RU on a neighboring RU, mediated through TnI, would be less plausible.
**Physiological models of Ca\(^{2+}\) regulation of contraction.** The first physiological model of striated muscle contraction was proposed by A.F.Huxley (Huxley, 1957). He introduced the rate constant \(f\) (rate of crossbridge attachment as a transition from weak to strong binding) and the reverse process defined as \(g\), which referred to the detachment from force-generating states.

Brenner modified Huxley’s model and added to the mechanism of the regulation of muscle contraction by Ca\(^{2+}\) valuable information as derived from the new technique that he developed (Brenner. 1986), known as \(k_{tr}\) (or rate of tension redevelopment). This technique was developed to be used in skinned muscle fibers originally. It measures the rate of crossbridge reattachment after forcibly detaching them by applying a rapid shortening, followed by a rapid restretch to the muscle original length. Brenner grouped together all the biochemical steps of the actomyosin ATP-ase cycle and the mechano-chemical transduction process resulting in force generation as a rate constant known as \(f_{app}\) and the reversal of the process as a rate constant known as \(g_{app}\). (\(app\) for apparent rate). The crossbridges belong to only two states: un-attached, non-force generating and attached or force-generating. The transition from un-attached to attached states was described by the rate constant \(f_{app}\) and the reverse transition, from attached to un-attached states, by the rate constant \(g_{app}\). The biochemical models suggest that each of these two states are described by a series of steps in the ATP-ase cycle, but the rates \(f_{app}\) and \(g_{app}\) estimate overall the transition back-and-forth from non-force generating to force-
generating, similarly to the rate constants proposed by Huxley as $f$ and $g$.

Brenner proposed the following equations to describe the force generating states, the stiffness and the ATP-ase activity:

$$\alpha_{Fs} = \frac{f_{app}}{f_{app} + g_{app}}$$

where $\alpha_{Fs}$ is the fraction of crossbridges in the force generating states

$$F = n F_0 \frac{f_{app}}{f_{app} + g_{app}}$$

where $F$ is the isometric force, $n$ is the number of attached crossbridges and $F_0$ is the average force produced by one crossbridge in the force generating states

$$S = n S_0 \frac{f_{app}}{f_{app} + g_{app}}$$

where $S$ is the isometric stiffness and $S_0$ is the mean stiffness of one crossbridge in the force generating states

If only one ATP molecule is assumed to be used per cycle, then the isometric ATP-ase activity, measured by the ADP and Pi production, is:

$$\text{ATPase} = n b \frac{g_{app} f_{app}}{f_{app} + g_{app}},$$

where $b$ is the number of half sarcomeres in the fiber.

The rate of force redevelopment, or $k_{tr}$, will be described as:

$$k_{tr} = f_{app} + g_{app}$$

Brenner measured $k_{tr}$, force, stiffness and ATP-ase as a function of $\text{Ca}^{2+}$ and he concluded that $\text{Ca}^{2+}$ correlates with $f_{app}$, such that increasing $\text{Ca}^{2+}$ would
increase $f_{app}$ and ultimately the rate of contraction. Correlated with information from biochemical studies, such as in vitro motility assays, and with skinned fiber experiments, the Pi release step did not depend on Ca$^{2+}$ and therefore Ca$^{2+}$ did not have a direct effect on the crossbridge cycle. It was concluded that Ca$^{2+}$ regulates the kinetics of the thin filament activation, which can influence the number of attached crossbridges.

Landesberg and Sideman (Landesberg & Sideman. 1994a, Landesberg & Sideman. 1994b) proposed a 4-state model of activation, which accounted for different states of thin filament activation kinetics and crossbridge kinetics in cardiac muscle. In state 1 (Ca$^{2+}$-free, no force), Ca$^{2+}$ begins activation of the thin filament and the rates of Ca$^{2+}$ binding to and dissociating from TnC are defined by $k_{on}$ and $k_{off}$ kinetics, respectively. In state 2 (Ca$^{2+}$-bound, no force), strong, force-generating cross-bridges can attach with a rate constant $f_{app}$ and have the potential to produce force but force is not generated yet. In state 3 (Ca$^{2+}$-bound, force), force is generated while Ca$^{2+}$ can dissociate from TnC even in the presence of strongly bound crossbridges and this transitions into state 4 (Ca$^{2+}$-free, force). From state 4, the crossbridges detach from actin with a rate equivalent to $g_{app}$ and the cycle repeats itself. This model takes into account factors that modify the crossbridge kinetics or Ca$^{2+}$ binding to TnC. The model approximated the evidence for the conditions affecting the thin filament activation, as one study reported that decreasing the Ca$^{2+}$ dissociation rate from TnC in the presence of calmidazolium (Regnier, et al, 1996) increased the rate
of contraction. A caveat of the model is that it cannot explain the mechanism of strongly bound, non-cycling crossbridges to activate the thin filament in the absence of Ca$^{2+}$ (NEM-S1 experiments).

Campbell (Campbell. 1997) introduced a six-state model to explain this phenomenon, by expanding the four-state model to include separate Ca$^{2+}$ binding and thin filament activation steps. In this model, a new state of unactivated, noncycling crossbridges is added to the previous states. Thus, the crossbridges can be found in two populations: non-cycling or cycling. The cycling crossbridges undergo transitions between un-attached, non-force generating states and attached, force-generating states with rate constants defined as $f_{app}$ and $g_{app}$. The population of non-cycling crossbridges is "recruited" to the cycling population as a result of either the thin filament activation or the strong crossbridge binding or both.

At low levels of Ca$^{2+}$, most of the crossbridges are in the non-cycling state. The crossbridges from the non-cycling pool have to become "recruited" in the cycling population, as a result of Ca$^{2+}$ binding to troponin C to activate the thin filament. Also the strong-binding crossbridges can further enhance the recruitment of the non-cycling population into the cycling states.

The recruitment process is slow at low Ca$^{2+}$ activation, because the level of the thin filament activation is small and only a few crossbridges are recruited from the non-cycling pool. Therefore, the progressive recruitment of
crossbridges at low levels of Ca\(^{2+}\) activation slows the rate of force redevelopment \((k_{tr})\). On the other hand, at high levels of Ca\(^{2+}\), most of the crossbridges are already recruited into the cycling state, which accelerates the rate of force development to the point that, at saturating Ca\(^{2+}\), the rate constant of force redevelopment \((k_{tr})\) will be approximated by the sum of the forward and reverse rate constants \(f_{app} + g_{app}\). This model was able to account for the effect of NEM-S1 to accelerate \(k_{tr}\) at submaximal activations. By strongly attaching to actin, NEM-S1 eliminated the slower recruitment of crossbridge binding at low levels of Ca\(^{2+}\) activation, by keeping the thin filament in a more “open” state and facilitating crossbridges entering into the cycling states.

Razumova and Campbell (2000) added to this model a new state, including the near-neighbor interactions along the thin and thick filaments. The new eight-state model proposed that the crossbridges could be cooperatively recruited not only within the regulatory unit (RU) of a thin filament (seven actins - 1 troponin complex -1 tropomyosin), but also in the neighboring regulatory units. The state of activation of a regulatory unit would be transmitted to the neighboring regulatory units via the tropomyosin end-to-end overlap between RUs. The state of activation could be increased as well by the attachment of force-bearing

Moreover, the probability of a crossbridge to enter force-bearing states would be increased if the neighboring crossbridges were in the same state. This
new model could explain the experimental data that showed that Ca\(^{2+}\) sensitivity of force production was increased when TnC was partially extracted from the thin filament (Moss et al, 1985). According to this model, extracting TnC disrupted the communication between the neighboring regulatory units, which eliminated the cooperative recruitment of crossbridges within the neighboring RUs.

This implies that the crossbridges were recruited into the cycling pool faster, as the slowing of recruitment within neighboring RUs was eliminated. Faster crossbridge recruitment into the cycling pool and into the force-bearing states increased the state of activation of the thin filament, as manifested by an increase in Ca\(^{2+}\) sensitivity of force development after partial TnC extraction (Moss et al, 1985).

In conclusion, all the models presented suggest that it is very challenging to be able to provide a complete picture of all the processes involved in the muscle contraction. An ideal model should include a complete description of the thin filament activation process in terms of the kinetics of Ca\(^{2+}\) binding to TnC and then the spread of activation along the regulatory units. The conformational changes in the troponin complex, propagated to tropomyosin, and resulting in movement of tropomyosin along the actin groove, are additional processes that might play an important role in the regulation of muscle contraction. The coupling of the chemical and mechanical energy and the complete
understanding of crossbridge cycling in different states of activation would provide additional information to account for the intricacies of interaction between the thin filament regulatory proteins and myosin.

**Specific aims of this study.** The main aim of this study was to investigate the influence of thin filament activation on cardiac muscle Ca\(^{2+}\) sensitivity of force development and the rate of cardiac contraction. The working hypothesis was that, increasing or decreasing the level of the thin filament activation could increase or decrease cardiac muscle Ca\(^{2+}\) sensitivity of force development and the rate of cardiac contraction, respectively. A novel approach in this study was to directly change the level of thin filament activation, by incorporating into rat skinned cardiac trabeculae cardiac TnC mutants with different Ca\(^{2+}\) binding properties.

**Aim 1:** Investigate the influence of altering the Ca\(^{2+}\) binding affinity of TnC on the muscle sensitivity of force development in cardiac muscle.

**Hypothesis 1:** Increasing or decreasing Ca\(^{2+}\) binding affinity of TnC could increase or decrease, respectively, the Ca\(^{2+}\) sensitivity of force development.

**Aim 2:** Investigate the influence of altering the Ca\(^{2+}\) binding affinity of TnC on the rate of cardiac muscle contraction, in the presence of un-altered crossbridge cycling.
Hypothesis 2: Increasing or decreasing Ca\(^{2+}\) binding affinity of TnC could increase or decrease the rate of cardiac muscle contraction when the crossbridge cycling is un-altered.

Aim 3: Investigate the influence of altering the Ca\(^{2+}\) binding affinity of TnC on the rate of cardiac muscle contraction in the presence of faster crossbridge cycling.

Hypothesis 3: Increasing or decreasing the Ca\(^{2+}\) binding affinity of TnC could increase or decrease the rate of cardiac muscle contraction when the crossbridge cycle faster in the presence of added Pi solutions.

The regulation of cardiac muscle contraction reflects the interplay between the thin and thick filament levels of activation. The cross talk and interdependence between the actin and myosin filaments is an intricate process, which various models attempt to explain in more or less detail. Nevertheless, experimental data represents the “building blocks” for elucidating the mechanisms responsible for the regulation of muscle contraction. The goal of the present study was to provide direct evidence of the role of thin filament activation in modulating the rate of cardiac muscle contraction and cardiac muscle sensitivity of force production. The main finding was that the rate of cardiac contraction was modulated by the level of thin filament activation only at sub-maximal levels of activation. This finding is important because the heart works as a twitch, mainly at sub-maximal levels of contraction, by modulating the intensity
and the speed of contraction on a beat-to-beat basis as responding to the body demands. This study suggests also possible clinical applications. In heart failure, sensitizing TnC mutants would increase the rate of contraction and improve contractility, whereas in hypertrophic cardiomyopathy, desensitizing TnC mutants would correct for the cardiac sensitization and/or the hypercontractile state.
CHAPTER 2

METHODS

*Rat skinned cardiac trabeculae preparation and experimental apparatus.* All protocols were approved by the Institutional Animal Care and Use Committee. Male LBN-F1 rats (175–200 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (Nembutal, 50mg/kg). The thoracic cavity was opened and heparin (0.1ml of 10,000u/ml bottle) was injected intracardially. The heart was rapidly excised and placed in relaxing solution (see Solutions) at room temperature. Unbranched trabeculae were harvested from the right ventricle and placed overnight at 4°C in relaxing solution containing 1% Triton-X. The skinned trabeculae were used within 48 hours. The skinned trabeculae were mounted between the arms of a high-speed length controller (model 322C, Aurora Scientific, Ontario, Canada) and an isometric force transducer (model 403A, Aurora Scientific) in the experimental chamber containing relaxing solution by means of aluminum T-clips, as previously described (Rall & Wahr, 1998). The resting sarcomere length was set at ~2.2 μm as determined by the first-order diffraction pattern from a HeNe laser directed through the trabeculae. A
reticule on the eyepiece of the dissecting microscope was used to measure the width and depth of the trabecula. Cross-sectional area was calculated from the depth and width measurements by assuming an elliptical circumference. Force per cross-sectional area (F/CSA) was calculated as an average of two maximal activations at the beginning of the experiment. Each trabecula was activated at the beginning of the experiments in a pCa 4.0 solution and a rapid slack (1ms, 20% of the total length) was applied when the isometric force reached a plateau. This resulted in a rapid drop in force below the resting force baseline. The total force developed was measured between the plateau and the new baseline after slack. Then the trabecula was returned to a pCa 9.0, held at the slack length for 3 s and re-stretched back to the original length in a 5 s ramp. The same procedure was utilized prior to the maximal activation in a pCa 9.0 solution to obtain the resting force. The active force generated by the trabeculae in various pCa solutions was calculated as the total force minus the resting force. The output of the force transducer was recorded using real-time data collection LabView software (edition 6.1) with in-house programming (see Figure 2.1). All experiments were performed at 15°C.

TnC extraction and reconstitution protocol. TnC was extracted by soaking the trabeculae for 30 minutes in an extraction solution containing 10 mM HEPES, 5mM EDTA and 0.5 mM trifluoperazine dihydrochloride (TFP) at pH 7.0. The trabeculae were then transferred to a pCa 9.0 solution and washed three times for 5 minutes to remove residual TFP.
Figure 2.1. LabView software interface for skinned cardiac experiments. Representative trace of activation at pCa 4.0 (as indicated on top of the screen) is shown.
The residual force in pCa 4.0 was $3.0 \pm 0.4\%$ of the maximal pre-extraction force for TnC$^{F27W}$ and its mutants used in this study ($n=72$). TnCs were reconstituted into the trabeculae by soaking the extracted trabeculae for 30 min in a pCa 9.0 solution containing 16.7 μM of TnC (control or mutant). The trabeculae were then activated in a pCa 4.0 solution and the force generated was expressed as a percent of the maximum pre-extraction force. This ratio represented the percent recovery of post-extraction force.

**Isometric force vs. pCa relationship.** The trabecula was activated in a pCa 4.0 solution and rapidly slackened after isometric force reached a plateau. The same procedure was utilized to obtain the resting force level of the trabeculae in a pCa 9.0 solution. The total force was measured between the plateau and baseline levels. The active force generated by the trabeculae in various pCa solutions was calculated as the total force minus the resting force. The un-extracted or reconstituted trabeculae were then exposed to a series of pCa solutions ranging from pCa 9.0 to pCa 4.0, to measure the Ca$^{2+}$ sensitivity of force development. The order of pCa activations was randomized throughout the experiment to ensure that no trend of activation is present. Representative traces of un-extracted rat skinned cardiac trabeculae activated at various pCas are shown in Figure 2.2 and in Figure 2.3. For trabeculae reconstituted with TnC mutants, the maximum force production was measured at pCa 4.0 prior to the extraction. Series of pCa 4.0 activations were performed at the beginning, middle and the end of each force vs. pCa experiment, averaged and used to normalize the
submaximal activations. The relationships between force and pCa was fitted with
a logistic sigmoid relationship mathematically equivalent to the Hill equation,
using the Fig. P Analysis software. The equation used to fit force vs.pCa traces
is: \( \text{min} + \frac{(\text{max} - \text{min})}{1 + \exp(-K*(X-X_{50}))} \), where min and max represent the
minimum and maximum force ( 0 and 1, respectively), K is a measure of the Hill
coefficient and \( X_{50} \) is a measure a \( \text{pCa}_{50} \).

**Protocol for measuring the rate of contraction in skinned trabeculae.** A
trabecula was rapidly slackened (1ms, 20% of the total length), held at the slack
length for 20ms, and then rapidly re-stretched (1ms) back to the original length.
This protocol measures the rate at which the detached crossbridges are able to
re-attach and develop force. The rate measured is referred to as \( k_{tr} \), where "K" is
the rate of contraction, and the subscript is the abbreviation for “tension (force)
redevelopment”. An in-house programming algorithm using LabView software
initiated the movement of the length controller arm. Force redeveloped to levels
similar to those before the slack-restretch (see Figure 2.4). This protocol was
repeated in pCa solutions ranging from pCa 6.2 to pCa 4.0. In order to average
out the rundown of the preparation throughout the experiment, maximum pCa
activations were repeated in the middle and at the end of the experiments. The
redeveloped force traces were imported into Figure P 2.7 analysis software
(Durham, NC) for further analysis.
Figure 2.2. Representative traces of un-extracted rat skinned cardiac trabeculae activated at various pCa.
Figure 2.3. Representative traces of un-extracted rat skinned cardiac trabeculae activated at various pCAs. Traces are superimposed to show that, at lower pCa activations, the force develops more slowly than at higher pCa activations.
The rate of force redevelopment estimated as time to half activation ($t_{1/2}$) was very similar to the rate fitted with a monoexponential relationship ($r^2=0.93$). Therefore, all the traces were fit with a monoexponential relationship. The fitted rate represented the rate of force redevelopment, $k_{tr}$. The $k_{tr}$ at levels of force less than 10% of the maximum force was difficult to measure accurately. Thus the cutoff for calculating the $k_{tr}$ values was set at levels of force generation more than 10% of the maximum force production. This protocol allowed us to calculate, for each pCa solution, the force produced before slack-restretch, which was used to generate the force vs. pCa relationships. Also, $k_{tr}$ values at each Ca$^{2+}$ activation were used to characterize the $k_{tr}$ vs. pCa relationships and $k_{tr}$ vs. force relationships.

The holding time (the time the preparation was maintained at a slack before being restretched at the original length) was set to 20 ms, similar to the holding time reported in $k_{tr}$ protocol by other investigators (Brenner, 1988; Palmer & Kentish, 1998; Regnier et al., 1996; Wolff et al., 1995). The holding time was varied from 10 ms to 100 ms to evaluate the time delay for the preparation to pick up the slack. It was noted that, after ~30 ms holding time, the trabecula was able to develop force. Therefore, the holding time was set to 20 ms, in order to minimize force redevelopment before the restretch. This protocol allowed us also to compare the rate of contraction in un-extracted trabeculae with the rates reported in the literature by other studies.
Figure 2.4. Top panel: representative $k_{fr}$ protocol. Bottom panel: representative $k_{fr}$ trace. This is a zoom-out of the red inset in the top panel, showing the slack, holding time and the redeveloped force.
**Standard solutions.** The solutions for skinned trabeculae experiments were prepared according to a computer program developed by R. Godt (Medical College of Georgia), as previously described (Rall & Wahr. 1998, see Solutions, table 2.1). 2 liter batches of pCa 9.0 and 4.0 were made from stocks, aliquoted in 5 ml tubes and kept at -80°C. From these stock solutions, varied amounts of pCa 9.0 and 4.0 solutions were thawed and mixed to make solutions with intermediate Ca\(^{2+}\) concentrations (intermediate pCa), which were used within one week (see Table 2.2). This allowed us the same buffer supply necessary to complete a set of experiments and minimized any variability in the solution composition. All added Pi solutions were made fresh daily at the beginning of the experiments in 1 ml pCa solution from a 0.5 M Pi stock (from potassium phosphate, monobasic, anhydrous, purchased from Sigma).

**Electron micrographs of rat skinned cardiac trabeculae.** Rat skinned cardiac trabeculae were isolated from the right ventricle and skinned overnight in a relaxing solution with 1% Triton-X. The trabeculae were attached to a force transducer and a stationary arm and the force production was measured at saturating levels of Ca\(^{2+}\) activation (pCa 4.0). The force per cross-sectional area was estimated for individual trabecula by averaging three Ca\(^{2+}\) activations at pCa 4.0. Samples were prefixed for three hours with cold 3.0% glutaraldehyde, 80 mM sodium phosphate buffer, 200 mM sucrose, 2 mM sodium azide, pH 7.3. The samples were washed three times with 50 mM K-phosphate buffer pH 7.3 for 10 minutes intervals to remove glutaraldehyde. Some of the samples were
observed under a Nikon inverted microscope with dark field illumination. Light micrographs were also taken. Post-fixation was done with 1% osmic acid (in H₂O) in ice overnight (~8 hrs). After dehydration in ethyl alcohol in series of increasing concentrations (15, 35, 50, 70, 90, 100% x3), and 100 % x3 acetone treatment, the samples were infiltrated with epon resin. The final treatment of the epon mixture was obtained by incubating the trabecula again with the same epon resin for 3 more days at 70°C. After the polymerization of the epon resin, the samples were cut into ultrathin sections (500-700 Å thickness) with an Ultratome Nova (LKB Bromma, Sweden) by using a diamond knife. Sections were double-stained with 1% aqueous uranyl acetate solution and lead citrate. Electron micrographs were obtained with a Phillip’s 300-transmission electron microscope at accelerating voltage of 60 kV. Photographs were taken on Kodak electron image films.

**Statistical analysis.** We determined the statistical significance by applying an unpaired two-sample t-test or a paired t-test (for Pi studies) using the statistical analysis software Minitab (State College, PA). The statistical significance was established at a p-value ≤ 0.05. All data is shown as a mean ± SEM.
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Table 2.1. **Standard solutions for skinned trabeculae experiments.** Concentrations are given in mM. Ionic strength =180 mM, pH=7.0 at 15°C. Free [Mg²⁺]= 1 mM, [Mg·ATP]= 4.4 mM.
Table 2.2. pCa mixing table.
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CHAPTER 3

CHARACTERIZATION OF RAT SKINNED CARDIAC TRABECULAE
PREPARATION

*Characterization of cardiac muscle sensitivity of force development.* The steady-state relationship between Ca\(^{2+}\) activation and force production has been fit with the Hill equation, which was originally used to describe the binding of O\(_2\) to hemoglobin. As many investigators report [Ca\(^{2+}\)] as pCa = -log [Ca\(^{2+}\)], the form of Hill equation to describe the force development as a function of [Ca\(^{2+}\)] becomes:

\[
 F = \min + \frac{(\max - \min)}{1 + \exp(-K^* (X - X_{50}))}
\]

In this equation, \(\min\) and \(\max\) represent the minimum and maximum force (0 and 1, respectively), \(K\) is a measure of the Hill coefficient and \(X_{50}\) is a measure of pCa\(_{50}\).

The pCa\(_{50}\) represents the [Ca\(^{2+}\)] at which the muscle develops half of the maximal force at saturating levels of Ca\(^{2+}\) and it is a measure of the Ca\(^{2+}\) sensitivity of activation. In the past years, investigators have developed and characterized many cardiac preparations, such as trabeculae, strips of papillary muscle, strips of free ventricular wall, isolated myocytes and isolated
cardiac myofibrils from various species (most common: rat, mouse, rabbit, dogs and pigs). In our laboratory, the cardiac preparation we developed is rat skinned cardiac trabeculae. Cardiac trabeculae have been isolated from the right ventricle from a region located under the tricuspid valve and towards the cardiac apex. This particular region has been chosen because in that location, there are usually 3-4 long, un-branched, medium–size trabeculae which are easy to isolate. The trabeculae in the left ventricle are usually shorter, branched and more often continue as ridges, going into the ventricular wall. We followed the protocol of dissection and isolation of trabeculae as described by Janssen and de Tombe (Janssen & de Tombe. 1997). The range of Ca\(^{2+}\) sensitivities of force development for various cardiac preparations from different species is summarized in Table 3.1. The pCa\(_{50}\) reported by our laboratory in rat skinned cardiac trabeculae is 5.70 ± 0.02 (n=30, un-extracted trabeculae) and it is comparable with pCa\(_{50}\)s reported by other laboratories (see Table 3.1). The Hill coefficient (represented by n in the Hill equation) is considered to provide an estimation of the minimum number of binding sites involved in the regulation of force, and in cardiac muscle it is typically ≥ 3. This apparent discrepancy between the number of Ca\(^{2+}\) binding sites in regulatory domain of TnC (one site) and the Hill coefficient suggests that the activation process is a cooperative process, which could be summarized by the cooperativity of Ca\(^{2+}\) binding to thin filament, crossbridge–induced increases in Ca\(^{2+}\) binding to TnC and/or crossbridge recruitment by strongly bound crossbridges. The Hill coefficient was reported to vary in cardiac muscle, over a broad range, from 2 to 9 (Gordon et al.
In this study, for an average of 30 un-extracted trabeculae, the Hill coefficient was $5.25 \pm 0.35$. Compared with previous experience from skeletal muscle experiments, the cardiac muscle preparation exhibits higher variability in terms of F/CSA and Ca$^{2+}$ sensitivity of force development.

In this study, 120 trabeculae have been used to characterize the force-pCa relationships for un-extracted trabeculae and TnC$^{F27W}$ mutants. A total of 90 trabeculae have been reconstituted with TnC$^{F27W}$ and its mutants. The cutoff for force rundown during the experiments was set at 25%, in accordance with the cutoff reported by other studies (Palmer & Kentish. 1998). All the trabeculae included in the study met this criterion, while for some fibers the rundown was minimal. The average dimensions of the fibers were: width = $138 \pm 3$ μm, depth = $138 \pm 3$ μm and length $1.5 \pm 0.5$ mm. There was a ~3.5-fold change variation in width, a ~6.3-fold change variation in depth and a ~5-fold change variation in length for all the trabeculae (see Table 3.2). There was over a 10-fold variation in F/CSA among all the trabeculae included in the study. The origin of this variation is not considered in the literature. There was no correlation between the F/CSA vs. the fiber length, width or depth or the resting tension (see Figure 3.1 and Figure 3.2). In skeletal muscle, three fibers are usually the typical sample size used for a set of experiments. However, in cardiac muscle, the minimal sample size was five or six trabeculae, in some cases, due to the variability in the force-pCa relationship, up to ten or eleven trabeculae were studied.

Light microscopy images of two skinned, un-extracted trabeculae manifesting a high F/CSA (59.7 mN/mm$^2$) and a low F/CSA (9.6 mN/mm$^2$)
suggest that the striated pattern is preserved throughout the preparation and no significant differences exist (see Figure 3.3). Also, the electron microscopy images of skinned, un-extracted trabeculae showed that the sarcomere structure was preserved throughout the preparation, in spite of a ~5-fold difference in F/CSA (M.Yamaguchi, personal communication; see Figure 3.4). We think that this variation in force might be due to the heterogeneity of the trabecula, as a multicellular preparation. Many factors can account for this variability of force production: the collagen content of the extracellular matrix, the orientation of the myocytes relative to the direction of force measurement, the relative ratio of myocytes vs. connective tissue content and the mitochondria content and/or the end-compliance due to the attachment in the experimental apparatus.

**Skinning striated muscle with Triton-X.** The skinned muscle preparation provides valuable information about the mechanism of Ca\(^{2+}\) activation of muscle contraction. The preparation allows free access to the contractile machinery of the muscle cell and the manipulation of activating solutions, in order to mimic the pathological and pathophysiologival states of activation. Triton-X has been traditionally used as the preferential detergent to destabilize the sarcolemma and the network of sarcoplasmic reticulum (for review, see Gordon, et al. 2000). Nevertheless, this protocol raised concerns about altering the sarcomeric structure and possible interfering with the functional properties of the muscle.
Figure 3.1. F/CSA vs. trabeculae depth or width (n=120).
Figure 3.2. F/CSA vs. trabeculae length or resting tension (n=120).
Figure 3.3. Light micrograph of rat skinned cardiac trabeculae. Magnification: 800X. Top panel: F/CSA=59.7 mN/mm$^2$. Bottom panel: F/CSA=9.6 mN/mm$^2$. 
Figure 3.4. Electron micrograph of rat skinned cardiac trabeculae. Magnification: 16,400X. Top panel: F/CSA=59.7 mN/mm$^2$. Bottom panel: F/CSA= 13.7 mN/mm$^2$. 
Table 3.1. Summary of pCa_{50} in various skinned cardiac preparations from different species, as reported in the literature.
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</tr>
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<td>Length (μm)</td>
<td>1497 ± 47</td>
<td>672 -3360</td>
<td></td>
</tr>
<tr>
<td>Width (μm)</td>
<td>138.0± 3.0</td>
<td>67.2 –201.6</td>
<td></td>
</tr>
<tr>
<td>Depth (μm)</td>
<td>138.0 ± 3.0</td>
<td>37.2 –235.2</td>
<td></td>
</tr>
<tr>
<td>% Resting tension</td>
<td>5.6 ± 0.4</td>
<td>0.5 -19.5</td>
<td></td>
</tr>
<tr>
<td>% Rundown</td>
<td>5.5 ± 0.6</td>
<td>0 - 25</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Summary of trabeculae F/CSA, trabeculae size, resting tension and rundown.
The “skinning” procedure was proven to be effective in skeletal muscle and also in cardiac muscle, even though the “skinning” time (exposure of the preparation to Triton-X) varies among different investigators, as short as 30 min (Fitzsimons, et al. 2001) and as long as 24 h (Olsson, et al. 2004).

Figure 3.5 shows the structure of an intact cardiac trabecula, which can be recognized by the abundance of mitochondria. By comparison, electron micrographs of cardiac preparations after skinning with Triton-X suggest that the mitochondria are absent, the sarcomere integrity is preserved (see Figure 3.4) but some disorganization might occur at the Z-lines (see Figure 3.6). The Z-lines of striated muscle connect thin filaments of the adjacent sarcomeres and play an important role in transmitting the tension developed upon the interaction of the thin and thick filaments within a sarcomere to the neighboring sarcomeres.

The main component of the Z-line is α-actinin, which is organized in filaments forming multiple layer lattices. The Z-line represents also a structural support for many proteins of the cytoskeleton and the morphology of the Z-line encompasses a plethora of proteins, whose functions are not elucidated yet (for review, see Pyle & Solaro. 2004). Triton-X treatment of the frog muscle fibers was associated with the retention of the native structure of the preparation, as revealed by the X-ray diffraction pattern (Magid & Reedy. 1980). The same study showed that swelling of the fibers has been observed either by manual skinning or chemical skinning, but the swelling has been restored by adding high molecular weight colloids (such as dextran). This observation suggested that the swelling was secondary to altering intracellular solution composition.
**Figure 3.5. Electron micrograph of intact cardiac trabeculae** (magnification 24,400X) showing the nucleus, t-tubules, sarcoplasmic reticulum (SR) and mitochondria.
The lattice spacing has been preserved, but the volume-sarcomere length ratio might have been changed and therefore a disorganization of the thin filament has been noticed (Magid & Reedy. 1980). The lipid content of the Z-discs in the skeletal muscle is considered distinct from the lipids from the sarcoplasmic reticulum and the mitochondria. The lipids might help stabilizing neighbouring Z-filaments, reinforce the Z-disc structure, and play a role in the force transmission of skeletal muscle myofibrils. The main lipids constituents of the Z-discs are phospholipids, triacylglycerols, cholesterol and free fatty acids. These are extracted from the Z-discs treated with Triton-X, but nevertheless intact configurations of Z-filaments composed of alpha-actinin were clearly visible under an electron microscope (Takahashi, et al. 2001).

**TnC extraction and reconstitution.** TnC was extracted from striated muscle using different extraction protocols. In skeletal muscle, TnC extraction using a solution of 5 mM EDTA as a Ca\(^{2+}\) chelator and 10 mM imidazole at pH 7.2 was reported to alter the structure of the Z-line (M. Yamaguchi, personal communication). Since α-actinin contains two EF-hands in the C-domain, similar to the EF-hand domains present in the TnC structure, it was proposed that the TnC extraction protocol might alter the Z-line structure by extracting α-actinin also.
Figure 3.6. Z-line on electron micrograph image (magnification 76,400X) in skinned cardiac trabeculae. Some variable degree of Z-line disorganization is present.
In our extraction protocol we used a different compound to extract TnC. Trifluoperazine (TFP) is a hydrophobic compound, which can diffuse in the cardiac trabeculae and destabilize the complex hydrophobic interactions between TnC and the other proteins anchoring TnC on the thin filament. The extraction solution contained 10 mM HEPES, 5 mM EDTA and 0.5 mM TFP, pH 7.0. The re-addition of TnC to the extracted trabeculae restored the force to ~60-80% of the pre-extraction force. This force recovery upon TnC reconstitution is in agreement with the reconstitution reported in other studies (for review, see Gordon et al., 2000). The partial recovery of force post-extraction can be due to incomplete and/or non-functional incorporation of TnC in the troponin complex, damage to the preparation during the extraction-reconstitution protocol, and/or extraction of other myofilament proteins.

The TnC extraction seems to occur preferentially at certain regions along the thin filament. Swartz, et al. 1997 showed that TnC extraction is not random along the length of the thin filament, but occurs mostly in the non-overlap region. Whole troponin exchange studies showed that troponin C incorporates along the thin filament in both overlap and non-overlap region, depending on the states of activation of the thin filament. (Swartz, et al. 2006). Moreover, our previous study in rabbit skinned psoas muscle showed that TnC mutants which did not support force were able to bind to the thin filament (Davis, et al. 2004). Additional exchange experiments showed that, when a fiber reconstituted with a TnC mutant which recovered force was soaked in a solution with a TnC mutant which did not recover force, the subsequent levels of force decreased in time. This
decrease in force was due to TnC replacement of the previously reconstituted TnC mutant. Vice-versa, replacement of a non-force generating TnC mutant with a force-generating TnC mutant resulted in an increase in the levels of force production with time (Davis et al., 2004).

The TnC mutants did not support force because their interaction with TnI was altered and, therefore, the inter-communication among the subunits of the troponin complex was perturbed, such as the Ca$^{2+}$ signaling pathway was not transmitted along the thin filament. Moreover, a fluorescent probe, IAEDANS, was attached to TnC mutants and their fluorescence observed upon TnC reconstitution into psoas myofibrils. According to the fluorescent images, IAEDANS-labeled TnC proteins incorporate into the myofibril at the myosin-actin filament overlap and non-overlap space (Davis et al., 2004). Overall, these studies suggest that TnC incorporation does occur along the thin filament.
CHAPTER 4

EFFECT OF ALTERING THE CA$^{2+}$ BINDING AFFINITY OF TROPONIN C ON THE CARDIAC MUSCLE SENSITIVITY OF FORCE DEVELOPMENT

**Cardiac TnC.** TnC is a part of the EF-hand Ca$^{2+}$-binding proteins family. It is shaped as a dumbbell, with N- and C-terminal globular domains connected by a flexible $\alpha$-helical linker (for review, see Finley et al, 2004). Each N- and C-domain contains Ca$^{2+}$ and Mg$^{2+}$ binding motifs, known as EF-hands. The EF-hand or helix-loop-helix motif consists of two flanking helices with an intervening 12-residue loop capable of binding either Ca$^{2+}$ or Mg$^{2+}$ via pentagonal bipyramidal (seven) or octahedral (six) coordination, respectively. Calcium is coordinated via oxygen ligands from highly conserved amino acid residues at positions 1(X), 3(Y), 5(Z), 7(-Y), 9(-X), and 12(-Z).

Troponin C can undergo different conformational changes when it binds Ca$^{2+}$. In skeletal muscle, the Ca$^{2+}$ binding to site I and site II of the regulatory domain of sTnC triggers a conformational change in the structure of the N-domain, which is stabilized in an open conformation. This open state is achieved by movement of the B and C helices away from the
N, A and D helices. Cardiac TnC has a 70% homology with the skeletal TnC. The site I in cardiac TnC is inactivated, and upon Ca\textsuperscript{2+} binding to site II, the N-domain of cTnC does not assume the open conformation as much as the skeletal counterpart does. Therefore, Ca\textsuperscript{2+}-bound cTnC is in nearly closed conformation, almost identical with the conformation of sTnC with no bound Ca\textsuperscript{2+}. The cTnC assumes the open conformation only in the presence of the switch peptide of Tnl. The additional driving force provided by Ca\textsuperscript{2+} binding to the site I of sTnC probably determines this difference between sTnC and cTnC activation.

The TnC in the troponin complex forms multiple interactions with Tnl and TnT. A recent crystal structure provided detailed information about these interactions (Takeda et al, 2003). The core domain of the troponin complex can be divided into two subdomains, the regulatory head and the IT arm (TnT-Tnl-C-TnC) which are connected by flexible linkers. The C lobe of TnC is integrated into the IT arm in the core Tn structure. The regulatory head consists of the N-domain of TnC and the switch region of Tnl. The switch region is situated between two actin-binding segments of Tnl (the inhibitory region and the C-terminus), and forms hydrophobic interactions with the hydrophobic patch on the surface of the “open” N-domain of TnC (Takeda et al, 2003).

After Ca\textsuperscript{2+} binds to the N-domain of TnC, the switch region of Tnl binds to the hydrophobic patch on the N-domain of TnC and it comes off actin, releasing the inhibition of myosin binding sites on actin. This event triggers complex conformational changes in the troponin complex and on actin, which initiates tropomyosin movement and allows myosin binding to actin and force
development. As a reverse process, a decrease in the intracellular Ca$^{2+}$ leads to dissociation of the switch region of TnI from the hydrophobic cleft of the N-domain of TnC, resulting in muscle relaxation (Li et al., 2004).

TnC Ca$^{2+}$ binding properties have been extensively investigated. A main approach is to design TnC mutants by substitution of selected amino acid residues in specific locations within the tertiary structure of the protein. In both skeletal and cardiac TnC, mutating the amino acids in the coordinating loop (Babu, et al. 1992, Gulati, et al. 1992) and also hydrophobic residues outside the loop, in the N-lobe, drastically change the Ca$^{2+}$ binding properties of TnC (Davis, et al. 2004, Gulati, et al. 1992, Hannon, et al. 1993, Tikunova & Davis. 2004, Tikunova, et al. 2002). The Ca$^{2+}$ binding properties can be characterized biochemically by titrating Ca$^{2+}$ binding to TnC to calculate the $k_d$. The rate of Ca$^{2+}$ dissociation from TnC can be measured using a stopped-flow technique (Johnson, et al.1979). To be able to characterize Ca$^{2+}$ binding and exchange with the regulatory domain of TnC, intrinsic fluorescent probes often are engineered to report the fluorescence changes upon Ca$^{2+}$ binding to TnC. In cardiac muscle, a Trp has been engineered at position 27 in the N-lobe of TnC, rendering TnC$^{F27W}$. This mutation is analogous to sTnC$^{F29W}$, which was used in previous skeletal muscle studies to investigate the Ca$^{2+}$ binding properties of skeletal TnC. By mutating hydrophobic residues with polar Gln in skeletal muscle, the Ca$^{2+}$ affinity of the regulatory domain of TnC$^{F29W}$ could increase or decrease, depending on the location of the residue within the tertiary structure of the protein.
Summary of TnC mutants Ca\(^{2+}\) binding properties in isolation and in the troponin complex.

**Ca\(^{2+}\) binding properties of troponin C mutants in isolation.** A series of cardiac TnC mutants have been designed in our laboratory (by JP Davis and SB Tikunova) to increase Ca\(^{2+}\) sensitivity of the regulatory N-domain, by individually mutating the hydrophobic residues Phe\(^{20}\), Val\(^{44}\), Met\(^{45}\), Leu\(^{48}\) and Met\(^{81}\) to polar Gln (Tikunova & Davis, 2004). All of these hydrophobic residues (with exception of Leu\(^{48}\)) are almost completely buried in the absence and presence of Ca\(^{2+}\), with their side chains involved in extensive hydrophobic interactions between helices B and C and helices N, A and D. The five TnC\(^{F27W}\) mutants studied in the present work possessed 2.1–15.2-fold higher Ca\(^{2+}\) affinities but only 1.2–2.9-fold slower Ca\(^{2+}\) dissociation rates (Tikunova & Davis, 2004). Calculation of the Ca\(^{2+}\) association rate using the equation \(k_{\text{on}}(\text{Ca}) = k_{\text{off}}(\text{Ca})/K_d(\text{Ca})\) suggested that the Ca\(^{2+}\)-sensitizing mutations should cause an increase in the Ca\(^{2+}\) association rates to the N-domain of cTnC\(^{F27W}\). Therefore, TnC\(^{F27W}\) mutants exhibited a 2.6–8.7-fold increase in Ca\(^{2+}\) association rate. All five mutants sensitized isolated TnC to Ca\(^{2+}\). These substitutions of Phe\(^{20}\), Val\(^{44}\), Met\(^{45}\), Leu\(^{48}\) and Met\(^{81}\) with polar Gln increased the Ca\(^{2+}\) affinity of the N-domain of TnC\(^{F27W}\) most likely by facilitating the movement of the BC unit away from the NAD unit, mimicking the effects of TnI binding. The increase in Ca\(^{2+}\) affinity of TnC\(^{F27W}\) in isolation was mainly due to faster Ca\(^{2+}\) association rates, rather than to slower Ca\(^{2+}\) dissociation rates.

**Ca\(^{2+}\) binding properties of troponin C mutants in troponin complex.**

As troponin C functions as a part of the troponin complex, it is important
to elucidate the Ca$^{2+}$ dependent interactions between TnC and TnI/TnT. The binding of TnI or its peptide to the Ca$^{2+}$ saturated N-domain of TnC leads to the loss of the hydrophobic interactions between NAD unit and BC unit, since the BC unit moves away from the NAD unit (Takeda et al, 2003, Sykes et al, 1999). Due to the presence of Trp residues in both TnI and TnT, the F27W substitution was not a good reporter of the fluorescence changes that occur upon Ca$^{2+}$ binding with the regulatory N-domain of TnC reconstituted into the Tn complex. Thus, a new mutation, S149C, has been introduced into the switch region of Cys-less TnI, and labeled with the fluorescent probe IAANS. All the Cys in TnI have been mutated so that the Cys that used to attach the probe was introduced at position 149 by substituting Ser to Cys. The fluorescence of TnI$_{F27W}^{S149C}$IAANS was sensitive to the Ca$^{2+}$ dependent interactions between TnI and the regulatory N-domain of TnC reconstituted into the Tn complex. Despite the fact that all five mutants sensitized isolated TnC to Ca$^{2+}$, only two of the mutations (V44Q and L48Q) led to significantly higher Ca$^{2+}$ sensitivity of the IAANS labeled Tn complexes. Furthermore, while the Ca$^{2+}$ affinity of isolated TnC$_{F27W}^{F27W}$ mutants was increased up to ~15.2-fold, the Ca$^{2+}$ sensitivity of the mutant Tn complexes was enhanced only up to ~2.6-fold. In addition, the F20Q and M45Q mutations, which increased Ca$^{2+}$ affinity of isolated TnC$_{F27W}^{F27W}$, actually produced significant decreases in the Ca$^{2+}$ sensitivity of the Tn complex (JP Davis, abstract, Biophysical Society 2005; JP Davis and SB Tikunova, personal communication). Therefore, individual substitution of Phe$_{20}$, Val$_{44}$, Met$_{45}$, Leu$_{48}$ and Met$_{81}$ with polar Gln increased the Ca$^{2+}$ affinity of the N-domain of TnC$_{F27W}^{F27W}$, most likely by facilitating the
movement of the BC unit away from the NAD unit, mimicking effects of TnI binding. The increase in Ca$^{2+}$ affinity was due to changes in both Ca$^{2+}$ association and Ca$^{2+}$ dissociation rates. Thus, mutations that sensitize the isolated TnC to Ca$^{2+}$ do not necessarily increase the Ca$^{2+}$ sensitivity of the Tn complex. The five unlabeled mutant Tn complexes had up to ~8.1-fold slower Ca$^{2+}$ dissociation rates, compared to that of TnC$^{F27W}$-Tn (see Table 4.1), a change in the dissociation rate much bigger than the change measured in TnC mutants in isolation (1.2–2.9-fold). Thus, mutations of hydrophobic residues had a larger effect on the rates of Ca$^{2+}$ dissociation from the Tn complex than observed from isolated TnC$^{F27W}$.

**Summary of Ca$^{2+}$ sensitivity of force development of rat skinned cardiac trabeculae reconstituted with the TnC mutants.** Trabeculae were extracted of endogenous TnC and then reconstituted with TnC$^{F27W}$ or its mutants. For un-extracted cardiac trabeculae, half-maximal force occurred at a pCa$_{50}$ of 5.64 ± 0.02, with a Hill coefficient of 4.7 ± 0.6 (see Table 4.2 and Figure 4.1). After TnC extraction, the average force generated by the trabeculae was 3.1 ± 0.5% (n=46) of the maximal pre-extraction force. After reconstitution of the trabeculae with TnC$^{F27W}$ or its mutants, force recovered to values higher than 60% of the maximal pre-extraction force at pCa 4.0 (Table 4.2). Trabeculae reconstituted with TnC$^{F27W}$ had a slightly higher Ca$^{2+}$ sensitivity of force development compared with the un-extracted trabeculae (Figure 4.1).

The Ca$^{2+}$ sensitivity of force production for all the other TnC$^{F27W}$ mutants was compared with TnC$^{F27W}$, which was the control in this study (see
Figure 4.2, Figure 4.3 and Table 4.2). For the rest of the mutants, the Ca$^{2+}$ sensitivities of force development ranged from pCa$_{50}$ of 5.54 ± 0.05 (with a Hill coefficient of 3.1 ± 0.4) for F20QTnC$^{F27W}$ to 5.92 ± 0.05 (with a Hill coefficient of 3.9 ± 0.4) for V44QTnC$^{F27W}$. The V44QTnC$^{F27W}$ and F20QTnC$^{F27W}$ mutations manifested the highest increase and decrease, respectively, in the Ca$^{2+}$ sensitivity of force development.

<table>
<thead>
<tr>
<th>Mutant Protein</th>
<th>pCa$_{50}$</th>
<th>Hill Coefficient</th>
<th>k$_{off(Ca)}$ Quin-2, s$^{-1}$</th>
<th>k$_{off}$ IAANS, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnC$^{F27W}$</td>
<td>6.22 ± 0.02</td>
<td>1.01 ± 0.03</td>
<td>30.8 ± 0.2</td>
<td>32.1 ± 0.2</td>
</tr>
<tr>
<td>F20QTnC$^{F27W}$</td>
<td>5.75 ± 0.02</td>
<td>1.43 ± 0.05</td>
<td>28.6 ± 0.5</td>
<td>37.6 ± 0.5</td>
</tr>
<tr>
<td>V44QTnC$^{F27W}$</td>
<td>6.62 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td>6.1 ± 0.1</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>M45QTnC$^{F27W}$</td>
<td>6.12 ± 0.01</td>
<td>0.85 ± 0.02</td>
<td>10.1 ± 0.2</td>
<td>17.4 ± 0.1</td>
</tr>
<tr>
<td>L48QTnC$^{F27W}$</td>
<td>6.64 ± 0.01</td>
<td>0.85 ± 0.01</td>
<td>3.8 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>M81QTnC$^{F27W}$</td>
<td>6.29 ± 0.03</td>
<td>1.00 ± 0.06</td>
<td>19.8 ± 0.4</td>
<td>18.1 ± 0.3</td>
</tr>
</tbody>
</table>

Table 4.1. Effect of TnC mutations on Ca$^{2+}$ binding properties of labeled (reported by IAANS) and unlabeled (reported by Quin 2) Tn complexes (From Tikunova SB and Davis JP, unpublished data).
Two other mutations, L48QTnC^{F27W} and M81QTnC^{F27W} were not significantly different from control. Therefore, mutations that sensitized isolated TnC to Ca^{2+} (Tikunova & Davis, 2004) do not necessarily increase the Ca^{2+} sensitivity of force development. In fact, the F20Q mutation desensitized both the Tn complex and cardiac trabeculae to Ca^{2+}. Furthermore, the M81Q mutation that sensitized isolated TnC to Ca^{2+} did not significantly affect the Ca^{2+} sensitivity of the Tn complex and muscle force development (see Figure 4.3). While the V44Q mutation sensitized the isolated TnC, the Tn complex and cardiac muscle to Ca^{2+}, the extent of sensitization was blunted in the Tn complex (~2.6-fold increase) and even more so in muscle (~1.5-fold increase), compared to isolated TnC (~15.2-fold increase, data not shown). Thus, in some cases, Tn complexes were better than isolated TnCs in predicting how modulation of Ca^{2+} binding to TnC affects the Ca^{2+} dependence of force production. However, the effect of the mutations on the Ca^{2+} sensitivity of force generation could not always be predicted from the Ca^{2+} sensitivities of the Tn complexes. For instance, the M45Q mutation caused a decrease in the Ca^{2+} sensitivity of the Tn complex, but an increase in the Ca^{2+} sensitivity of force development, while the L48Q mutation caused an increase in the Ca^{2+} sensitivity of the Tn complex, but did not affect the Ca^{2+} sensitivity of force development (see Figure 4.3).

**Interpretation of the results - significance and further directions.** One of the most important events in the regulation of muscle contraction is the Ca^{2+}-dependent interaction between the troponin subunits, which triggers a series of conformational transitions among the thin filament proteins that activate the
thin filament and facilitate actin-myosin binding. A model of the thin filament regulation of muscle contraction describes three states of the thin filament, blocked (B-state), closed (C-state) and open (M-state), which reflect various degrees of interaction between actin and the myosin head (McKillop & Geeves, 1993). In the B-state, present when Ca$^{2+}$ levels are very low such that N-domain of TnC is not binding Ca$^{2+}$, TnI blocks the interaction of myosin heads with actin. Two TnI actin-binding domains have been described (Takeda, et al. 2003). In the B-state, these domains establish extensive interactions with actin. When Ca$^{2+}$ binds to TnC, a hydrophobic patch is exposed in the N-terminal domain, and particularly in the case of cardiac TnC, this structural change requires TnI binding (Li et al. 1999). This hydrophobic patch interacts with the regulatory region of TnI, which weakens the interaction of the C-terminal actin-binding site of TnI with actin. This new state is called the C-state, because it is triggered by Ca$^{2+}$ binding to TnC. According to structural studies, this is equivalent to tropomyosin movement along the groove of the actin strand, which allows the myosin heads to form weak attachments with actin. In the C-state, only weak–binding, non-force generating crossbridges are formed. In the M-state, strong–binding, force–generating crossbridges are formed, which induce further movement of tropomyosin down the actin groove.

The interactions between the troponin subunits, tropomyosin and actin ultimately influence the state of activation of the thin filament by facilitating transitions among the three states described.
<table>
<thead>
<tr>
<th>Mutant protein</th>
<th>pCa$_{50}$</th>
<th>Hill coefficient</th>
<th>% Force recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnC$^{\text{endog}}$</td>
<td>5.64 ± 0.02</td>
<td>4.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>TnC$^{F27W}$</td>
<td>5.73 ± 0.01</td>
<td>3.8 ± 0.4</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>F20QTnC$^{F27W}$</td>
<td>5.54 ± 0.05</td>
<td>3.1 ± 0.4</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>V44QTnC$^{F27W}$</td>
<td>5.92 ± 0.05</td>
<td>3.9 ± 0.4</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>M45QTnC$^{F27W}$</td>
<td>5.85 ± 0.02</td>
<td>3.0 ± 0.4</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>L48QTnC$^{F27W}$</td>
<td>5.74 ± 0.03</td>
<td>3.2 ± 0.5</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>M81QTnC$^{F27W}$</td>
<td>5.80 ± 0.03</td>
<td>2.7 ± 0.2</td>
<td>66 ± 2</td>
</tr>
</tbody>
</table>

Table 4.2: Effect of TnC mutations on the Ca$^{2+}$ sensitivity of force development (pCa$_{50}$), Hill Coefficient, and % force recovery in skinned cardiac trabeculae. n= 6-10 for each mutant.
Figure 4.1. Force vs pCa for un-extracted trabeculae and TnC$^{F27W}$ reconstituted trabeculae. Un-extracted trabeculae are shown in black (as TnC$^{endog}$ traces) and TnC$^{F27W}$ reconstituted trabeculae are shown in blue.
Figure 4.2. Force vs pCa for TnC$^{F27W}$, V44QTnC$^{F27W}$ and F20QTnC$^{F27W}$ reconstituted trabeculae. TnC$^{F27W}$ is shown in blue, V44QTnC$^{F27W}$ in red and F20QTnC$^{F27W}$ in green.
Figure 4.3. Force vs pCa for TnC$^{F27W}$, M81QQTnC$^{F27W}$, M45QTnC$^{F27W}$ and L48QTnC$^{F27W}$ reconstituted trabeculae. TnC$^{F27W}$ is shown in blue, M81QTnC$^{F27W}$ in orange, M45QTnC$^{F27W}$ in cyan and L48QTnC$^{F27W}$ in magenta.
This model of activation takes into consideration that muscle force production is a comprehensive process, which requires not only Ca\(^{2+}\) binding to TnC to initiate the transition from the B-state to the C-state, but also myosin heads binding to further activate the thin filament by favoring the transition from the C-state to the M-state. This model of regulation of cardiac muscle contraction can explain the discrepancies between the observed Ca\(^{2+}\)-binding properties of TnC mutants in isolation and in the troponin complex and the muscle Ca\(^{2+}\) sensitivity of force production.

According to a recent crystal structure of the human cardiac troponin complex (Takeda, et al. 2003), the five mutated hydrophobic amino acids can account for a significant part of the contact area between TnC and TnI interaction. They are located at the interface of the NAD and BC subunits of TnC in the absence of TnI. The residues in TnI that come in contact with the five residues in TnC mutated in this study are all hydrophobic. Thus, mutating the residues in TnC to polar Gln sensitized the isolated TnC to Ca\(^{2+}\), probably by facilitating the movement of the BC unit away from the NAD unit, thus decreasing the energetic cost of Ca\(^{2+}\) binding. Moreover, mutating hydrophobic amino acids to polar amino acids would destabilize the hydrophobic interaction between TnC and TnI, thus decreasing the affinity of N-domain of TnC for the regulatory domain of TnI. Nevertheless, the TnI-TnC interaction in the troponin complex cannot solely predict the sensitivity of force production. This happens because the troponin complex is an incomplete model to study the thin filament regulation of cardiac muscle contraction, as it does not take into account the M-
state of activation and the transition between the states of activation described as B-, C-, and M-states.

The results suggest that mutations in the regulatory domain of TnC alter the TnC-TnI interaction, which triggers conformational changes in TnT-TnI, TnT-actin and TnI-actin interactions, ultimately affecting the equilibrium of the thin filament differently. This relates to the transition of the thin filament from the “off” states (B- and C-states) to the “on” states (M-states), which happens when the TnI inhibition is released upon binding of the switch region of TnI to TnC. The increased or decreased muscle sensitivity of force production reflects transitions of the thin filament from the B-and C-states to M-states, or from the M-states to C- and B-states, respectively. For example, TnC mutations that, upon Ca$^{2+}$ binding, induce conformational changes that destabilize the B-state and favor the C-state would sensitize the cardiac muscle to Ca$^{2+}$.

The molecular basis for the protein-protein interactions critical to the regulation of muscle force production is not entirely elucidated. Ultimately, myofilaments can be sensitized to Ca$^{2+}$ by enhancing the Ca$^{2+}$ affinity of the regulatory site of cardiac troponin and / or by enhancing myosin binding to actin. These two processes are inter-dependent, as different studies showed that myosin binding to the thin filament increases the Ca$^{2+}$ binding affinity to the regulatory site of cardiac troponin C (Pan and Solaro, 1987, Hofmann & Fuchs, 1987). Recently, reports have shown that troponin I can be a major player in both the TnC - TnT- actin - tropomyosin- myosin binding pathway (as a forward sequence of events) and the crossbridge feedback on TnC
pathway (as a backward sequence of events). Many cardiomyopathy–related mutations in cardiac troponin I have been identified. A recent study (Kobayashi & Solaro, 2006) suggests that the effect of cardiomyopathy related TnI mutantions on increasing the Ca$^{2+}$ affinity of the thin filaments could be explained by shifting the equilibrium of the thin filament states. Moreover, a region of N-terminal domain of TnI was identified to be responsible for the direct effect of crossbridges on activating the thin filaments (Engel, et al. 2007). According to this study, a localized N-terminal region of TnI (which establishes interaction with the C-terminal region of TnC) is of particular significance in transducing the effect of thin filament activation by strongly attached crossbridges.

As the experimental evidence suggests, TnI establishes extensive molecular interactions with the troponin subunits, actin, and tropomyosin and indirectly with the crossbridges, through alterations in tropomyosin and/or TnT structures (Takeda et al. 2003). These interactions can be studied in a system that can reproduce and mimic closely a physiological system (such as cardiac muscle preparation) but it is yet simple enough that it is able to incorporate basic players in the regulation of cardiac muscle contraction.

As this study suggests, both troponin C in isolation or the troponin complex are reductionist systems, providing incomplete answers to elucidate the modulation of contraction. Ultimately, the answer to how changes in the Ca$^{2+}$ binding properties of TnC can alter the muscle Ca$^{2+}$ sensitivity of force production can be provided by intact or skinned cardiac preparations. Nevertheless, the cardiac muscle preparation allows less insight into the molecular
mechanisms that regulate cardiac contraction. In the recent years, the regulated thin filaments have proven to be a simpler, yet more insightful system which allows determination of the effects of TnI, Tm, actin, and/or myosin on Ca\(^{2+}\) binding and exchange with the regulatory domain of TnC (Davis et al. 2007). This approach allows one to better relate the physiological function of cardiac TnC to the biochemical behavior, as troponin C functions as a part of the troponin complex under the influence of both Ca\(^{2+}\) binding and crossbridge binding.

The regulated thin filaments represent a valuable tool in monitoring changes in the Ca\(^{2+}\) binding properties of the troponin complexes reconstituted with structural components of the thin and/or thick filament. For example, neither Tm or actin alone affected the Ca\(^{2+}\) binding properties of the troponin complexes, but when the troponin complex was reconstituted with actin and Tm, Ca\(^{2+}\) sensitivity was decreased due to an increase in the Ca\(^{2+}\) dissociation rate from troponin complexes. The attenuation of the Ca\(^{2+}\) sensitizing effects of TnI on TnC in the presence of actin and Tm suggests that TnC and actin compete for binding to the C-terminal domain of TnI. This competition for the TnI binding might be the basis for a reduced Ca\(^{2+}\) sensitivity of the troponin complexes when incorporated into the reconstituted thin filaments. Nevertheless, this mechanism is not solely responsible for explaining changes in the Ca\(^{2+}\) binding properties of the troponin complex. TnI establishes various contacts with actin, TnT, Tm, and even myosin crossbridges (Engel et al. 2007), interactions which can propagate a series of conformational changes and structural re-arrangements at the myosin – actin interface, which ultimately affect the Ca\(^{2+}\) activation of the thin filament.
and re-orient Tm on actin in positions either favoring (C- to M-states) or inhibiting (B-states) crossbridge binding and force development.

The rate of Ca\(^{2+}\) dissociation from TnC can be further decreased by myosin S1 binding to reconstituted thin filaments (Davis et al. 2007), which points out that the activation of the thin filament which subsequently initiates changes in the binding state of the thick filament are not separate processes, but interrelated. A further approach to elucidate the molecular mechanism for each TnC mutant would be to incorporate them in the reconstituted thin filaments and to investigate the Ca\(^{2+}\) binding properties under different conditions (only actin or Tm binding or actin + Tm both in the absence and in the presence of myosin heads). This comprehensive approach would take into account that all the major filament proteins (TnI, TnT, Tm, actin and myosin) can have direct or indirect effects on the Ca\(^{2+}\) binding affinity of TnC.

**Characterization of the Ca\(^{2+}\) sensitivity of force development for the mutation G159D located in C-domain of TnC related with dilated cardiomyopathy.** Dilated cardiomyopathy (DCM) is a disease that causes the heart to become enlarged and weak. For the majority of cases of DCM, the cause is not known. The condition is then termed idiopathic (no identifiable cause). Other identifiable factors which may trigger the development of DCM can be genetic/familial, viral infection, auto-immune diseases, excessive alcohol consumption, exposure to toxic compounds, pregnancy and so on. In some cases, patients present with a familial form of the disease, with mutations in the sarcolemmal, cytoskeletal or sarcomeric proteins.
To date, only two cardiomyopathy-related mutations in TnC have been identified: L29Q (in N-domain of TnC) and G159D (in C-domain of TnC), as shown in Figure 4.4. The L29Q mutation was found in a patient with FHC (familial hypertrophic cardiomyopathy), but the G159D has been linked with the development of DCM. The G159 residue is located in the H-helix of the C-domain of TnC, and it is involved in tight binding of TnI$_{34-71}$. The effects of this mutation on the contractile parameters of the cardiac muscle have not been reported yet by any studies (Li et al. 2004). We studied the Ca$^{2+}$ sensitivity of force development for G159D in rat skinned cardiac trabeculae, using the extraction-reconstitution protocol (see Methods). The percent recovery of force was 80% of the maximum force pre-extraction (n=6). The pCa$_{50}$ for G159DTnC$^{F27W}$ reconstituted trabeculae was 5.69 ± 0.02 (n=6), which was not significantly different from pCa$_{50}$ of the un-extracted trabeculae (5.68 ± 0.01, n=9, Figure 4.9). From this study, we conclude that the G159D mutation itself does not change the Ca$^{2+}$ binding properties of the heart. There must be other mechanisms responsible for the manifestation of the disease, and these mechanisms are still to be elucidated.
Figure 4.4. Force vs pCa for $\text{TnC}^{\text{endog}}$ and $\text{TnC}^{G159D}$ reconstituted trabeculae.
CHAPTER 5

INFLUENCE OF ALTERING THE THIN FILAMENT CALCIUM ACTIVATION
ON THE RATE OF CARDIAC MUSCLE CONTRACTION

Background. In both skinned cardiac and skeletal muscle, it is well established that the rate of contraction, as measures by $k_{fr}$, becomes faster with increasing levels of activation by Ca$^{2+}$ (Campbell. 1997, Chase, et al. 1994, Gordon, et al. 2000, Palmer & Kentish. 1998, Wolff, et al. 1995). Many studies have investigated the role Ca$^{2+}$ plays in the activation dependence of $k_{fr}$ (Gordon, et al. 2000). It has been proposed that the effects of Ca$^{2+}$ on $k_{fr}$ occur either as a direct effect of Ca$^{2+}$ on the crossbridges or indirectly by activating the thin filament, which subsequently allows crossbridges to cycle from non-force generating states to force-generating states. Studies in skeletal muscle investigated the hypothesis that Ca$^{2+}$ has a direct effect on the crossbridge cycle. Caged Pi experiments suggest that Ca$^{2+}$ does not regulate the kinetics of Pi release but rather the distribution of crossbridges between non-force and force- generating states (Millar & Homsher. 1990, Walker, et al. 1992). Similarly, in
vitro motility assays have shown that Ca\textsuperscript{2+}, through binding to TnC, controls the number of crossbridges interacting with actin, rather than directly the rate of ATP hydrolysis or the filament sliding speed (Gordon, et al. 1997, Homsher, et al. 1996). Moreover, Ca\textsuperscript{2+} does not control k\textsubscript{tr} through binding to the regulatory light chains (Metzger & Moss. 1992). Overall, these studies suggest that Ca\textsuperscript{2+} does not influence the rate of contraction through a direct effect on the crossbridge cycling. Alternatively, it was suggested that in skeletal muscle Ca\textsuperscript{2+} influences the rate of contraction by modulating the level of thin filament activation. For instance, calmidazolium sensitizes the muscle to Ca\textsuperscript{2+} by increasing the Ca\textsuperscript{2+} binding affinity of TnC without any direct effect on crossbridges (Regnier, et al. 1996). In the presence of calmidazolium, k\textsubscript{tr} was increased at submaximal Ca\textsuperscript{2+} activation but it was unchanged at maximal activation. Thus, modulating the thin filament Ca\textsuperscript{2+} activation by changing the TnC Ca\textsuperscript{2+} binding properties can ultimately influence the rate of contraction.

In cardiac muscle, interesting results have been observed by measuring on the same preparation the rate of contraction using two protocols, k\textsubscript{tr} and k\textsubscript{Ca}. The k\textsubscript{Ca} protocol measures the rate of contraction by using caged Ca\textsuperscript{2+} compounds to rapidly activate the thin filament (Rall & Wahr, 1998). It is considered that the rate of contraction estimated by k\textsubscript{tr} is a measure of crossbridge cycling whereas the rate of contraction assessed by k\textsubscript{Ca} is influenced by both thin filament activation and the rate of crossbridge cycling. One study did not find any difference between k\textsubscript{tr} and k\textsubscript{Ca} and concluded that the activation rate
is determined solely by the kinetics of crossbridge cycling (Palmer & Kentish, 1998). Another study reported that the rate of contraction measured by $k_{Ca}$ was slower than $k_{tr}$ (Regnier et al, 2004). This study suggested that the slower rate of contraction during $k_{Ca}$ protocol results from the dynamics of Ca$^{2+}$ binding and activating the thin filament, especially at low Ca$^{2+}$ concentrations, in addition to the kinetics of the crossbridge cycling. Nevertheless, both studies indirectly estimated the role of thin filament activation on the rate of cardiac contraction.

Our novel approach was to directly change the level of thin filament Ca$^{2+}$ activation by incorporating into rat skinned cardiac trabeculae TnC mutants with varied Ca$^{2+}$ binding affinities. We assessed the rate of contraction as measured by $k_{tr}$. Our hypothesis is that directly increasing or decreasing the Ca$^{2+}$ sensitivity of the thin filament would increase or decrease the Ca$^{2+}$ sensitivity of force and $k_{tr}$. As described in chapter 4, the V44QTnC$^{F27W}$ and the F20QTnC$^{F27W}$ mutations increased and decreased, respectively, the Ca$^{2+}$ sensitivity of steady-state force development. Thus, V44QTnC$^{F27W}$ and F20QTnC$^{F27W}$ were used to increase and decrease the level of thin filament activation, respectively.

Effect of TnC mutants on Ca$^{2+}$-sensitivity of force development. Rat skinned cardiac trabeculae were reconstituted with TnC mutants V44QTnC$^{F27W}$ or F20QTnC$^{F27W}$. For the control TnC$^{F27W}$, V44QTnC$^{F27W}$ and F20QTnC$^{F27W}$ in the experiments with no added Pi, the percent recovery of force was 86% ± 7, 68% ± 5 and 66% ± 9, respectively (n=6). Compared to TnC$^{endog}$, the TnC$^{F27W}$ mutant increased the Ca$^{2+}$ sensitivity of force development ~1.3-fold (see Figure
5.1). In agreement with the Ca\(^{2+}\) binding affinities of the TnC-TnI complex, V44QTnC\(^{F27W}\) and F20QTnC\(^{F27W}\) increased ~1.6-fold and decreased ~1.5-fold respectively the Ca\(^{2+}\) sensitivity of force development (pCa\(_{50}\) = 6.00 ± 0.02 and pCa\(_{50}\) = 5.61 ± 0.03) compared with control TnC\(^{F27W}\) (pCa\(_{50}\) = 5.80 ± 0.03, Figure 5.2). Thus, it was possible to sensitize or desensitize cardiac muscle to Ca\(^{2+}\) upon incorporating TnC mutants with different Ca\(^{2+}\) binding affinities into skinned cardiac trabeculae.

**Figure 5.1.** Force vs. pCa for un-extracted trabeculae and TnC\(^{F27W}\) reconstituted trabeculae. Un-extracted trabeculae are shown in black (as TnC\(^{endog}\) traces) and TnC\(^{F27W}\) reconstituted trabeculae are shown in blue (as TnC\(^{F27W}\) traces). pCa\(_{50}\) for TnC\(^{endog}\) : 5.70 ± 0.03, pCa\(_{50}\) for TnC\(^{F27W}\) : 5.80 ± 0.03, n≥5.
Figure 5.2. Force vs. pCa for TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae. TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae are shown in blue, red and green, respectively. (n=6-10).
**Effect of Ca\(^{2+}\) on \(k_{\text{tr}}\).** \(k_{\text{tr}}\) became progressively faster as the level of Ca\(^{2+}\) activation increased from pCa 6.2 to pCa 4.0. For this range of Ca\(^{2+}\) concentrations, the \(k_{\text{tr}}\) values ranged from 3.0 ± 0.4 s\(^{-1}\) to 9.3 ± 0.8 s\(^{-1}\) for TnC\(^{\text{endog}}\), from 1.8 ± 0.1 s\(^{-1}\) to 11.0 ± 0.9 s\(^{-1}\) for TnC\(^{F27W}\), from 3.6 ± 0.2 s\(^{-1}\) to 10.5 ± 0.9 s\(^{-1}\) for V44QTnC\(^{F27W}\) and from 3.7 ± 0.5 s\(^{-1}\) to 9.3 ± 0.9 s\(^{-1}\) for F20QTnC\(^{F27W}\) (n=6). Representative traces from three trabeculae reconstituted with TnC\(^{F27W}\), V44QTnC\(^{F27W}\) or F20QTnC\(^{F27W}\) are shown in Figure 5.3, Figure 5.4 and Figure 5.5, respectively. At the same submaximal pCa activations, \(k_{\text{tr}}\) for the TnC mutants was increased for V44QTnC\(^{F27W}\) or decreased for F20QTnC\(^{F27W}\) compared with control TnC. The intercept of the \(k_{\text{tr}}\) vs. relative force relationship, approximated from the \(k_{\text{tr}}\) vs. relative force plot (Figure 5.8), was used to adequately fit the \(k_{\text{tr}}\) vs. pCa relationships and characterize the \(k_{\text{tr}}\) dependence on Ca\(^{2+}\). The intercept values were 0.94 ± 1.33 s\(^{-1}\) for TnC\(^{\text{endog}}\), 0.56 ± 1.08 s\(^{-1}\) for TnC\(^{F27W}\), 1.20 ± 0.66 s\(^{-1}\) for V44QTnC\(^{F27W}\) and 2.03 ± 0.84 s\(^{-1}\) for F20QTnC\(^{F27W}\), n≤6. Figure 5.6 shows that the TnC mutants exhibited different \(k_{\text{tr}}\) vs. pCa relationships. The pCa for half-maximal \(k_{\text{tr}}\) was 5.77 ± 0.01 for TnC\(^{F27W}\), 6.00 ± 0.03 for V44QTnC\(^{F27W}\) and 5.61 ± 0.01 for F20QTnC\(^{F27W}\). Thus, V44QTnC\(^{F27W}\) increased the Ca\(^{2+}\) dependence of \(k_{\text{tr}}\) ~1.6-fold and F20QTnC\(^{F27W}\) decreased the Ca\(^{2+}\) dependence of \(k_{\text{tr}}\) ~1.4-fold. For control TnC\(^{F27W}\), V44QTnC\(^{F27W}\) and F20QTnC\(^{F27W}\), maximum \(k_{\text{tr}}\) values were not significantly different. Therefore, by sensitizing or desensitizing cardiac muscle to Ca\(^{2+}\), the rates of contraction at submaximal levels of Ca\(^{2+}\) activation increased or decreased, respectively.
Figure 5.3. Representative $k_{tr}$ traces for TnC$^{F27W}$ reconstituted trabeculae at pCa 4.0, pCa 5.8 and pCa 6.0. All traces are fitted with a monoexponential relationship (shown in black), which is superimposed on the unfitted trace.
Figure 5.4. Representative $k_{tr}$ traces for V44Q TnC$^{P27W}$ reconstituted trabeculae at pCa 4.0, pCa 5.8 and pCa 6.0. All traces are fitted with a monoexponential relationship (shown in black), which is superimposed on the unfitted trace.
Figure 5.5. Representative $k_{tr}$ traces for F20Q TnC$^{F27W}$ reconstituted trabeculae at pCa 4.0, pCa 5.6 and pCa 5.8. All traces are fitted with a monoexponential relationship (shown in black), which is superimposed on the unfitted trace.
Figure 5.6. $k_{tr}$ vs. pCa relationships for TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae. TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae are shown in blue, red and green, respectively.
**Relationship between $k_{tr}$ vs. relative force.** Figure 5.7 shows that, when TnC reconstituted trabeculae generated similar amounts of relative force at different pCa activations, similar rates of contraction were observed. Figure 5.8 shows the relationship between average $k_{tr}$ values and average relative force at each pCa activation for TnC$^{endog}$, TnC$^{F27W}$, V44QTnC$^{F27W}$ and F20QTnC$^{F27W}$. Table 5.1 indicates the average levels of force (with SEM) versus the average $k_{tr}$ (with SEM) at each corresponding pCa, values used to generate the scatterplot in Figure 5.8. The $k_{tr}$ vs. force relationship was fitted with a linear relationship.

The $k_{tr}$ vs. force relationship is the same for all reconstituted and un-extracted trabeculae regardless of changes in the thin filament Ca$^{2+}$ activation. In the presence of V44QTnC$^{F27W}$ or F20QTnC$^{F27W}$ reconstituted trabeculae, the rates of contraction were significantly increased or decreased, respectively, but only at submaximal levels of Ca$^{2+}$ activation compared to TnC$^{F27W}$. The maximum $k_{tr}$ was not different among all TnCs (see Figure 5.6). Also, the intercept of the $k_{tr}$ vs. relative force relationship was not different. In spite of sensitizing or desensitizing the muscle to Ca$^{2+}$, the $k_{tr}$ vs. relative force relationship did not change. The linear relationship of $k_{tr}$ vs. relative force shows that, at higher levels of force production, the rate of contraction becomes faster. Increasing the thin filament Ca$^{2+}$ activation increased the force production at any Ca$^{2+}$ activation compared with control. This resulted in a faster rate of contraction.

In summary, these results are consistent with the interpretation that altering the thin filament Ca$^{2+}$ activation does not change directly the crossbridge
cycling kinetics during sub-maximal or maximal contraction. This observation is in agreement with previous studies, which showed that Ca\(^{2+}\) does not influence directly the crossbridge cycle (Brenner. 1986, Millar & Homsher. 1990, Regnier, et al. 2004, Wolff, et al. 1995).

Moreover, this study shows that directly altering the thin filament Ca\(^{2+}\) binding properties modifies the level of fractional force, which modulates \(k_{tr}\) only at sub-maximal levels of force production. Consistent with our results, a study in skeletal muscle previously showed that sensitizing the thin filament to Ca\(^{2+}\) increased \(k_{tr}\) at submaximal activation, similar to our results in cardiac muscle (Regnier, et al. 1996). In view of the two-state crossbridge model presented by Brenner as \(f_{app}\) and \(g_{app}\) (Brenner. 1988), our data implies that sensitizing the muscle to Ca\(^{2+}\) facilitates more crossbridge binding to actin and increases the number of crossbridges in the attached state. Therefore, increasing or decreasing thin filament Ca\(^{2+}\) activation will subsequently increase or decrease the number of force generating crossbridges, which will increase or decrease, respectively, the rate of contraction.
Figure 5.7. $k_{fr}$ traces for TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae at matched levels of force. TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ reconstituted trabeculae are shown in blue, red and green, respectively.
Figure 5.8. \( k_{tr} \) vs. relative force relationships for un-extracted trabecuale, TnC\(^{F27W}\), V44Q TnC\(^{F27W}\) and F20Q TnC\(^{F27W}\) reconstituted trabeculae. TnC\(^{endog}\), TnC\(^{F27W}\), V44Q TnC\(^{F27W}\) and F20Q TnC\(^{F27W}\) reconstituted trabeculae are shown in black, blue, red and green, respectively. The intercept (I) and slope (S) values for all TnCs are: TnC\(^{endog}\), I= 0.94 ± 1.33, S= 9.28 ± 1.70, TnC\(^{F27W}\), I= 0.56 ± 1.08, S= 10.5 ± 1.30, V44Q TnC\(^{F27W}\), I= 1.20 ± 0.66, S= 10.07 ± 0.87, F20Q TnC\(^{F27W}\), I= 2.03 ± 0.84 and S= 8.08 ± 1.07. The intercepts and slopes are not significantly different for all TnC mutants.
Table 5.1. Summary of average relative force and average $k_{tr}$ at corresponding pCa activation for each TnC mutant and TnC$^{endog}$. The $k_{tr}$ values corresponding to the relative levels of force less than 10% of the maximum force generated at pCa 4.0 were not included, as at low levels of force the $k_{tr}$ measurements become inconclusive.
Interpretation of the results - significance and further directions. Cardiac muscle force generation is a dynamic process, reflecting the cross-talk among thick and thin filament proteins as Ca\(^{2+}\) initiates the process by binding to TnC. Full activation of the thin filament requires Ca\(^{2+}\) binding to the troponin complex and also subsequent strong crossbridge binding to the thin filament. The rate of contraction as estimated by \(k_{tr}\) measures the transition of crossbridges from weakly bound states to strongly bound states, and the overall process can be summarized as the sum of the attachment and detachment transitions: \(k_{tr} = f_{app} + g_{app}\). Many studies reported that \(k_{tr}\) is calcium-sensitive, and the dependence of \(k_{tr}\) of calcium is different for skeletal and cardiac muscle. In both cardiac and skeletal muscle, \(k_{tr}\) increases with increasing levels of Ca\(^{2+}\) activation, which implies that Ca\(^{2+}\) regulates the transition of crossbridges from weak binding, non-force generating states to strong binding, force-generating states (Brenner, 1988). The relationship between \(k_{tr}\) and Ca\(^{2+}\) is different in cardiac versus skeletal muscle, which reflects that \(k_{tr}\) is dependent on the type of the troponin C isoform present. The relationship between \(k_{tr}\) vs. Ca\(^{2+}\) is curvilinear in skeletal muscle and it has a ~10-fold change in rate when Ca\(^{2+}\) is varied, whereas in cardiac muscle, the fold-change in \(k_{tr}\) is much lower (~ 3-fold change) and it is described as either linear (in this study and by Wolff et al, 1995) or slightly curvilinear relationship (Palmer & Kentish, 1998). Interestingly, extraction of endogenous skeletal TnC from psoas fibers and reconstitution with the cardiac TnC isoform or the skeletal TnC with a mutated, inactive site I of the regulatory
domain, reproduced the smaller fold-change in $k_{tr}$ as seen in cardiac muscle (Morris et al. 2001). Extraction–reconstitution of psoas fibers with cardiac TnC showed that $k_{tr}$ becomes faster with higher $Ca^{2+}$ activation regardless of the type of TnC isoform, but nevertheless, the TnC isoform bound to the thin filament modulates the rate of tension redevelopment, by influencing the fold change and the shape of the $k_{tr}$ vs. relative force relationship. Even though $k_{tr}$ depends of $Ca^{2+}$ and the type of myosin isoform, in case of the protocol utilized by Morris et al. (2001), the crossbridge cycle and the myosin isoform was unchanged. Therefore, the variation in $k_{tr}$ should be caused by TnC-dependent effects. The most likely explanation is derived from the differences in TnC interaction with $Ca^{2+}$ in skeletal versus cardiac muscle and a different signaling pathway to the other subunits of the troponin complex, actin, and tropomyosin. The structural differences between cardiac TnC and skeletal TnC may alter the ability of TnC to interact with TnI and ultimately to affect the tropomyosin position, which will dictate the state of crossbridge attachment to the thin filament. Therefore, the rates at which TnC can undergo the required conformational changes to induce structural modifications on the thin filament may be isoform dependent.

Our study directly investigated the change in $k_{tr}$ at various levels of $Ca^{2+}$ activation by incorporating cardiac TnC mutants with altered $Ca^{2+}$ binding properties into skinned cardiac preparations. In the view of the three-states model introduced by Geeves and his collaborators as B-, C-, and M-states, this study suggests that the probability that Tm is in a position that favors strong
crossbridge binding depends on both Ca\(^{2+}\) and strong crossbridge formation. At submaximal levels of activation, Ca\(^{2+}\) is recruiting crossbridges into strong attached states by increasing the transition into C-states. When muscle generates more than 50% of the maximal force, a sufficient number of strong crossbridges are already bound and these crossbridges can recruit the non-attached or weakly attached crossbridges and facilitate the transition into strong, force-generating M-states.

This model can explain why, at submaximal activation, the \(k_{fr}\) can be modulated by the level of thin filament activation and also at maximal activation, \(k_{fr}\) is unchanged regardless of the type of TnC mutant. We suggest that, increasing or decreasing the level of the thin filament activation by incorporating TnC mutants with different Ca\(^{2+}\) binding properties can speed up or slow down, respectively, the transition of tropomyosin from the B-states into C-states. This transition is governed by conformational changes induced in the troponin complex upon Ca\(^{2+}\) binding, with TnI being a major player responsible for coordinating the Ca\(^{2+}\) signaling pathway between the Tn complex and actin and Tm (Davis et al, 2007, Swartz et al. 2006, Engel et al, 2007, Kobayashi & Solaro, 2006). Thus, the slower the transition into C-states (as in case of the F20Q TnC\(^{F27W}\) mutant), the lower the probability of crossbridges to attach to actin and enter the strong crossbridge formation (M-state) and further recruit more crossbridges. This results in slower rates of attachment of crossbridges to actin, less force generation, and slower \(k_{fr}\). As for V44QTnC\(^{F27W}\) mutant, the transition
into C-states is faster; therefore, more crossbridges are recruited into strong, force-generating states. As force is proportional to the number of attached crossbridges (Brenner, 1998), more force is generated for the same amount of Ca$^{2+}$ activation (cardiac muscle sensitization) and as more crossbridges attach, $k_{tr}$ increases. However, there is cooperativity of activation between crossbridges, which implies that strong, force-generating crossbridges can recruit non-attached or weak-binding crossbridges into force-generating states (Campbell, 1997). This study suggests that there is a threshold of activation of the thin filament beyond which Tm position can readily switch into M-states. Above the threshold, $k_{tr}$ is independent of the TnC mutant present and the level of activation of the thin filament, and it is only dependent on the rate of crossbridge cycling or the myosin isoform. This means that strong crossbridge binding stabilize Tm in a position that makes available further myosin binding sites along the thin filament.

Future studies to further elucidate the influence of the thin filament activation on the rate of contraction would be to measure the rate of contraction by using caged Ca$^{2+}$ compounds ($k_{Ca}$) and compare $k_{Ca}$ and $k_{tr}$. As $k_{Ca}$ represents both the thin and thick filament activation processes and $k_{tr}$ is a measure of the thick filament activation, differences between $k_{Ca}$ and $k_{tr}$ would further suggest that the dynamics of Ca$^{2+}$ binding to TnC on the thin filament can influence the rate of contraction. A very elegant, direct approach would be to measure the affinity of Ca$^{2+}$ binding to cardiac TnC in muscle, along with force measurements. This approach has been attempted by Bell et al, 2006. A rhodamine–derived probe
(5’-TMR)IA) reported the changes in TnC structure during activation in skinned cardiac trabeculae. The authors have used fluorescence polarization to monitor changes in the TnC structure reported by the probe, which was attached to Cys 84 on TnC. This technique assesses modifications in the underlying TnC conformation by monitoring changes in the probe orientation, analyzed as peak angle and dispersion. It was assumed that the changes in probe orientation would be sensitive to both Ca$^{2+}$ binding to TnC and crossbridge binding to actin and enhancing further Ca$^{2+}$ binding to TnC. However, due to the technical difficulties and complexity of interaction of the fluorescent probes on TnC with the environment, the kinetics of Ca$^{2+}$ binding to TnC in muscle fibers requires more elaborate characterization.
CHAPTER 6

INFLUENCE OF ALTERING THE CROSSBRIDGE CYCLING KINETICS ON
THE RATE OF CARDIAC MUSCLE CONTRACTION

*Effects of Pi on cardiac muscle contractile performance.* Up to now, the majority of Pi studies have been performed in skeletal muscle (Cooke, et al. 1988, Cooke. 1997, Kerrick & Xu. 2004, Millar & Homsher. 1990, Pate & Cooke. 1989, Tesi, et al. 2000). In striated muscle, the release of the byproducts of ATP split by the actomyosin ATPase, ADP and Pi, is associated with important steps in the crossbridge cycle and force production. The release of Pi is associated with the transition from weak binding, non-force generating crossbridges to the strong binding, force generating crossbridges and, therefore, with the power stroke. In solutions with added Pi, there is a shift in the population of crossbridges from force generating states to non-force generating states, and the crossbridges are cycling faster from strong-binding states to weak-binding states. Force production is proportional to the number of force-generating crossbridges, which reflects a balance between the rates of transition into force-generating states and the rates of detachment of strongly bound
crossbridges from actin filaments. Therefore, in the presence of added Pi solutions, the maximum force is depressed and the rate of contraction is increased at all levels of activation. The depression of force is manifested as a right shift of the force vs. pCa relationship compared to solutions with no added Pi, equivalent to the effect of Pi to desensitize striated muscle to Ca\(^{2+}\) (Cooke & Pate. 1985, Cooke, et al. 1988, Pate & Cooke. 1989, Tesi, et al. 2000, Walker, et al. 1992).

As reported by Hinken et al, in skinned cardiac myocytes at 13\(^\circ\)C, in the presence of 2.5 mM Pi, 5.0 mM Pi and 10.0 mM added Pi, force decreased progressively to 66%, 57% and 30% values from the maximum force in the absence of added Pi (Hinken & McDonald. 2004). The \(k_{tr}\) rates at 2.5 mM Pi, 5.0 mM Pi and 10.0 mM added Pi increased with 40%, 94% and 379% more than in the absence of added Pi. Kentish (1986) investigated the effects of increasing Pi concentrations on rat skinned cardiac trabeculae at room temperature. In average, the 2.5 mM Pi, 5.0 mM Pi and 10.0 mM added Pi decreased the level of maximum force to ~ 70%, 50% and 40%, respectively, compared with the maximum force in the absence of added Pi (Kentish. 1986). Also increasing the Pi concentration shifted the force vs. pCa relationships to higher Ca\(^{2+}\) concentrations, consistent with the effect of high Pi to reduce the Ca\(^{2+}\) sensitivity of force development, as previously reported in skeletal muscle. Araujo et al. estimated the influence of Pi on the rate of contraction in skinned cardiac myocytes at 15\(^\circ\)C using caged phosphate and caged calcium compounds (Araujo & Walker. 1996). In their study, the rate of contraction was increased
almost four-fold in the presence of 10 mM Pi and force was depressed more than half of the maximum force when no Pi was added.

Our experiments in rat skinned cardiac trabeculae at 15°C showed that increasing Pi concentration progressively decreased force production in un-extracted trabeculae, desensitized muscle to Ca\(^{2+}\), and increased \(k_{tr}\). Our results are similar to the observations from the previous studies in cardiac muscle, in spite of differences in temperature, species, solutions composition, and cardiac preparation.

**Influence of altering crossbridge kinetics on \(k_{tr}\).**

**Effect of Pi on force in un-extracted trabeculae and trabeculae reconstituted with TnC\(^{F27W}\), F20Q TnC\(^{F27W}\) and V44Q TnC\(^{F27W}\) mutants.** We wanted to investigate if the effect of accelerating or slowing the rate of contraction when the thin filament activation was increased or decreased, respectively, is still present when the kinetics of crossbridge cycling is modified. It has been shown that, in the presence of added Pi solutions, force is depressed in cardiac muscle preparations (Araujo & Walker. 1996, Hinken & McDonald. 2004, Kentish. 1986). Figure 6.1 demonstrates the effect of two Pi concentrations on the amount of relative force in un-extracted trabeculae. At 2.5 mM Pi and 5.0 mM Pi, the force generated at maximal activation was 79.7 ± 5.0 % and 65.4 ± 5.0%, respectively, of the force generated at maximal activation in the absence of added Pi. For the un-extracted trabeculae, the Ca\(^{2+}\) sensitivity of force production in the presence of 2.5 mM Pi (pCa\(_{50}\) = 5.78 ± 0.02) was similar to that in the
Figure 6.1. Force vs. pCa relationships for un-extracted trabeculae in the absence of added Pi and in the presence of 2.5 mM and 5.0 mM added Pi. The maximum force production in 2.5 mM Pi and 5.0 mM Pi is normalized to the maximum force production in no added Pi.
Figure 6.2. Force vs. pCa relationships for un-extracted trabeculae in the absence of added Pi and in the presence of 2.5 mM and 5.0 mM added Pi. The maximum force production in the absence of added Pi and in the presence of 2.5 mM Pi and 5.0 mM Pi is normalized to 1.
absence of added Pi (pCa_{50}=5.79\pm 0.04) and significantly decreased (pCa_{50}=5.68 \pm 0.02) in the presence of 5.0 mM Pi (Figure 6.2).

In the presence of 2.5 mM added Pi, the maximum force generation for trabeculae reconstituted with TnC^{F27W}, V44QTnC^{F27W}, or F20QTnC^{F27W} decreased to 75.8\% \pm 3.2, 76.3\% \pm 3.0 and 64.5\% \pm 2.9 from maximum force in the absence of added Pi (Figure 6.3). However, in the presence of 2.5 mM added Pi, V44QTnC^{F27W} and F20QTnC^{F27W} still sensitized or desensitized, respectively, the myofilaments to Ca^{2+} (pCa_{50} =5.86 \pm 0.04 and 5.62 \pm 0.01) in relationship with the control TnC^{F27W} (pCa_{50} = 5.70 \pm 0.03, see Figure 6.3). Thus, in accordance with the literature (Araujo & Walker. 1996, Kentish. 1986), adding Pi to the activation solution decreased force production and desensitized cardiac muscle to Ca^{2+} in both un-extracted and V44QTnC^{F27W} reconstituted trabeculae.

Effect of 2.5 mM Pi on k_{tr} vs. pCa relationship for un-extracted and trabeculae reconstituted with TnC^{F27W}, V44QTnC^{F27W}, or F20Q TnC^{F27W}. We used Pi solutions to increase the crossbridge cycling and we assessed the k_{tr} values for TnC^{endog}, TnC^{F27W} (control), V44QTnC^{F27W} and F20QTnC^{F27W}. Only the effects of 2.5 mM Pi on k_{tr} were studied since 5.0 mM Pi decreased the amount of force at submaximal Ca^{2+} activations to levels at which k_{tr} values would have been difficult to measure accurately. The k_{tr} vs pCa relationships for TnC^{F27W}, V44QTnC^{F27W} and F20Q TnC^{F27W} in the absence of added Pi and in the presence of 2.5 mM added Pi are shown in Figure 6.4.
Figure 6.3. Force vs. pCa relationships for trabeculae reconstituted with TnC<sub>F27W</sub>, V44Q TnC<sub>F27W</sub> and F20Q TnC<sub>F27W</sub> in the presence of 2.5 mM added Pi. Maximum force in the presence of 2.5 mM Pi is normalized to maximum force in the absence of added Pi.
Figure 6.4. $k_{tr}$ vs. pCa relationship for trabeculae reconstituted with TnC$^{F27W}$, V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$ in the absence and in the presence of 2.5 mM added Pi. No added Pi: open squares; added Pi: solid squares. Color code: blue: TnCF27W, red: V44Q TnC$^{F27W}$ and green: F20Q TnC$^{F27W}$. A line was manually drawn to outline the $k_{tr}$ vs pCa relationship for each TnC mutant.
The $k_{tr}$ values were increased for TnC$^{endog}$, TnC$^{F27W}$, V44Q TnC$^{F27W}$, and F20Q TnC$^{F27W}$ at all levels of Ca$^{2+}$ activation in the presence of added Pi solutions (see Figure 6.4). However, in the presence of 2.5 mM added Pi, only the $k_{tr}$ values at sub-maximal levels of Ca$^{2+}$ activation were significantly faster or slower for V44Q TnC$^{F27W}$ and F20Q TnC$^{F27W}$, respectively, compared to TnC$^{F27W}$, whereas the maximum $k_{tr}$ values were not significantly different (see Figure 6.4). We can conclude that, even in the presence of faster crossbridge cycling, the rates of contraction at submaximal Ca$^{2+}$ activations were influenced by thin filament Ca$^{2+}$-activation.

**Effect of 2.5 mM Pi on $k_{tr}$ vs. relative force relationship for un-extracted and trabeculae reconstituted with TnC$^{F27W}$, V44Q TnC$^{F27W}$ or F20Q TnC$^{F27W}$**. The average $k_{tr}$ values were plotted versus the average relative force at each pCa activation for all TnC$^{F27W}$ mutants. Figure 6.5 shows that $k_{tr}$ was increased at all relative force values by the addition of 2.5 mM Pi for all TnC$^{F27W}$ mutants. In the presence of 2.5 mM Pi, the same $k_{tr}$ vs. relative force relationship is observed for un-extracted trabeculae and trabeculae reconstituted with TnC$^{F27W}$, V44Q TnC$^{F27W}$, or F20Q TnC$^{F27W}$ (see Figure 6.6). The relationship between $k_{tr}$ vs. relative force is linear for all TnCs in this study in the absence of added Pi, and in the presence of added Pi, the same linear relationship is observed, but the intercepts are higher, according to Pi accelerating the transition of crossbridges from force-generating into non-force generating states. The intercepts and the slopes are shown in Figure 6.5. Table 6.1 presents a summary of the average $k_{tr}$ and the average relative force at each corresponding pCa. In
spite of increasing the crossbridge transition rate from force to non-force generating states in the presence of Pi, $k_{tr}$ values at matched levels of relative force were similar for trabeculae reconstituted with TnC$^{F27W}$, V44QTnC$^{F27W}$ or F20Q TnC$^{F27W}$. This data suggests that $k_{tr}$ is correlated with the level of force, which is proportional to the number of attached crossbridges at any calcium activation. In conclusion, increasing the crossbridge cycling by added Pi does not change the $k_{tr}$ vs. relative force relationship for un-extracted and reconstituted trabeculae. Even in the presence of faster crossbridge cycling, the rates of contraction at submaximal Ca$^{2+}$ activations were increased or decreased, respectively, for the trabeculae reconstituted with the high or low Ca$^{2+}$ affinity TnC mutant compared with trabeculae reconstituted with TnC$^{F27W}$. This data implies that sensitizing or desensitizing the muscle to Ca$^{2+}$ increases or decreases, respectively, the number of crossbridges in the attached state, even when crossbridges cycle faster between force-generating states and non-force generating states. The opposite process occurs when myofilaments are desensitized to Ca$^{2+}$. The intercept of the $k_{tr}$ vs. relative force relationship increased ~5-fold for trabeculae reconstituted with TnC$^{F27W}$, V44QTnC$^{F27W}$, or F20Q TnC$^{F27}$. According to Brenner’s model, the rate of force redevelopment ($k_{tr}$) is described as the sum of forward and backward rates: $k_{tr} = f_{app} + g_{app}$, where $f_{app}$ is the sum of the forward transition of crossbridges from detached, non-force generating states to attached, force-generating states and $g_{app}$ represents the backward transition and return to non-force generating states. The intercept on the $k_{tr}$ vs. relative force plot represents $g_{app}$ (Brenner. 1988,
the intercept in the presence of faster cycling crossbridges suggests that the $g_{app}$
value reflects only intrinsic properties of the crossbridges, which are not
dependent on the level of activation of the thin filament. Furthermore, the $k_{tr}$ vs.
relative force relationships were similar for trabeculae reconstituted with TnC$^{F27W}$,
V44QTnC$^{F27W}$, or F20Q TnC$^{F27W}$ in the presence of 2.5 mM added Pi. These
observations suggest that $k_{tr}$ is correlated with the amount of force production,
either in the presence of un-altered or faster crossbridge cycling (Figure 6.6). At
submaximal force production, the level of the thin Ca$^{2+}$ activation modulates $k_{tr}$,
but at maximal force production, the kinetics of crossbridge cycling governs the
rate of contraction. Moreover, this result might have physiological significance as,
on a beat-to-beat basis, the heart is able to modulate the rate of contraction and
the force generated (or the ejection fraction) to meet the body needs. Also, the
ability to increase or decrease, respectively, the rate of contraction by altering the
Ca$^{2+}$ binding properties of the intrinsic TnC might prove beneficial in
pathophysiological states, such as heart failure or hypertrophic cardiomyopathy.
Figure 6.5. $k_tr$ vs. relative force relationship for un-extracted trabeculae and trabeculae reconstituted with $\text{TnC}^{F27W}$, $\text{V44Q TnC}^{F27W}$, and $\text{F20Q TnC}^{F27W}$ in the absence and in the presence of 2.5 mM added Pi. No added Pi: open squares; added Pi: solid squares. Color code: blue: $\text{TnC}^{F27W}$, red: $\text{V44Q TnC}^{F27W}$ and green: $\text{F20Q TnC}^{F27W}$. $I = \text{intercept}$; $S = \text{slope for the linear regression relationship for each TnC mutant and TnC}^{\text{endog}}$. 
Figure 6.6. Average $k_{tr}$ vs. average relative force relationship for trabeculae reconstituted with TnC$^{F27W}$, V44Q TnC$^{F27W}$, and F20Q TnC$^{F27W}$ in the absence and in the presence of 2.5 mM added Pi. No added Pi: open squares; added Pi: solid squares. Color code: blue: TnC$^{F27W}$, red: V44Q TnC$^{F27W}$, and green: F20Q TnC$^{F27W}$.
Table 6.1. $k_t$ and relative force in the presence of 2.5 mM added Pi at various pCa activations for TnC$^{\text{endog}}$, TnC$^{F27W}$, V44QTnC$^{F27W}$, and F20QTnC$^{F27W}$. Values are shown as mean ± SEM.
<table>
<thead>
<tr>
<th>TnC</th>
<th>pCa</th>
<th>K_{tr} (s(^{-1}))</th>
<th>Rel. Force</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnC(\text{endog})</td>
<td>5.8</td>
<td>4.21 ± 0.67</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>No added Pi</td>
<td>5.6</td>
<td>5.99 ± 0.62</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>n=9</td>
<td>5.2</td>
<td>6.29 ± 0.47</td>
<td>0.92 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>7.47 ± 0.62</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>TnC(\text{endog})</td>
<td>5.8</td>
<td>8.12 ± 0.04</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>2.5mM added Pi</td>
<td>5.6</td>
<td>10.78 ± 1.12</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>n=9</td>
<td>5.2</td>
<td>11.49 ± 0.89</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>11.37 ± 1.12</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td>TnC(^{F27W})</td>
<td>6.0</td>
<td>3.74 ± 0.96</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>No added Pi</td>
<td>5.8</td>
<td>4.54 ± 0.55</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>n=6</td>
<td>5.6</td>
<td>7.51 ± 0.72</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>7.47 ± 0.62</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>8.28 ± 0.75</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>TnC(^{F27W})</td>
<td>5.8</td>
<td>11.13 ± 0.64</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>2.5mM added Pi</td>
<td>5.6</td>
<td>13.11 ± 1.24</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>n=6</td>
<td>5.2</td>
<td>13.65 ± 1.22</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>12.89 ± 1.02</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>V44Q TnC(^{F27W})</td>
<td>6.2</td>
<td>2.93 ± 0.27</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>No added Pi</td>
<td>6.0</td>
<td>5.50 ± 0.67</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>n=10</td>
<td>5.8</td>
<td>6.98 ± 0.73</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>7.47 ± 0.62</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>8.25 ± 0.71</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>7.63 ± 0.62</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>V44Q TnC(^{F27W})</td>
<td>6.0</td>
<td>9.56 ± 0.79</td>
<td>0.23 ± 0.04</td>
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<tr>
<td>2.5mm added Pi</td>
<td>5.8</td>
<td>10.76 ± 0.59</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>n=10</td>
<td>5.6</td>
<td>13.52 ± 0.92</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>12.22 ± 1.07</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>11.57 ± 0.92</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>F20Q TnC(^{F27W})</td>
<td>5.8</td>
<td>3.08 ± 0.35</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>No added Pi</td>
<td>5.6</td>
<td>5.29 ± 0.43</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>n=6</td>
<td>5.2</td>
<td>7.06 ± 0.56</td>
<td>0.89 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>7.43 ± 0.59</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>F20Q TnC(^{F27W})</td>
<td>5.6</td>
<td>11.47 ± 0.84</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>2.5mM added Pi</td>
<td>5.2</td>
<td>12.38 ± 0.95</td>
<td>0.67 ± 0.04</td>
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<tr>
<td>n=6</td>
<td>5.0</td>
<td>13.77 ± 1.01</td>
<td>0.65 ± 0.03</td>
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<tr>
<td></td>
<td>4.0</td>
<td>13.38 ± 0.73</td>
<td>0.65 ± 0.03</td>
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</table>
The effect of Pi to increase the rate of contraction and to depress force production is independent of the order of activation for matched experiments. For either un-extracted or mutant TnC reconstituted trabeculae, the Pi activations were repeated on the same fiber at identical pCas (matched experiments). For 2.5 mM Pi, six un-extracted trabeculae were activated first in no added Pi solutions, and then in added Pi solution at the same pCa.

To verify if the order of activation can have an impact on the effect of Pi to speed up $k_{tr}$ and desensitize the myofilaments, three additional experiments were performed in un-extracted trabeculae, in which the order of activation was reversed. First, the trabecula was activated in the presence of added Pi followed by the activation in no added Pi at the same pCa. Figure 6.7 shows that, in the presence of added Pi solutions, the force was depressed to similar levels and the myofilaments were desensitized similarly, independent of the order of activation (see Table 6.3).

The $k_{tr}$ vs. relative force relationship was the same in both experimental protocols (Figure 6.8), which showed the same linear relationship, or the increase in $k_{tr}$ was proportional with the number of attached crossbridges and the level of force production at each pCa activation, independent of the order of activation in Pi solutions.

Effect of 15 mM Pi on maximal force production and $k_{tr}$ in un-extracted trabeculae. Additional experiments were performed in un-extracted trabeculae to further investigate the effect of Pi to depress force production and increase $k_{tr}$. The trabeculae were activated at saturating levels of Ca$^{2+}$ (pCa 4.0) in the
<table>
<thead>
<tr>
<th></th>
<th>no Pi / Pi activation n=6</th>
<th>Pi / no Pi activation n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>no Pi (pCa\textsubscript{50})</td>
<td>5.79 ± 0.04</td>
<td>5.78 ± 0.01</td>
</tr>
<tr>
<td>Pi (pCa\textsubscript{50})</td>
<td>5.79 ± 0.04</td>
<td>5.78 ± 0.01</td>
</tr>
<tr>
<td>(F_{4.0P}/F_{4.0})</td>
<td>79.7 ± 5.0</td>
<td>5.78 ± 0.02</td>
</tr>
</tbody>
</table>

Table 6.2. Summary of pCa\textsubscript{50} and force production in the presence of added Pi for two different protocols of activation.

Figure 6.7. Force vs. pCa relationships for un-extracted trabeculae in the presence of added 2.5 mM Pi compared with no added Pi. The order of activation was no Pi, then Pi (black traces) and Pi, then no Pi (blue traces).
Figure 6.8. The relationship between $k_{fr}$ vs. relative force is the same for both protocols of activation. The average $k_{fr}$ is plotted against the average level of relative force production at pCa activation in a range from 6.0 to 4.0.
Table 6.3. Summary of force production and $k_{tr}$ in the absence of added Pi and in the presence of 15 mM added Pi in un-extracted trabeculae. Individual activations at pCa 4.0 are shown for each trabecula.

<table>
<thead>
<tr>
<th>Trabecula</th>
<th>$K_{tr}, 15$ mM Pi $\left(\text{s}^{-1}\right)$</th>
<th>$K_{tr}, \text{no Pi} \left(\text{s}^{-1}\right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>20.2</td>
<td>8.02</td>
</tr>
<tr>
<td></td>
<td>20.14</td>
<td>8.53</td>
</tr>
<tr>
<td>#2</td>
<td>16.27</td>
<td>7.02</td>
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<td>15.93</td>
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<td>9.37</td>
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<td>28.3</td>
<td>10.16</td>
</tr>
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</table>
Figure 6.9. Representative traces of force generated by un-extracted trabeculae in the absence of added Pi and in the presence of 15 mM added Pi. A: Traces are not normalized to show the effect of 15 mM added Pi to depress force production. B: Force traces in the presence of 15 mM added Pi are normalized to force traces in the absence of added Pi to show the effect of Pi to speed up $k_{fr}$. 
absence of added Pi and in the presence of 15 mM added Pi. The results are summarized in Table 6.4. The average force production at pCa 4.0 in 15 mM added Pi was $0.39 \pm 0.07$ (n=4) of the average force production in the absence of added Pi at pCa 4.0. Figure 6.9 shows the effect of 15 mM Pi on force production and $k_{tr}$. The $k_{tr}$ in 15 mM added Pi ($20.72 \pm 1.5$ s$^{-1}$, n=4) increased almost 2.4-fold compared to $k_{tr}$ in the absence of added Pi ($8.68 \pm 0.32$ s$^{-1}$, n=4, Table 6.4).

Interpretation of the results – significance and future directions. The role of Ca$^{2+}$ in regulation of muscle contraction was first considered to be like an “on/off” switch, which opens/closes the myosin binding sites on actin. This mechanism was first proposed by H.E. Huxley as the “steric blocking model” (H.E. Huxley, 1971). Further studies by Brenner showed that this is not entirely true, because crossbridges can bind very rapidly and weakly to actin even in the absence of Ca$^{2+}$ (Brenner et al., 1982). Brenner showed that, in the presence of ATP, myosin S1 can bind to regulated actin independent of Ca$^{2+}$. Also, in skinned relaxed muscle fibers at low ionic strength, stiffness of the relaxed muscle studied at very rapid stretches was still high. The biochemical and physiological evidence provided by Brenner suggested that, even in the absence of Ca$^{2+}$, a population of crossbridges could attach/detach very rapidly, but do not sustain force. This population of crossbridges is known as weakly-bound, non-force generating crossbridges. It was hypothesized that Ca$^{2+}$ would modulate the rate of Pi release from the actomyosin, but the independence of the rate of Pi release in muscle contracting isometrically at various pCa suggested that Ca$^{2+}$ would regulate a transition prior to the Pi release. Therefore, in the series of steps of the
ATPase cycle (Pate & Cooke, 1989), Ca^{2+} would regulate the crossbridge transition from weakly-bound, non-force generating state to a strongly-bound, non-force generating state. The Pi release would occur during the transition from a strongly-bound, non-force generating state to a strongly-bound, force generating state. A representation of the biochemical and physiological events that correlates the ATPase cycle and force production is shown in Figure 6.10. The Ca^{2+} regulation of contraction occurs at the transition from the B-state to the C-state, where B and C-states represent the three states of the thin filament activation, as introduced by Geeves (Geeves and Conibear, 1995).

\[
\begin{align*}
\text{M.ADP.Pi} & \quad \text{AM.ADP.Pi} & \quad \text{AM.ADP} \\
\text{weakly attached XB} & \quad \text{strongly attached XB} & \quad \text{strongly attached XB} \\
\text{non-force generating} & \quad \text{non-force generating} & \quad \text{force generating} \\
B (\text{Blocked}) & \quad C (\text{Calcium}) & \quad M (\text{Myosin})
\end{align*}
\]

**Figure 6.10. Transition of crossbridges through different states during muscle activation.** XB: crossbridge; top: biochemical steps in the ATPase cycle; bottom: thin filament activation states.
Our study showed that, even when crossbridges cycle from the strongly-attached, force-generating states to strongly-attached, non-force generating states in the presence of added Pi, Ca\textsuperscript{2+} is able to modulate the transition from weakly-attached, non-force generating to strongly-attached, non-force generating states. Moreover, there are TnC-dependent effects which influence the Ca\textsuperscript{2+} signaling process within the troponin complex, such as, at submaximal activation, the transition from B to C state is enhanced by the high Ca\textsuperscript{2+} binding affinity V44QTnC\textsuperscript{F27W} and it is diminished by the low Ca\textsuperscript{2+} binding affinity F20QTnC\textsuperscript{F27W}. This study provides direct evidence that the thin filament activation properties are able to modulate the rate of contraction in various conditions of crossbridge cycling.
CHAPTER 7

DISCUSSION

*Molecular interactions in the troponin complex and the mechanism of troponin regulation of muscle contraction.* The solving of the crystal structures of the troponin complex (Takeda et al., 2003; Vinogradova et al., 2005) together with the NMR data on the mobility of the troponin complex in the relaxed and activated states (Murakami et al., 2005; Hoffman et al., 2006) advanced the understanding of the molecular interactions among the proteins in the troponin complex and suggested novel mechanisms of regulation of muscle contraction by the troponin complex. These structures visualize many protein-protein interactions not elucidated by previous studies in smaller fragments of the troponin complex.

The crystal structure showed the organization of the core domain of the troponin complex in two subdomains, the IT domain and the regulatory head. The orientation of the N-domain of TnC relative to the IT arm and the relative movement of TnI in relationship with TnC or actin defined the "drag and release"
mechanism of activation of muscle contraction (Li et al., 2004). The IT arm is comprised of N-TnI, C-TnT and C-TnC, where C-TnT and N-TnI are visualized as two pairs of alpha-helical “chopsticks” which grab TnC by the C-domain, and the C-TnI, which contains the inhibitory and the switch regions, extends away from the IT arm to interact with N-TnC, forming the regulatory head. During muscle activation, the opening of the hydrophobic pocket of N-TnC and subsequent binding of the switch peptide of TnI extends the central helix of TnC (DE linker) and tugs on the inhibitory region of TnI, which detaches from actin and re-positions along the extended central helix of TnC. The DE linker bound with inhibitory peptide was visualized only in the Ca\(^{2+}\)-activated structure.

When Ca\(^{2+}\) comes off TnC, the closing of the hydrophobic pocket expels the switch region, the TnC central helix loses the helical conformation and it “melts”, re-organizing into a more disordered structure. In the inactivated state, the DE linker and the switch peptide were crystallized, but the structure was not detailed enough to be resolved. Because the TnI inhibitory peptide is not bound and stabilized by the central helix, it is released from TnC and assumes an alpha-helical configuration, establishing interactions with actin and inhibiting myosin binding sites on actin. The recent NMR data, taking advantage of the flexibility of the troponin molecule, was able to describe the relative “swinging” movement of the regulatory head around the IT arm and defined the mobile domain (Md) of TnI (see Figure 7.1B).
As $Ca^{2+}$ binds to TnC, the relative movement of the mobile domain “tumbles independently of the core domain around possible “hinge” regions in the alpha-helix 4 of the C-domain of TnI. The proposed role of the mobile domain is to modulate the binding of the inhibitory peptide of TnI to actin. The mobile domain has a fluctuating conformation, alternating between compact and extended conformations (see Figure 7.1A). Hoffman et al., (2006) proposed that Md is folded on actin in the absence of $Ca^{2+}$, binding to actin with a low affinity, stabilized mostly through salt-bridge interactions, and it becomes unfolded in the presence of $Ca^{2+}$. The mobile domain alternates between folded and unfolded states and moves outside the troponin core to reach to actin. The movement of the Md through the gap between the actin and the troponin core is initiated mainly by conformational fluctuations due to protein folding, and this behavior was interpreted in the view as the “fly-casting” mechanism of molecular recognition (Shoemaker et al., 2000). Thus, new aspects of the molecular and dynamic aspects of the steric blocking mechanism of contraction were revealed.

In the inhibited state in the absence of $Ca^{2+}$ binding, tropomyosin is held by troponin in a “blocking” position, preventing myosin to bind to actin. The core domain of the troponin complex is tethered to actin through the TnT2 segment of TnT, anchoring the IT arm to tropomyosin and actin. Both the inhibitory region
and the mobile domain have net positive charges and tend to establish strong
 electrostatic interactions with the outer domain of actin, to which they bind in a C-
 clamp configuration. The inhibitory and mobile domain of N-TnI, though the TnT1
domain of TnT, holds Tm in the “blocking” position. The switch peptide is
oriented towards N-TnC, in the space between actin and N-TnC, which is
connected to the rest of the troponin complex through the unstructured DE linker.
In the activated state, the N-TnC exposes the hydrophobic pocket, which is a
binding site for the switch peptide of TnI. When the switch peptide binds to TnC,
the mobile domain dissociates from actin, which cooperatively promotes the
dissociation of the inhibitory peptide from actin. The binding of the inhibitory
peptide to the central helix of TnC reduces the conformational fluctuations of N-
TnC by restructuring the DE linker, which becomes more rigid and extended.
Therefore, the inhibitory and switch peptides are TnC-bound, the mobile domain
is unstructured and flexible and away from actin. The only contact between the
troponin complex and the thin filament is through TnT2. Without the inhibitory
action of TnI on actin, tropomyosin is free to move on the surface of the actin
filament, allowing the myosin heads to bind to the uncovered outer actin
domains. This series of events is summarized in Figure 7.1B. The return to the
relaxation state is initiated by Ca$^{2+}$ coming off TnC. Nevertheless, the switch
peptide is still bound to the hydrophobic pocket, and in order to detach, it has to
overcome an activation barrier. The mobile domain was proposed to facilitate the
Figure 7.1. Interactions of the troponin complex with tropomyosin and actin. A: Relationship of actin and troponin in the relaxed state. TnI: blue, TnT: orange, TnC: red, mobile domain: yellow, actin monomers: green, light blue and pink. B: Proposed mechanism of Ca\(^{2+}\) regulation of contraction by troponin. Tropomyosin is depicted as two continuous grey lines along the actin monomers. For detailed explanations, see text. (Reprinted from J.Mol.Biol 9; 352 (1): 178-201, Structural basis for Ca\(^{2+}\) regulated muscle relaxation at interaction sites of troponin with actin and tropomyosin, pages 192 and 196, Copyright 2005, with permission from Elsevier\(^{\text{TM}}\))
switch peptide overcoming this barrier, as in the absence of Ca\(^{2+}\), the Md can reach to actin again, establishing electrostatic interactions and undergoing the movement described by the “fly-casting” mechanism. Then, the switch peptide dissociates from TnC and this event destabilizes the DE linker - inhibitory peptide interaction, the DE linker begins to melt and the inhibitory peptide resumes its binding actin, blocking myosin interaction.

**Proposed molecular mechanisms of altering the muscle force production by cardiac TnC mutants.** Substituting polar Gln for hydrophobic amino acids, which are constituents of the hydrophobic pocket that binds to the switch peptide of TnI, proved to sensitize the isolated TnC to calcium (Tikunova & Davis, 2004). The mechanism of sensitization of isolated TnC to calcium was hypothesized to involve a destabilization of the NAD and BC unit, the polar Gln disrupting the hydrophobic interaction between NAD and BC unit, thus favoring a more “open” the structure of TnC, mimicking the TnI binding (see Figure 7.3). All five amino acids substitutions sensitized the isolated TnC to calcium. The kinetic studies suggested that Ca\(^{2+}\) binding properties were altered due to changes in both Ca\(^{2+}\) association and dissociation rates (Tikunova & Davis, 2004). Reconstitution of rat skinned cardiac trabeculae with these mutants showed that three of them did not alter the muscle sensitivity of force production, whereas the other two mutants significantly increased and decreased, respectively, the cardiac muscle sensitivity of force production. This finding suggested that not all the hydrophobic amino acids constituents of the hydrophobic pocket are equally critical for
establishing and maintaining the interaction with the switch peptide of TnI. This study suggests that Val 44 and Phe 20 might be critical for this interaction.

**V44Q TnC\(^{F27W}\) mutant alters the affinity of TnC for the switch peptide of TnI.** Ca\(^{2+}\) binding studies suggest that V44Q TnC\(^{F27W}\), M45 TnC\(^{F27W}\), V44Q TnC\(^{F27W}\), L48 TnC\(^{F27W}\), and M81TnC\(^{F27W}\) decreased the Ca\(^{2+}\) dissociation rate from TnC, which implied that the TnC assumes a more “open” conformational change, enhancing TnI binding. Val 44, Met 45, Leu 48 and Met 81 are all constituents of the hydrophobic pocket that binds the switch peptide of TnI. The fact that only the V44Q TnC\(^{F27W}\) mutation sensitized muscle to Ca\(^{2+}\) suggests that not all of the hydrophobic amino acids in the hydrophobic pocket are required for maintaining the interaction with the switch peptide of TnI. According to structural studies (Li et al., 1999), the switch region binds to a hydrophobic core formed mainly by helices B and C, where all four TnC mutants mentioned above are located. A study by Buscemi et al. (2002) reported that the phosphorylation of S149 in the switch region of TnI by PAK (p21-activated kinase) sensitized myofilaments to Ca\(^{2+}\). Thus, introducing a negative charge at the interaction site between TnC and TnI increased the affinity of TnC for TnI together with a decrease in the affinity of TnI for actin, similar to our approach of substituting hydrophobic amino acids with polar Gln. Thus, the mechanism of sensitization of cardiac muscle by V44Q TnC\(^{F27W}\) could be to favor the TnC-TnI interaction over the TnI-actin interaction, similar to NMR binding studies of S149 phosphorylated TnI to the N-domain of TnC (Buscemi et al., 2002). It would be
relevant to perform similar studies with the V44QTnC\textsuperscript{F27W} mutation to determine the binding affinity of the switch peptide for the N-domain of V44Q TnC\textsuperscript{F27W}.

\textit{F20Q TnC\textsuperscript{F27W} mutant mimics the effect of the interaction of the N-terminal, PKA phosphorylated fragment of TnI with the N-domain of TnC.}

The 1-32 amino acids, cardiac specific N domain of TnI establishes contacts with the N-domain of cTnC in the absence of phosphorylation (Vassylyev et al., 1998). In the presence of TnI and Ca\textsuperscript{2+}, the regulatory domain of TnC exists in a more "open" conformation. TnI phosphorylation causes TnC to adopt a more "closed" conformation. PKA phosphorylation at Ser\textsuperscript{23}/Ser\textsuperscript{24} of TnI alters the TnC regulatory domain conformation but does not affect the C terminus conformation of TnC. High resolution NMR has been useful in understanding the interactions within the troponin complex as well as conformational changes induced by phosphorylation of TnI (Gaponenko et al., 1999; Sakthivel at al., 2005). Phosphorylation or introduction of negative charge at Ser\textsuperscript{23}/Ser\textsuperscript{24} in the cardiac specific amino terminus shifts the open-closed conformational equilibrium in the N-domain of TnC towards the closed state, which may explain the molecular basis for a decrease in the Ca\textsuperscript{2+} affinity of site II in TnC. NMR studies have previously identified the region around the inactive site I in the N-lobe of TnC and the A-helix as the primary interaction sites with the cardiac specific amino terminus of TnI (Vassylyev et al, 1998). Thus, the introduction of a negative charge at position 20 in the structure of the A-helix would suggest that this modification behaves similarly to the effect of phosphorylation of N-TnI to
desensitize the myofilaments to Ca$^{2+}$. Nevertheless, this could change the interaction between the switch peptide of TnI and TnC through propagated structural changes, which could decrease the interaction of TnI-TnC, as well. Similarly, previous studies in skeletal muscle revealed the importance of the N-helix in transmitting the signal of Ca$^{2+}$ activation to the TnI and TnT in the troponin complex (Smith et al., 1999). Again, TnI-TnC affinity binding studies would provide valuable insight to elucidate the mechanism of TnC mutants in altering the sensitivity of force production.

**Proposed physiological mechanism of altering the force production by cardiac TnC mutants.** Force production in striated muscle is correlated with the number of available myosin binding sites on the thin filament (or the level of the thin filament activation), which structurally is dependent on the position of tropomyosin on the thin filament. Tropomyosin overlays 7 actin monomers along each strand, with a head-to-tail overlap between neighboring Tm. Each Tm binds in the overlap region with a troponin complex, which consists of TnC, TnI and TnT.

The tropomyosin position on the thin filament is influenced by both structural changes induced in the troponin complex when Ca$^{2+}$ binds to TnC and by structural changes at the interface of the actin-myosin interaction. In the absence of Ca$^{2+}$, TnI is bound to actin and holds Tm in a position that inhibits myosin binding to actin (B-state or the “blocked” state). When Ca$^{2+}$ binds to TnC, TnI binding to actin is decreased, therefore Tm can move along actin to a more
permissive position (C-state or the "closed" state), in which force is not yet produced, even though weak crossbridge binding to actin has been reported. As the crossbridge binding becomes stronger, Tm is further displaced on the actin filament, exposing strong myosin binding sites on actin (see Figure 7.4). This event represents the force generating process, and it is referred to as the "open" state or the M-state. This model accounts for the availability of the thin filament binding sites for myosin as determined by the tropomyosin movement, which is under the influence of both Ca\(^{2+}\) binding to the troponin complex and the crossbridge binding to the thin filament.

This study outlines a global mechanism that couples the crossbridge kinetics with the thin filament activation properties: the Ca\(^{2+}\) binding properties of TnC can influence thin filament activation dynamics by modulating the transition from the B-state to the C-state, predominantly at submaximal levels of force production. The fact that the thin filament activation kinetics is not influencing the rate of maximum force generation correlates with the observation that thin filament activation is enhanced by the strongly-bound, force generating crossbridges (Gillis et al., 2007).

This means that, when a "threshold" number of strongly-bound crossbridges is met, the thin filament activation is further increased, promoting more crossbridge binding, such that the rate of the maximum force generation becomes dependent only on the intrinsic rate of crossbridge cycling.
**Plans for future research.** To elaborate a complete model of how thin filament regulation can modulate crossbridge cycling during contraction and relaxation, more questions still need to be answered. The fact that thin filament activation dynamics can influence the rate of sub-maximal force production seems relevant with regard to the modulation of heart function *in vivo*, an a beat-to-beat basis. Nevertheless, this observation can be further investigated by different approaches, which will be proposed below. Moreover, modulating the rate of contraction by altering TnC-Ca$^{2+}$ binding properties would have more physiological significance if the rate of relaxation is, at minimal, not changed or, desirably, even enhanced.

**Investigate the influence of TnC mutants with increased or decreased Ca$^{2+}$ binding affinity on the rate of contraction induced by photolysis of NP-EGTA.** The use of caged Ca$^{2+}$ compounds (such as NP-EGTA) to initiate muscle contraction proved to be a valuable tool that provides information about both thin and thick filament Ca$^{2+}$ activation. The rate of contraction obtained by photolysis of caged Ca$^{2+}$ compounds is known as $k_{Ca}$. Contraction would be induced by photolysis of NP-EGTA in rat skinned cardiac trabeculae at 15°C. At submaximal levels of Ca$^{2+}$ activation, $k_{Ca}$ would be different than $k_{tr}$, due to the Ca$^{2+}$ binding properties of various TnC mutants. In particular, $k_{Ca}$ would be longer than $k_{tr}$, but nevertheless, $k_{Ca}$ measured in the presence of the high Ca$^{2+}$ affinity V44QTnC$^{F27W}$ mutant will be faster then the $k_{Ca}$ measured in the presence of the low Ca$^{2+}$ affinity F20QTnC$^{F27W}$ mutant. At
maximal levels of Ca\(^{2+}\) activation, \(k_{Ca}\) would be the same as \(k_{tr}\), as the crossbridge cycling kinetics would be a primary determinant of the rate of relaxation. At maximal levels of Ca\(^{2+}\) activation, it is hypothesized that \(k_{Ca}\) would be the same as \(k_{tr}\), as the crossbridge cycling kinetics would be a primary determinant of the rate of relaxation, independent of the Ca\(^{2+}\) binding properties of different TnC mutants.

**Investigate the influence of TnC mutants with increased or decreased Ca\(^{2+}\) binding affinity on the rate of relaxation induced by photolysis of diazo-2.** The rate of relaxation would be induced by photolysis of caged Ca\(^{2+}\)-chelator, diazo-2, in rat skinned cardiac trabeculae at 15°C. Two processes can determine the rate of relaxation: the rate of thin filament inactivation and/or the rate of crossbridge detachment.

It is hypothesized that the rate of relaxation might be unchanged or prolonged in trabeculae reconstituted with the high Ca\(^{2+}\) binding affinity V44QTnC\(^{F27W}\) and unchanged or shortened in trabeculae reconstituted with the low Ca\(^{2+}\) binding affinity F20QTnC\(^{F27W}\). In skeletal muscle, fibers reconstituted with a TnC mutant with a faster rate of Ca\(^{2+}\) dissociation from TnC did not speed up relaxation, whereas relaxation was prolonged in the presence of a TnC mutant with a slower Ca\(^{2+}\) dissociation rate from TnC (Luo et al., 2002).

In cardiac muscle, it might be that the Ca\(^{2+}\)-off rate by itself is not predicting the change in the relaxation rate, as the Ca\(^{2+}\) signaling pathway which takes place in the troponin complex involves complex interactions among the
troponin subunits, interactions which are further propagated to the thick filament. Therefore, to better correlate the observed changes in the relaxation rate with the dynamics of troponin subunit interactions within the troponin complex and with actin and/or myosin, additional experiments need to be performed in a system which allows gradual reconstruction of interactions among the “players” of muscle contraction. This system would be cardiac reconstituted thin filaments.

*Investigate the effects of troponin I, tropomyosin, actin and myosin on Ca\(^{2+}\) binding and dissociation from the regulatory domain of troponin in reconstituted thin filaments.* The novel fluorescent probe which reports Ca\(^{2+}\) binding properties of troponin complexes reconstituted into thin filaments in the absence and in the presence of myosin S1 (Davis et al., 2007) will be used to study the Ca\(^{2+}\) binding properties of V44QTnC\(^{F27W}\) and F20QTnC\(^{F27W}\) in systems of increasing complexity. This approach will help to elucidate differential effects of thin filament proteins and myosin S1 on the Ca\(^{2+}\) binding affinity of troponin C. It is predicted that a major role in modulating the propagation of the Ca\(^{2+}\) signal to the thick filament might be the interaction between TnC and TnI in the troponin complex. It is hypothesized that V44QTnC\(^{F27W}\) would increase the affinity of the switch region of TnI for the N-domain of TnC, whereas F20QTnC\(^{F27W}\) would decrease the affinity of the switch region of TnI for the N-domain of TnC. The corresponding increase or decrease in the TnI affinity for TnC would ultimately increase or decrease the available myosin binding sites on actin. Subsequently, the muscle will be sensitized or desensitized, respectively, to Ca\(^{2+}\).
Limitations of the study.

The skinned cardiac trabecula preparation has proven to be a good tool to investigate the Ca\(^{2+}\) sensitivity of force production and the rates of contraction and relaxation under different experimental conditions. However, the preparation could have some limitations. The trabeculae harvested from the right ventricle are assumed to be representative of the whole myocardium. The characteristics of force production and rates of contraction in trabeculae are considered to be equivalent to the function of the whole heart. Trabeculae are thought to play a role in synchronizing the contraction of the endocardial layer of the myocardium during systole. The pressures developed in the left ventricle are much higher than on the right side. Therefore, the trabeculae from the left ventricle are shorter, thicker and end mostly as ridges into the ventricular wall, being unsuitable for tissue harvesting.

The skinned muscle preparation is supposed to approximate the contractile properties of the intact cardiac muscle. Similar standard buffers used by various laboratories mimic the intracellular composition of the intact myocyte, with regard to ionic strength, ionic composition and pH. However, the Ca\(^{2+}\) sensitivity of force production in cardiac muscle is different in the skinned preparation compared with the intact preparation. Even though changes induced by skinning may alter the properties of myofilaments, the observed effects of different experimental conditions on the contractile parameters are assumed to
be qualitatively similar in skinned and intact cardiac muscle.

Traditionally, the skinned muscle preparation was studied at sub-physiological temperatures. Our laboratory investigated the contractile properties of skinned trabeculae at 15°C, for the following reasons: 1) ease of comparison of our results with the results reported by other laboratories at the same temperature, 2) biochemical measurements were performed at the same temperature and 3) the preparation is more stable at 15°C than at physiological temperature.

Moreover, the cardiac TnC mutants are considered to represent functional proteins, which are able to assume the proper tertiary structure and correct folding. Evidence that the proteins are functional is the ability of the muscle to generate force when reconstituted with the TnC mutants. As discussed in Chapter 3 and Appendix B, fluorescent studies and displacement of a TnC mutant with another TnC mutant suggest that TnC is able to incorporate into the thin filament.

The percent of TnC reconstitution in skinned trabeculae can be variable from preparation to preparation. Variable levels of force recovery could be due to various factors: differences in the passive tension, different degrees of compliance, different degrees of viability and integrity of the preparation, and differences in the buffer capacity to diffuse into thinner preparations compared to thicker preparations.
APPENDIX A

CARDIAC MUSCLE
Low Molecular Weight Gel of rat skinned cardiac trabeculae.

**Gel preparation.** Separating gel solution was de-gased for 30 min, and then placed on stirrer and ammonium persulfate (APS) and TEMED were added. The separating gel was poured up the 1.5 inches from the top of the plate and the water overlay added, allowing polymerizing for 1.5 hours. The stacking gel solution was de-gased for 30 min. After the separating gel polymerized, the water overlay was poured off and the top rinsed with water three times. The comb was inserted part of the way and the stacking gel poured and allowed to polymerize for 1 hour. Then the comb was removed, the wells in the stacking gel were rinsed with upper buffer three times, then the gel was loaded, the upper buffer was poured up the electrode and the gel was run at 30 mA constant current and starting voltage of 213 V, for ~ 4 hours. When the gel was stopped, the current was 21 mA and the voltage was 505V.

**Gel fixation and silver staining.** Immediately after the gel is terminated, it is placed in a solution of 150 ml of anhydrous methanol (or 100% ethanol as a substitute), 120 ml of water and 30 ml of glacial acetic acid in a square plastic container (e.g., Rummermaid) that is large enough that the gel is laying flat (i.e., not folded over). The gel is gently rocked in this solution on a rotating table for 30-60 min. It is then immediately transferred (i.e., without washing) to another plastic container with 24 ml of 50% glutaraldehyde and 240 ml of water and gently rocked for 30-60 min (or overnight if this is more convenient). The overnight-soak in the glutaraldehyde solution markedly increases the ability to detect trace amounts of isoforms of protein. The next day, the gel is washed six
times with water. The first time, the glutaraldehyde is discarded; the gel gently transferred to a clean container with water, and gently and manually rocked for about 30 seconds, then the water is discarded. Fresh water is added and the gel is rocked for about 15 minutes on a rotating table in a fume hood. This double-wash step is repeated five more times, each spaced 10-15 minutes from the previous double-wash. After all the washing is completed, the staining and inactivating solutions are prepared. The gel is gently transferred to the stain solution and laid flat to ensure uniform staining, and gently rocked for 6 minutes. Very gently, the gel is transferred to a plastic tray with water and washed three times, with each wash including about 30 seconds of manual rocking. The gel is placed then in a developing solution and the bands of interest are allowed to appear. Then the gel is transferred to a second developing solution. This two-way method for developing keeps the background to a minimum. When the bands of interest are developed, the gel is transferred to the inactivator solution and manually rocked for about 15 seconds. The gel is washed three times (each wash being about 30 seconds with manual rocking). The gel is then scanned and the images captured.

**Solutions.** 12% acrylamide (200:1) cross-linking ratio, 0% glycerol separating gel. This separating gel contains 10 ml water, 7.5 ml 4X 3M separating buffer (pH=9.3), 0.3 ml of 30% acrylamide (200:1), 240 μl of 10% ammonium persulfate and 24 μl of TEMED.
4% acrylamide (50:1), 0% glycerol stacking gel. This gel contains 2.67 ml of 30% acrylamide (50:1), 2.8 ml of 0.5M stacking buffer (pH=6.8), 0.8 ml of 0.1 M EDTA, 12.73 ml of water, 300 μl of 10% ammonium persulfate and 30 μl TEMED.

Stacking gel buffer. To make 250 ml of 4X buffer with 0.5 M Tris, pH=6.8 at 18°C, 15 g of Trizma base is added to ~ 125 ml of water, stirred to dissolve and cooled to 18°C. Then it is titrated to pH 6.8 with HCl, brought to 250 ml in a volumetric flask, filtered and stored at 4°C.

Separating gel buffer. To make 100 ml of 4X buffer with 3.0 M Tris, pH=9.3 at 18°C, 36.34 g of Trizma base is added to ~ 60 ml of water, stirred to completely dissolve before cooling at 18°C (it might be needed to slightly warm it to get it completely into solution). After it was cooled at 18°C, it is titrated to pH 9.3 with HCl, brought to 100 ml in a volumetric flask, filtered and stored at room temperature.

Stain solution. 300 ml of water it is added to a 500 ml glass beaker, which is placed on a stirrer. Then 2.32 g of silver nitrate (Sigma S=0139), 4.2 ml of ammonium hydroxide (from a 14.8 M stock) and 32 ml of 180 mM NaOH are added. The ammonium hydroxide is added rapidly, as solution turns brown for a few seconds and then becomes clear. After mixing completely, the solution is poured into a 9 in X 9 in tray.

Developing solution. In a 9 in X 9 in tray, it is added 500 ml of water, 2.5 ml of 50 mM citric acid and 0.25 ml of formaldehyde.
Gel sample preparation. Samples are weighed and 30 µl sample buffer/ mg tissue are added to 1.5 or 2.0 pre-weighed and labeled microcentrifuge tubes. The tubes are centrifuged very briefly to ensure that the entire sample is in the buffer, homogenized 5-10 seconds, and chilled immediately on ice. Then, the tubes are centrifuged again very briefly, heated for 2 minutes at ~ 65-95°C, then chilled on ice for 5 minutes to stop the heating. The tubes are then centrifuged for 3-4 minutes. The supernatant is transferred to another similarly labeled tube and the pellet is discarded. The sample is diluted 1:10 with sample buffer and stored at –70°C in another tube marked accordingly.

Rat skinned cardiac trabeculae. Six trabeculae were skinned according to the protocol described in Methods, and then each trabecula was cut in half. One half was kept in relaxing solution and the other half was placed in extraction solution, for 30 min. This method provided matched samples for further gel analysis. Five trabeculae previously reconstituted with TnC mutants and kept frozen at –80°C were thawed and the samples prepared as described above. First lane of the gel was loaded with wild-type human cardiac TnC as a control, and the last two lanes were loaded with significant amounts of two TnC mutants. The trabeculae dimensions for the matched pairs and the percent recovery for the trabeculae reconstituted with TnC mutants are indicated in Table A.1.
**Table A.1** Characteristics of the rat skinned cardiac trabeculae used for gel analysis.
<table>
<thead>
<tr>
<th>No.</th>
<th>Length ((\mu)m)</th>
<th>Width/Thickness ((\mu)m)</th>
<th>Length ((\mu)m)</th>
<th>Width/Thickness ((\mu)m)</th>
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<td></td>
<td>un-extracted trabeculae</td>
<td>un-extracted trabeculae</td>
<td>extracted trabeculae</td>
<td>extracted trabeculae</td>
</tr>
<tr>
<td>1</td>
<td>1710</td>
<td>180 / 180</td>
<td>1440</td>
<td>180 / 180</td>
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<td>180 / 144</td>
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<td></td>
<td>Length ((\mu)m)</td>
<td>Width/Thickness ((\mu)m)</td>
<td>Human cardiac TnC mutant and percent of force recovery after reconstitution</td>
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<td>M45QTnC\textsuperscript{F27W} ; 71.7% reconstitution</td>
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<td>F20QTnC\textsuperscript{F27W} ; 78.3% reconstitution</td>
<td></td>
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<tr>
<td>4</td>
<td>840</td>
<td>134.4 / 134.4</td>
<td>L48QTnC\textsuperscript{F27W} ; 58.1% reconstitution</td>
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<tr>
<td>5</td>
<td>1680</td>
<td>100.8 / 100.8</td>
<td>TnC\textsuperscript{F27W} ; 73% reconstitution</td>
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Figure A.1. 12% Low molecular weight gel. NE and E represents non-extracted and extracted trabeculae, respectively (adjacent lanes contain half of the same trabecula). Numbers 1 through 15 represent the lane number. Number 1 contains wild-type (Wt) TnC, numbers 2 through 13 contain un-extracted and extracted trabeculae and lanes 14 and 15 trabeculae reconstituted with TnC mutants. The reconstituted trabeculae were not freshly isolated and some sample deterioration might have occurred. Only in lane 15 it was possible to identify the appearance of a TnC band. Abbreviations used: MHC: myosin heavy chain, TnT: troponin T, TnI: troponin I, TnC: troponin C, Tm: tropomyosin, LC1: myosin light chain 1, LC2: myosin light chain 2.
MHC
C-protein
alpha-actinin
desmin
actin
TnT
Tm
TnI
LC1
TnC
LC2
**Gel analysis.** Gel was scanned and the peak height, peak width and the area under each peak were calculated for actin, TnT, Tm, TnI, LC1, TnC and LC2. To test whether there were differences in the content of the proteins of interest, paired t-tests were applied to both the areas under the peak and to the peak height. The statistical analysis software Minitab was used to perform the statistics on the gel analysis.

The areas corresponding to actin in both un-extracted and extracted trabeculae were not significantly different (paired t-test, p=0.142). Therefore, all the other areas corresponding to the proteins of interest were normalized to actin and a paired t-test applied. There was not a significant statistical difference between TnT areas (p=0.427), Tm areas (p=0.616), LC1 areas (p=0.149) and TnI areas (p=0.318). Because LC2 is not different between un-extracted and extracted trabeculae, areas for LC2 and TnC were added for both un-extracted and extracted trabeculae. This area summation was performed as the TnC band is very close to the LC2 band and it was difficult to distinguish two bands separately. Thus, the areas for TnC and LC2 for un-extracted trabeculae were significantly different from the areas in extracted trabeculae (p=0.038).

The peak height for actin in un-extracted and extracted trabeculae was not significantly different (p=0.407), thus all the other peak heights corresponding to the proteins of interest were normalized to actin. There was not significant statistical difference between peak heights for other proteins, such as LC1 (p=0.13) or LC2 (p=0.146). The only statistical difference was between TnC
peak heights ($p=0.008$). The un-extracted/extracted area ratios for TnC is 0.56, the un-extracted/extracted area ratios for TnC normalized to actin is 0.58, and the cumulative area ratios for TnC + LC2 is 0.61. In conclusion, the similarity of results using various comparisons suggests that the extraction protocol is extracting only TnC from cardiac trabeculae. The myofibrillar composition, apart from TnC, it is not changed.

**Characterization of physiological properties of TnC$^{T53C}$ IAANS, a fluorescent TnC.** In order to study thin and thick filament effects on Ca$^{2+}$ binding to TnC, we developed a novel fluorescent TnC, TnC$^{T53C}$ IAANS. Thr 53 was mutated to Cys because Cys can be selectively labeled with environmentally sensitive fluorophores, such as IAANS. The Thr to Cys mutation is relatively conservative, and Thr 53 does not bind Ca$^{2+}$ directly and it is spatially separated from the Ca$^{2+}$-binding loop, thus upon mutation should not directly interfere with Ca$^{2+}$ binding. Also Thr 53 does not interact with residues within TnC that contribute to the core of the hydrophobic pocket utilized to bind TnI, and Thr 53 itself does not establish any interactions with TnI or TnT, as it can be observed from the Ca$^{2+}$-saturated TnC structures (Takeda & Maeda, 2003). Thr 53 is located in the BC subdomain (helices B and C), which moves away from the NAD subdomain (helices N, A and D) when the regulatory domain binds Ca$^{2+}$ and interacts with TnI. This location confers Thr 53 the potential to report changes in the environment if a fluorescent probe is attached to it. Therefore, substitution of Thr 53 with Cys and subsequent labeling with IAANS was expected to minimally affect TnC function,
and report the structural changes that occur in the regulatory domain of TnC upon Ca\textsuperscript{2+} binding and dissociation. In solution, biochemical studies showed that the fluorescence of TnC\textsuperscript{T53C\textsubscript{IAANS}} follows and minimally affects the Ca\textsuperscript{2+} binding properties of the regulatory domain of TnC (Davis et al., 2007). It is important to investigate if this probe, when reconstituted into rat skinned cardiac trabeculae, affects the Ca\textsuperscript{2+} muscle sensitivity of force production.

Consistent with this idea, skinned trabeculae reconstituted with TnC\textsuperscript{T53C\textsubscript{IAANS}} developed force with nearly identical Ca\textsuperscript{2+} sensitivity (pCa\textsubscript{50}= 5.68 ± 0.03) as wild -type TnC (pCa\textsubscript{50}= 5.70 ± 0.02) and endogenous TnC (pCa\textsubscript{50}= 5.70 ± 0.02, n=6 (Figure 8.2). The fluorescent TnC TnC\textsuperscript{T53C\textsubscript{IAANS}} was also able to recover force (79 ± 4 %) to a similar extent as the wild-type (79 ± 7 %). Thus, TnC\textsuperscript{T53C\textsubscript{IAANS}} behaved biochemically and physiologically similar to wild-type and endogenous cardiac TnC.
Figure A.2. Force vs. pCa relationship of TnC\textsuperscript{IAANS}\textsuperscript{53C} in comparison with TnC\textsuperscript{endog} or wild-type TnC. TnC\textsuperscript{IAANS}\textsuperscript{53C} is shown in blue.
APPENDIX B

SKELETAL MUSCLE
Investigation of the Ca\(^{2+}\)-sensitivity of muscle force production for skeletal TnC mutants reconstituted into rabbit skinned psoas fibers. In this study, we investigated the effect of mutating hydrophobic residues in the N-terminal domain of TnC on the Ca\(^{2+}\)-binding affinities of the TnC-TnI (inhibitory peptide) and on muscle force production. We wanted to determine if TnC-TnI Ca\(^{2+}\) binding properties are a better predictor of the Ca\(^{2+}\)-sensitivity of force production than the Ca\(^{2+}\)-binding properties of isolated TnC. The Phe to Trp mutation at position 29 (TnC\(^{F29W}\)) was introduced to follow the fluorescence changes correlated with the conformational changes in the N-domain upon Ca\(^{2+}\)-binding. When Ca\(^{2+}\) binds the N-domain of TnC, a large conformational change occurs in the regulatory domain, which is known as an open state. Hydrophobic side chains are exposed and favors the binding of TnI to the N-domain of TnC, lessening the binding of TnI to actin. Subsequently, the myosin binding sites on actin are exposed and ultimately myosin can bind to actin, filaments slide, and the muscle contracts. The binding of TnI to TnC enhances the TnC Ca\(^{2+}\)-binding affinity, by maintaining TnC in the open state. Mutation of hydrophobic residues in the regulatory domain of TnC to polar Gln had various effects on the Ca\(^{2+}\) binding affinity of the isolated TnC or TnC-TnI binding. Some of them stabilized the open state of TnC and increased Ca\(^{2+}\) binding affinity (e.g., V45QTnC\(^{F29W}\), M46QTnC\(^{F29W}\) or M82QTnC\(^{F29W}\)) while others impeded the formation of the open state and decreased Ca\(^{2+}\) binding affinity (e.g., I62QTnC\(^{F29W}\), I73TnC\(^{F29W}\)).
The hydrophobic mutation which decreased the Ca\textsuperscript{2+} binding affinity of TnC-TnI are located in a \(\beta\)-sheet in between the two EF-hand Ca\textsuperscript{2+} binding sites, and do not establish a direct interaction with TnI, according to the NMR structure. Thus, the maintenance of the \(\beta\)-sheets interaction seems to be very important for enabling TnC to bind Ca\textsuperscript{2+} with high affinity. Ultimately, the ability of the TnC mutants to support force and the Ca\textsuperscript{2+} sensitivity of force production were investigated by reconstituting TnC mutants into rabbit skinned psoas fibers.

Endogenous TnC in psoas muscle fibers was extracted and the replaced with TnC\textsuperscript{F29W} or its mutants and steady-state force measurements at various Ca\textsuperscript{2+} activations were performed to define the Ca\textsuperscript{2+} sensitivity of force production for each mutant. After TnC extraction, the average force generated by single skinned muscle fibers was 2.3 ± 0.5% of the maximal force. Subsequent reconstitution of the muscle fibers with V45QTnC\textsuperscript{F29W}, M46QTnC\textsuperscript{F29W}, TnC\textsuperscript{F29W}, M81QTnC\textsuperscript{F29W}, or F78QTnC\textsuperscript{F29W} recovered 82 ± 5, 73 ± 4, 90 ± 3, 65 ± 8, and 80 ± 2% of the maximal force at pCa 4, respectively.

The Ca\textsuperscript{2+} dependence of force generation with TnC\textsuperscript{F29W} or its mutants followed qualitatively more closely to the Ca\textsuperscript{2+} sensitivities of the mutant TnC\textsuperscript{F29W}-Tnl\textsubscript{96–148} complexes and not to that of the isolated TnC\textsuperscript{F29W} proteins. In comparison with TnC\textsuperscript{F29W}, two mutants increased (V45Q TnC\textsuperscript{F29W} and M46QTnC\textsuperscript{F29W}) and two mutants decreased (M81QTnC\textsuperscript{F29W} and F78QTnC\textsuperscript{F29W}) the Ca\textsuperscript{2+}-sensitivity of muscle force production. Comparison of the effect of these mutations on the force vs pCa relationship with the effects on Ca\textsuperscript{2+} binding to
isolated TnC or TnC-TnI suggest that the TnC-TnI Ca\(^{2+}\) binding properties are predicting much better how changes in Ca\(^{2+}\) binding to TnC would affect the Ca\(^{2+}\) sensitivity of force production.

Another effect observed in the reconstituted fibers and which did not correlate with the Ca\(^{2+}\) binding properties of the isolated TnC, was the maximal amount of force produced by each mutant. In particular, three TnC mutants, I73QTnC\(^{F29W}\), I62QTnC\(^{F29W}\), I37QTnC\(^{F29W}\) and F26QTnC\(^{F29W}\) recovered very poorly at 45 ± 6, 12 ± 4, 15 ± 1 and 13 ± 15% of the maximal force, respectively. For the majority of these mutants, the large decrease in TnI binding affinity is the possible reason why the mutants were not able to support force. It is possible that Ca\(^{2+}\) binding to these mutants impedes TnI binding to TnC; therefore TnI is bound to actin, keeping the myosin binding sites on actin un-exposed and rendering the thin filament more inactive.

A possible concern was the ability of TnC mutants, which were unable to support force, to reconstitute into skinned fibers. To address this concern, additional exchange experiments were performed in fibers. At time 0, the maximal force recovered by TnC\(^{F29W}\) at pCa 4.0 in a reconstituted muscle fiber. The fiber was then transferred to a relaxing solution containing 16.7μM I62QTnC\(^{F29W}\), and the force generated at pCa 4.0 was measured at several time intervals. The amount of force production decreased with time, eventually reaching a value similar to that generated by fibers solely reconstituted with I62QTnC\(^{F29W}\). This decrease in force suggests that I62QTnC\(^{F29W}\) was able to
bind to the thin filaments and competitively displace TnC\textsuperscript{F29W} from the thin filament. Also, when a fiber was initially reconstituted with I62QTnC\textsuperscript{F29W} and then soaked in relaxing solution with TnC\textsuperscript{F29W} for various time intervals, the maximal force increased with a time course similar to that at which I62QTnC\textsuperscript{F29W} inhibited the force generated with TnC\textsuperscript{F29W}. Similar displacement studies were performed with I37QTnC\textsuperscript{F29W} and F26QTnC\textsuperscript{F29W}. Thus, the displacement studies suggest that the TnC\textsuperscript{F29W} mutants that minimally support force (<15%) do bind to thin filaments and incorporate into the troponin complex.

**Investigation of the effect of skeletal TnC mutants reconstituted into rabbit skinned psoas fibers on the rate of contraction at submaximal Ca\textsuperscript{2+} activation.** The level of Ca\textsuperscript{2+} activation correlates with the rate of striated muscle contraction. Ca\textsuperscript{2+} binding to TnC activates the thin filament, which subsequently allows myosin to bind to actin and crossbridge cycling, resulting in muscle force generation. Therefore, the kinetics of activation of the thin and thick filaments can both contribute to modulating the rate of contraction. At maximal activation of rabbit skinned skeletal muscle fibers, the rate of contraction, estimated as k\textsubscript{Ca}, is determined solely by the kinetics of crossbridge cycling. A study by Luo et al. (2006) showed that reconstitution of psoas fibers with mutants of TnC with increased Ca\textsuperscript{2+} binding affinity due to a decreased Ca\textsuperscript{2+} dissociation rate (M82Q), or with mutants of TnC with decreased Ca\textsuperscript{2+} binding affinity, due to an increased Ca\textsuperscript{2+} dissociation rate (NHdel TnC), did not affect maximal k\textsubscript{Ca}. We investigated the effect of reconstituting rabbit skinned psoas fibers with TnC mutants with
various Ca\textsuperscript{2+} binding affinities due to changes in the Ca\textsuperscript{2+} association/dissociation rate to/from TnC at sub-maximal Ca\textsuperscript{2+} activation. The k\textsubscript{Ca} experiments have been performed similarly to the protocol described by Luo at al. (2002). V45QTnC\textsuperscript{F29W} sensitized the muscle to Ca\textsuperscript{2+} and F78QTnC\textsuperscript{F29W} desensitized the muscle to Ca\textsuperscript{2+}, respectively.

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<tr>
<th>Rate of contraction at submaximal force</th>
<th>TnC</th>
<th>Force pre-flash</th>
<th>Force post-flash</th>
<th>K\textsubscript{Ca} (s\textsuperscript{-1})</th>
<th>% Force recovery</th>
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<tr>
<td>TnC\textsuperscript{endog}</td>
<td>0</td>
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<td>5.4 ± 0.7</td>
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<tr>
<td>TnC\textsuperscript{endog}</td>
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<td>60.6 ± 6.0</td>
<td>7.8 ± 1.8</td>
<td>N/A</td>
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<tr>
<td>TnC\textsuperscript{F29W}</td>
<td>23.0 ± 3.2</td>
<td>69.6 ± 15.2</td>
<td>12.5 ± 3.1</td>
<td>82.4 ± 0.6</td>
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<td>F78Q TnC\textsuperscript{F29W}</td>
<td>2.7 ± 1.3</td>
<td>36.0 ± 1.6</td>
<td>12.5 ± 3.1</td>
<td>94.6 ± 5.4</td>
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<tr>
<td>G34D TnC\textsuperscript{F29W}</td>
<td>3.4 ± 2.0</td>
<td>64.5 ± 8.2</td>
<td>5.5 ± 0.3</td>
<td>42.3 ± 0.1</td>
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Table B.1. Summary of the levels of pre- and post-photolysis and the rate of contraction for TnC mutants.
Preliminary results investigating the rate of contraction for TnC mutants at various levels of Ca\textsuperscript{2+} activation are presented in Table B.1. Ideally, the pre-photolysis force should be minimal or less than 10-20% of the force the muscle generated in pCa 4.0 before photolysis. For the high Ca\textsuperscript{2+} binding affinity V45QTnC\textsuperscript{F29W}, the level of force pre-photolysis was more than 50% of the force at saturating Ca\textsuperscript{2+}. Therefore, the data was not used in this study.

Preliminary data suggests that the rate of contraction at submaximal Ca\textsuperscript{2+} activation for TnC mutants with various on and off rates of Ca\textsuperscript{2+} binding to TnC is not different from the rate of contraction in un-extracted fibers. It seems that, in skeletal muscle, the rate of crossbridge cycling is the major determinant of the contraction rate.

**Characterization of physiological properties of fluorescent probes attached to skeletal TnC.** In order to monitor the Ca\textsuperscript{2+}-binding properties of the N-regulatory domain of TnC, two fluorescent TnC probes have been characterized. TnC was labeled with two different environmentally sensitive fluorescent probes, IAANS and MIANS, respectively. The intrinsic threonine was mutated to cysteine to minimize the interference in fluorescence, and serine has been substituted for cysteine to attach the fluorescent probe IAANS or MIANS, respectively. In solution and in skeletal myofibrils, both IAANS and MIANS reported similar N-terminal Ca\textsuperscript{2+} dissociation rates (personal communication, J.Davis), which made them valuable tools to follow Ca\textsuperscript{2+} -binding to TnC. However, it was important to verify that both TnC constructs were biologically
active. Both IAANS and MIANS fluorescent TnC were individually reconstituted into rabbit skinned psoas fibers to test the biological function as measured by the force vs. pCa relationship. After TnC extraction, the average force generated was $3 \pm 2\%$ of the maximal force before extraction. Subsequent reconstitution with TnC$^{T54C, C101S}$, TnC$^{IAANS}$, or TnC$^{MIANS}$ recovered $90 \pm 7\%$, $87 \pm 6\%$, and $85 \pm 8\%$ of the maximal force, respectively. Table B.2 shows that fibers reconstituted with fluorescent probes IAANS or MIANS had similar Ca$^{2+}$ sensitivity of force development as endogenous TnC or the TnC$^{T54C, C101S}$. Thus, both fluorescent TnC manifested nearly identical biological activity when reconstituted in muscle fibers (Figure 9.5). Therefore, attaching the fluorescent probes to TnC did not affect the physiological properties of TnC.

<table>
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<tr>
<th>TnC</th>
<th>PCa$_{50}$</th>
<th>Hill coefficient</th>
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<tr>
<td>Endogenous (n=6)</td>
<td>$5.97 \pm 0.03$</td>
<td>$3.00 \pm 0.43$</td>
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<tr>
<td>TnC$^{T54C, C101S}$ (n=4)</td>
<td>$5.90 \pm 0.02$</td>
<td>$2.51 \pm 0.32$</td>
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<tr>
<td>TnC$^{IAANS}$ (n=3)</td>
<td>$5.97 \pm 0.02$</td>
<td>$3.29 \pm 0.48$</td>
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<tr>
<td>TnC$^{MIANS}$ (n=3)</td>
<td>$6.02 \pm 0.01$</td>
<td>$3.12 \pm 0.27$</td>
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</table>

Table B.2. Summary of PCa$_{50}$ and Hill coefficient for endogenous TnC, unlabeled TnC and TnC labeled with fluorescent probes IAANS and MIANS skeletal TnC reconstituted into psoas fibers.
Figure B.1. Force versus pCa relationship for endogenous TnC, un-labeled TNC and skeletal TnC labeled with fluorescent probes IAANS and MIANS.
BIBLIOGRAPHY


Brenner B. 1986. The cross-bridge cycle in muscle. mechanical, biochemical, and structural studies on single skinned rabbit psoas fibers to characterize cross-bridge kinetics in muscle for correlation with the actomyosin-ATPase in solution. Basic Res. Cardiol. 81 Suppl 1 : 1-15


Finley NL, Howarth JW, Rosevear PR. 2004. *Structure of the Mg2+-loaded C-lobe of cardiac troponin C bound to the N-domain of cardiac troponin I: Comparison with the Ca2+-loaded structure.* Biochemistry. 43 : 11371-9

Fitzsimons DP, Patel JR, Moss RL. 2001. *Cross-bridge interaction kinetics in rat myocardium are accelerated by strong binding of myosin to the thin filament.* J. Physiol. 530 : 263-72


Geeves MA, Conibear PB. 1995 *The role of three-state docking of myosin S1 with actin in force generation.* Biophys J. Apr;68(4 Suppl):194S-199S;


Gordon AM, Ridgway EB. 1987. *Extra calcium on shortening in barnacle muscle. is the decrease in calcium binding related to decreased cross-bridge attachment, force, or length?* J. Gen. Physiol. 90 : 321-40


Hofmann PA, Fuchs F. 1987. *Effect of length and cross-bridge attachment on Ca2+ binding to cardiac troponin C*. Am. J. Physiol. 253 : C90-6


Kawai M, Saeki Y, Zhao Y. 1993. *Crossbridge scheme and the kinetic constants of elementary steps deduced from chemically skinned papillary and trabecular muscles of the ferret*. Circ. Res. 73 : 35-50


Kerrick WG, Xu Y. 2004. Inorganic phosphate affects the pCa-force relationship more than the pCa-ATPase by increasing the rate of dissociation of force generating cross-bridges in skinned fibers from both EDL and soleus muscles of the rat. J. Muscle Res. Cell. Motil. 25 : 107-17


Mehegan JP, Tobacman LS. 1991. Cooperative interactions between troponin molecules bound to the cardiac thin filament. J. Biol. Chem. 266 : 966-72


Palmer S, Kentish JC. 1998. Roles of Ca2+ and crossbridge kinetics in determining the maximum rates of Ca2+ activation and relaxation in rat and guinea pig skinned trabeculae. Circ. Res. 83 : 179-86


Swartz DR, Moss RL, Greaser ML. 1997. *Characteristics of troponin C binding to the myofibrillar thin filament: Extraction of troponin C is not random along the length of the thin filament*. Biophys. J. 73 : 293-305

Szczesna D, Fajer PG. 1995. *The tropomyosin domain is flexible and disordered in reconstituted thin filaments.* Biochemistry. 34 : 3614-20


Tikunova SB, Davis JP. 2004. *Designing calcium-sensitizing mutations in the regulatory domain of cardiac troponin C.* J. Biol. Chem. 279 : 35341-52

Tikunova SB, Rall JA, Davis JP. 2002. *Effect of hydrophobic residue substitutions with glutamine on ca(2+) binding and exchange with the N-domain of troponin C.* Biochemistry. 41 : 6697-705


Wolff MR, McDonald KS, Moss RL. 1995. Rate of tension development in cardiac muscle varies with level of activator calcium. Circ. Res. 76 : 154-60