Troponin I Isoforms and Chimeras: Tuning the Molecular Switch of Cardiac Contraction

Margaret V. Westfall1,2 and Joseph M. Metzger1

Departments of Physiology1 and Surgery,2 University of Michigan, Ann Arbor, Michigan 48109-0686

Troponin I, a key myofilament protein, plays a critical role in regulating force generation in striated muscle by acting as a Ca2+-dependent molecular switch. Domains contributing to the functional properties of troponin I have recently been defined in intact myofilaments of adult cardiac myocytes. This has been attained using gene transfer of chimeras derived from two troponin I isoforms expressed during cardiac development.

The primary function of the heart is to pump blood, and this function depends directly on the capacity of cardiac muscle to generate force. Parallel arrays of thick and thin myofilaments organized into sarcomeres within cardiac myocytes are directly responsible for force generation. Specifically, myosin cross-bridges on thick filaments attach to actin on thin filaments to produce force. This actomyosin interaction is regulated by Ca2+ binding to troponin, a heterotrimeric complex on thin filaments that is comprised of the Ca2+-binding subunit troponin C (TnC), inhibitory subunit troponin I (TnI), and tropomyosin-binding subunit troponin T (TnT). A fundamental goal of research on the troponin regulatory complex is to understand the unique functions of each subunit within intact myofilaments of muscle cells.

Changes in TnI function are linked to alterations in cardiac performance

The influence of TnI on cardiac function has received increasing attention due to correlations between modification of cardiac TnI (cTnI) and changes in cardiac function. Normally, TnI behaves as a molecular switch within the myofilament by toggling between actin and TnC in a Ca2+-dependent manner (14). In cardiac muscle, events such as the developmental transition from slow skeletal to cardiac TnI isoform expression (12), as well as β-adrenergic-activated protein kinase A (PKA)-mediated phosphorylation of cTnI, modulate the tension response (Fig. 1; Refs. 11 and 14). Several clinically important pathophysiological conditions known to compromise heart function have also been linked to TnI (Fig. 1). For example, release of cTnI from the heart is now used clinically as a key indicator of myocardial ischemia (3). Ischemia and other cardiovascular conditions are also associated with changes in TnI phosphorylation state (20), and changes in cellular ions that can accompany myocardial ischemia, such as acidic pH (10), are associated with TnI dysfunction. Moreover, work in transgenic animals has shown that removal of 17 amino acids at the carboxy terminus of cTnI is sufficient to produce myocardial stunning. Myocardial stunning is a clinical event resulting from reduced perfusion (9) in which TnI degradation occurs and causes decreased myocardial function. In addition, mutations within cTnI are linked to the human disease state hypertrophic cardiomyopathy (8). Together, these points underscore the importance of investigating the function of TnI within the myofilaments of cardiac myocytes.

TnI influences myofilament Ca2+ sensitivity of tension

Insights into the function of TnI have been gained by studying cardiac development. In comparative studies of fetal/neonatal and adult myocardium, developmental transitions in TnI isoform expression correlated with significant differences in Ca2+-activated force development (13). Results from these studies indicated that expression of the slow skeletal isoform of TnI (ssTnI) in fetal/neonatal myocardium correlated with heightened Ca2+ sensitivity compared with adult hearts, which exclusively express the cTnI isoform. This developmental relationship is complicated by near-simultaneous isoform transitions in numerous sarcomeric proteins, making it challenging to assign a specific physiological function to an isoform transition for a single contractile protein (12).

Another approach to examining TnI function has been to use purified protein or fragments reconstituted into actomyosin preparations (6, 11, 14). Important strides toward understanding the fundamental functions of this protein have been achieved using this approach. Biochemical studies provided the first evidence supporting the idea that TnI acted as a molecular switch. At low Ca2+ levels, such as in diastole, TnI bound tightly to actin and inhibited actomyosin ATPase activity, whereas increasing Ca2+ levels, as during systole, resulted in high-affinity binding of TnI for TnC and reduced affinity for actin (6, 11, 14). Experiments with purified full-length protein and fragments of TnI have revealed important information about TnI structure and function by showing that TnI interacts with TnC in an antiparallel arrangement (6) and contains multiple actin- and TnC-binding regions (15). Despite these advances in our understanding of TnI, contractile protein stoichiometry within these preparations cannot be duplicated in biochemical studies, and actomyosin ATPase activity measured in biochemical studies may not reflect the Ca2+-activated myofilament tension response under some ionic conditions (5). Thus it is critical to investigate TnI function within the intact myofilaments of cardiac myocytes to determine how changes in TnI function influence force output.

To study TnI function within the intact myofilament requires specific manipulation of TnI within sarcomeres, followed by
measurement of force produced by the myocyte. Gene transfer into myocytes represents a new tool for the precise genetic dissection of individual contractile proteins in the adult cardiac sarcomere. Rod-shaped adult myocytes are used for these studies because they display the required parallel arrays of myofilaments necessary for the measurement of force. Recombinant adenoviral vectors facilitate rapid and efficient expression and myofilament incorporation of individual contractile proteins within sarcomeres of adult myocytes (18). Gene sequences up to ~7 kb can be engineered into first-generation, replication-deficient adenovirus vectors, whereas longer sequences up to ~30 kb can be included with gutted viral vectors (reviewed in Ref. 4). After gene delivery, myocytes are cultured under serum-free conditions to prevent dedifferentiation of the contractile apparatus (Fig. 2; Refs. 16–18). This approach results in nearly complete replacement of endogenous cTnI with newly expressed TnI in the myofilaments of adult cardiac myocytes without detected changes in the stoichiometry and/or isoform expression of the contractile proteins. As a result, the influence of TnI isoforms and novel TnI proteins on Ca$^{2+}$-activated myofilament tension can be followed in individual adult cardiac myocytes as the nascent TnI gene products are expressed and incorporated into the myofilaments of living myocytes.

In studies using gene transfer, TnI function has been examined by comparing Ca$^{2+}$-activated tension in myocytes expressing cTnI vs. the ssTnI isoform (18). Increased Ca$^{2+}$ sensitivity of tension and decreased myofilament cooperativity are observed in ssTnI-expressing compared with cTnI-expressing adult cardiac myocytes. These results provide direct evidence that TnI isoform expression prominently contributes to the differences in Ca$^{2+}$-activated myofilament tension observed during cardiac development, a finding subsequently confirmed in experiments with transgenic mice (7). An important question arising from these results is whether one or more domain(s) within the TnI protein are responsible for these isoform-specific differences in myofilament tension generation.

Identification of TnI domains influencing myofilament Ca$^{2+}$ sensitivity

Functional domains within TnI have been widely studied using biochemical approaches. Multiple TnC and actin binding domains within TnI have been localized to the carboxy region of the protein with these approaches (6, 15). The minimum sequence necessary to inhibit actomyosin ATPase activity has been similarly defined using biochemical analysis of TnI fragments, and this carboxy portion of TnI is designated the inhibitory peptide (IP) region (Fig. 3A; Ref. 6). Binding studies indicate that the IP region toggles between actin and TnC in the absence and presence of Ca$^{2+}$, respectively. Secondary actin and TnC binding regions lying further downstream in TnI also have been identified using TnI fragments (15). Thus sequence differences within the IP and other actin/TnC binding regions within the carboxy portion of TnI may be responsible for isoform-specific differences in Ca$^{2+}$-activated force between cTnI- and ssTnI-expressing myocytes.

To test this hypothesis, two chimeras of the cTnI and ssTnI isoforms were designed and individually expressed in adult myocytes following gene transfer (16, 17). One chimera, N-slow/card-C TnI, was formed by joining the amino terminal 68 amino acids from ssTnI with the carboxy terminal 110 amino acids from cTnI (Fig. 3A). A second chimera, N-card/slow-C TnI, was constructed from the amino terminal 100 amino acids from cTnI and the carboxy terminal 120 amino acids from ssTnI.

Interestingly, the functional phenotypes of myocytes expressing these chimeras did not follow predictions derived from earlier biochemical results. Instead, the Ca$^{2+}$ sensitivity of tension in myocytes expressing N-slow/card-C TnI decreased relative to cTnI, and N-card/slow-C TnI expression increased myofilament Ca$^{2+}$ sensitivity of tension above that shown for adult cardiac myocytes expressing ssTnI (16, 17). Thus a single domain in the
least two isoform-specific functional domains within TnI were required to explain the chimaera phenotypes. The predicted carboxy terminal domain was clearly present, with the sequence present in ssTnI causing a greater increase in Ca\(^{2+}\) of tension than the cTnI sequence. Also, the leftward shift in Ca\(^{2+}\) sensitivity of tension caused by N-card/slow-C TnI vs. ssTnI, as well as the decreased sensitivity observed with N-slow/card-C TnI vs. cTnI-expressing myocytes, provided evidence for a second isoform-specific, myofilament Ca\(^{2+}\) sensitivity domain within the carboxy terminus of TnI could not fully explain these results. At least two isoform-specific functional domains within TnI were required to explain the chimaera phenotypes. The predicted carboxy terminal domain was clearly present, with the sequence present in ssTnI causing a greater increase in Ca\(^{2+}\) of tension than the cTnI sequence. Also, the leftward shift in Ca\(^{2+}\) sensitivity of tension caused by N-card/slow-C TnI vs. ssTnI, as well as the decreased sensitivity observed with N-slow/card-C TnI vs. cTnI-expressing myocytes, provided evidence for a second isoform-specific, myofilament Ca\(^{2+}\) sensitivity domain within the carboxy terminal portion of TnI (Fig. 3A). In contrast to domain(s) within the carboxy region, the amino terminal domain was not predicted from biochemical findings. Results with TnI chimeras indicate that the amino terminal domain of cTnI results in increased Ca\(^{2+}\) sensitivity of tension compared with the amino terminal domain of ssTnI.

Further studies are now needed to identify the amino acids involved in defining isoform-specific differences in myofilament Ca\(^{2+}\) sensitivity within the amino and carboxy terminal domains of TnI. Several regions reported to bind actin and/or TnC in the carboxy portion of TnI encompass isoform-specific amino acid sequences within TnI. These sequence differences could contribute to isoform-specific differences in myofilament Ca\(^{2+}\) sensitivity. One or more amino acid regions in the amino terminal region of TnI may also contribute to the isoform-specific function of this domain. In the future, myocytes expressing unique TnIs containing well-defined isoform-specific sequence substitutions can be used to advance our understanding of the molecular switch functions of TnI in working myocytes and/or hearts under physiological and pathophysiological conditions.

**TnI acts as a pH sensor in the myofilaments**

Cellular acidosis is an important pathophysiological condition in which TnI significantly influences the Ca\(^{2+}\)-activated tension response in cardiac myocytes (10). Acidosis develops during myocardial ischemia and is thought to be an important contributor to the contractile dysfunction associated with ischemia (10). Adult cardiac muscle develops more severe contractile dysfunction in response to acidosis than fetal/neonatal myocardium (13). Expression of the ssTnI isoform in developing myocardium has been proposed to provide a significant protective effect on force in fetal/neonatal hearts during ischemia based on the correlation between ssTnI expression and relative pH insensitivity in fetal myocardium (see references in Ref. 18). This correlation has given rise to the idea that TnI isoforms significantly influence the myofilament pH response in myocardium. To test this hypothesis directly, the tension response to acidosis was compared in adult cardiac myocytes expressing cTnI and ssTnI. Compared with myocytes expressing cTnI, expression of ssTnI significantly blunted the acidosis-insuenced decrease in Ca\(^{2+}\) sensitivity of tension (18). These results demonstrate that TnI acts as an important pH sensor in an isoform-specific manner. In other experiments, myocytes expressing the N-slow/card-C TnI chimera responded to acidic pH in a manner comparable with cTnI-expressing myocytes and myocytes expressing ssTnI or N-card/slow-C TnI responded to acidic pH in an equivalent manner (Fig. 3C; Refs. 16 and 17). This chimera analysis provides evidence that the isoform-specific, pH-sensitive domain lies in the carboxy portion of TnI.

**cTnI is a phosphoprotein**

The regulatory properties of cTnI can also be modified by PKA-mediated phosphorylation of Ser23/Ser24 (11, 14) located within the unique 32-amino acid extension of cTnI. Phosphorylation of myosin binding protein C and both serines on TnI have been implicated in causing decreased myofilament...
ment Ca\textsuperscript{2+} sensitivity of tension, but the contribution of each protein to the functional response has been controversial (14). The amino terminal extension of cTnI is absent from ssTnI, and no detectable PKA-mediated phosphorylation of ssTnI is observed following gene transfer of this isoform into adult myocytes (19). The Ca\textsuperscript{2+} sensitivity of tension also does not change significantly in response to PKA in the ssTnI-expressing adult cardiac myocytes (Fig. 3C). These results, together with comparable findings in transgenic mice expressing ssTnI (7), provide conclusive evidence that phosphorylated cTnI is the major myofilament phosphoprotein contributing to the PKA-mediated decrease in myofilament Ca\textsuperscript{2+} sensitivity of tension.

The mechanism whereby PKA-mediated cTnI phosphorylation decreases the Ca\textsuperscript{2+} sensitivity of tension is not well understood. Phosphorylation of cTnI may cause direct conformational changes in the amino terminal extension to inhibit cTnI interactions with TnC and lead to reduced myofilament Ca\textsuperscript{2+} sensitivity (1). Alternatively, PKA-mediated cTnI phosphorylation may decrease myofilament Ca\textsuperscript{2+} sensitivity via long-range conformational changes in downstream TnI domains that interact with TnC (14). Evidence to support both of these possibilities has been obtained using purified TnI (1, 2, 11). To gain insight into this problem, the magnitude shift in Ca\textsuperscript{2+} sensitivity of tension caused by PKA phosphorylation was measured in myocytes expressing the N-card/slow-C TnI chimera. This protein was phosphorylated to levels comparable with cTnI in permeabilized myocytes treated with PKA and in intact myocytes stimulated with isoproterenol, and the Ca\textsuperscript{2+}-activated myofilament tension response to PKA was comparable in myocytes expressing either cTnI or N-card/slow-C TnI (19). Thus isoform-specific differences between cTnI and N-card/slow-C TnI that were previously shown to influence baseline myofilament Ca\textsuperscript{2+} sensitivity did not influence the magnitude of the Ca\textsuperscript{2+}-sensitive functional response to PKA. Further studies also indicated that the decreased Ca\textsuperscript{2+} sensitivity of tension observed in response to PKA phosphorylation of cTnI was independent from the pH-sensitive decrease in sensitivity previously localized to the carboxy portion of TnI. These findings are consistent with the idea that PKA phosphorylation causes a direct conformational change in the amino terminus of cTnI, although further experiments are needed to exclude the possibility that isoform-independent conformational changes in the carboxy portion of TnI contribute to the PKA-mediated myofilament functional response.

Future directions

Gene transfer of TnI isoforms and chimeras into adult cardiac myocytes has opened an exciting path for investigating the relationship between TnI structure and myofilament function. New insights into TnI function and its response to important cellular conditions such as acidosis and/or modulation by phosphorylation illustrate the usefulness of viral-based gene transfer for studying protein function within the contractile apparatus of adult cardiac myocytes. Future research could lead to the identification of precise isoform-specific functional domains within TnI. Charge and/or folding differences may be important for the isoform-dependent effects on basal as well as pH- and phosphorylation-dependent changes in performance. These hypotheses can now be tested to determine the motifs or sequences that lead to TnI isoform-dependent differences in function. In the future, the development of genetically engineered TnIs may offer a new approach to redesign sarcomeric function in failing or cardiomyopathic hearts.

References