Covalent and Noncovalent Modification of Thin Filament Action: The Essential Role of Troponin in Cardiac Muscle Regulation

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Abstract—Troponin is essential for the regulation of cardiac contraction. Troponin is a sarcomeric molecular switch, directly regulating the contractile event in concert with intracellular calcium signals. Troponin isoform switching, missense mutations, proteolytic cleavage, and posttranslational modifications are known to directly affect sarcomeric regulation. This review focuses on physiologically relevant covalent and noncovalent modifications in troponin as part of a thematic series on cardiac thin filament function in health and disease. (Circ Res. 2004;94:146-158.)

Key Words: calcium | phosphorylation | myocardium | contractility

Since its discovery some 40 years ago, troponin has been a focal point for studies on the mechanisms of calcium regulation of striated muscle function. Over 6000 articles have been published on troponin and thin filament regulation, and appropriately, there have been numerous outstanding reviews on troponin and striated muscle regulatory function. The scope of the present review is on posttranslational modifications of the thin filament, with a primary focus on troponin. Specifically, the first and major part of this review discusses how covalent modification of troponin, largely through phosphorylation by protein kinases A and C, affects troponin and muscle performance in vitro and in vivo. In the second part, we overview how noncovalent modification of troponin, focusing on muscle acidification, alters troponin’s function at the cellular, organ, and whole animal levels. This review is intended to complement the companion articles in this series that address additional vital aspects of troponin and thin filament regulatory function in health and disease.

Thin Filament Regulatory System

The thin filament regulatory system of cardiac and skeletal muscle consists of a highly ordered assembly of proteins (Figure 1). This regulatory machinery is directly responsible for governing the force-generating interactions between myosin and actin. The thin filament structure of mammalian skeletal and cardiac muscle is dominated by actin, a globular protein that, under physiological conditions, polymerizes into elongated filaments of double helical strands. Residing in the groove formed between actin strands is tropomyosin (Tm), an elongated protein that spans seven actin monomers and is polymerized head-to-tail along the actin filament. Associated with each Tm is troponin, a globular protein complex con-
Tropomyosin. In the absence of Ca\(^{2+}\) and (3) troponin T (TnT), the subunit that binds to tropomyosin.1,2 In the absence of Ca\(^{2+}\), tropomyosin is thought to sterically hinder strong, force-producing interactions between actin and myosin.8 Addition of Ca\(^{2+}\) is thought to dis inhibit crossbridge formation by binding to troponin C, resulting in altered conformation of troponin and movement of tropomyosin deeper into the groove between actin strands, thus revealing actin binding sites for myosin attachment.8 Support for this model comes from time-resolved x-ray diffraction studies that show apparent movement of tropomyosin before crossbridge attachment and tension development.8 Direct evidence in support of this idea has been provided in studies using 3-dimensional reconstruction of electron micrographs of interacting filaments.9,10 The steric blocking model has been challenged by investigators who showed that under some conditions (eg, low ionic strength and high actin concentration), the thin filament system does not inhibit crossbridge formation but rather regulates a kinetic step among attached crossbridges.11,12 Recently, a 3-state model of thin filament regulation has been developed.13 Briefly, the model proposes three states of the thin filament (blocked, closed, and open), and that transitions between these states are governed by myosin and Ca\(^{2+}\) as activating ligands. In this model, force production is permitted only in the open state.

### Figure 1. Schematic representation of cardiac sarcomere (left) showing atomic structure of troponin core regulatory domain (expanded panel).

Troponin core domain is from Takeda et al.15 The structure (PDB 1J1E) was solved in the Ca\(^{2+}\)-saturated form using the trimeric complex of truncated cTnT (T2 region, residues 183 to 288), partial truncations of cTnI (residues 31 to 163 and 31 to 210), and full-length cTnC (residues 1 to 161). In the sarcomere structure (left), myosin is yellow, actin is blue, tropomyosin is red, and the heterotrimeric troponin complex is white/green.

Troponin I Is a Molecular Switch

It has long been appreciated that TnI plays an indispensable role in Ca\(^{2+}\) regulation of the thin filament. TnI can be considered as the molecular switch of the thin filament regulatory system. Biochemical studies have shown that the inhibitory domain of TnI (roughly from residues 90 to 121 in skeletal TnI) contains sufficient structure to cause about 50% inhibition of the actomyosin ATPase in solution. Full inhibition of ATPase activity can be achieved using a larger fragment of TnI that extends into the C-terminal domain of the molecule.5 This provides evidence that the inhibitory/C-terminal region of TnI interacts with the N-terminus of TnC, and that this interaction depends on the [Ca\(^{2+}\)]. Thus, it has been proposed that the inhibitory/C-terminus of TnI serves as a molecular switch, such that at low [Ca\(^{2+}\)], it is anchored to actin, and at high [Ca\(^{2+}\)] it interacts with the regulatory domain of TnC,3 possibly via interaction with an exposed hydrophobic “sticky patch” of TnC.14

Atomic Structure of the Core Domain of Troponin

The atomic structure of the core domain of human cardiac troponin has just recently been proposed based on x-ray crystallography analysis of the troponin C, T, and I complex.15 The structure was solved in the Ca\(^{2+}\)-saturated form using the trimeric complex of truncated cTnT (T2 region, residues 183 to 288), partial truncations of cTnI (residues 31 to 163 and 31 to 210), and full-length cTnC (residues 1 to 161). The model reveals multiple \(\alpha\)-helices and flexible linkers in the core domain. In this model, cTnI consists of two rigid helixes (H1, residues 43 to 79; H2, residues 90 to 135) that wrap around TnT, adjacent to the C-terminal region of troponin (IR, residues 137 to 148, not well resolved in crystal structure), helixes H3 (residues 150 to 159) and H4 (residues 164 to 188), and finally, the immediate C-terminal cTnI domain (residues 192 to 210; Figure 2). The lack of structure for the IR domain suggests this linker between H1/H2 and H3/H4 is flexible, and that in the presence of Ca\(^{2+}\) H1 attaches to the C-terminal region of TnC.15 Key elements of the structure are in keeping with NMR studies of fragments of the troponin complex.16–18 The NMR and x-ray crystallography studies support a model in which TnI H3 serves as a molecular switch by dissociating from actin and binding to the N-terminus of TnC on increased [Ca\(^{2+}\)] in the myoplasm15,19 (Figure 2). Takeda et al15 further propose that the release of TnI H3 from actin must, in turn, cause a large portion of the TnC C-terminus to detach from actin (shown schematically in Figure 2B). Future NMR and x-ray studies will facilitate detailed structure-function analysis of troponin as modified by posttranslational modifications relevant in health and disease (discussed later).

### Functional Significance of Covalent Modification of Troponin

Cardiac contractile performance is markedly enhanced during \(\beta\)-adrenergic stimulation.7,20–22 The molecular mechanisms underlying the enhanced contractile function are of great interest, yet despite intensive efforts, are not fully understood. The effects of \(\beta\)-adrenergic stimulation on cardiac function
a broad functional range by mammalian heart has the capacity to alter pump performance over cardiac output can increase to greater than 30 L/min with the TnI inhibitory region; IT is the rigid TnT is light blue, tropomyosin is green, and actin is blue. IR is formed between TnT and TnI. 15

Figure 2. Model of calcium-dependent alterations of the troponin core in the context of the thin filament regulatory apparatus. Model is adapted from the calcium-saturated core structure reported by Takeda et al.15 Schematic of the core structure in the absence (A; diastole) and presence (B; systole) of activating calcium. Vertical doubled-headed arrows represent points of strong interaction between the troponin core and tropomyosin/actin as indicated. Dashed line with single-headed arrow (B) is to conceptually represent weakening of the C-terminal elements of cTnI with actin and strengthening interactions with the N-terminus of TnC as derived from Takeda et al.15 Schematic of the troponin core complex is based on the atomic model of Takeda et al.15 Structures not included in the core structure (TnT N-terminus, TnI N-terminus) are represented by dotted lines. Structures unresolved in the crystal structure are represented by ellipsoids, and resolved helixes (H) are identified and numbered according to Takeda et al.15 TnC is yellow, TnI is red, TnT is light blue, tropomyosin is green, and actin is blue. IR is the TnI inhibitory region; IT is the rigid α-helical coiled-coil formed between TnT and TnI.15

can be broadly summarized as increases in cardiac pressure generation (inotropy), relaxation rate (lusitropy), and cardiac contractile frequency (chronotropy). For example, in humans under resting conditions, left ventricular cardiac output is about 4 to 5 L/min, and heart rate is about 70 to 80 bpm.20 However, in response to maximum β-adrenergic stimulation, cardiac output can increase to greater than 30 L/min with heart rates exceeding 220 bpm in humans. Thus, the mammalian heart has the capacity to alter pump performance over a broad functional range by β-adrenergic stimulation. This common feature of mammalian heart performance is of central physiological significance to the overall biological fitness of mammals from rodents to humans as it is required to meet the changing metabolic demands of the organism going from the resting state to the maximal “fight or flight” stress response state.

To accomplish the marked positive inotropic and chronotropic responses of the heart to β-adrenergic stimulation requires that the myocardium must be able to relax from a higher force, and over a shorter time interval in order to preserve the proper diastolic function of the ventricles.20 Indeed, this increase in myocardial relaxation rate (positive lusitropy) is readily achieved in response to β-adrenergic stimulation. In the hypothetical case of a lack of positive lusitropy on β-adrenergic stimulation, left ventricular pressure would fall too slowly during diastole, cause a reduction in ventricular filling, and consequently reduce the stroke volume. In fact, altered lusitropy is one of the hallmarks of the failing myocardium.22 It is evident that the increased relaxation rate of the myocardium plays a central role in the β-adrenergic stimulation–mediated enhanced cardiac pump performance in both normal and diseased heart. With the advent of genetic technologies directed at the heart, significant inroads have been made toward the molecular mechanisms of accelerated relaxation kinetics of the myocardium during β-adrenergic stimulation. An overview of progress in this area is considered below.

Cardiac phosphoproteins play a central role in the altered 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.23 The increased cAMP concentration causes a direct activation of protein kinase A (PKA) within the cardiac cell.23 The activated catalytic subunit of PKA is known to have multiple intracellular targets in cardiac muscle: the sarcolemmal Ca2+ channel, phospholamban of the sarcoplasmic reticulum, and two key myofilament proteins, TnI of the thin filament and C-protein of the thick filament.23 The precise role(s) of these target phosphoproteins in causing altered cardiac performance during β-adrenergic stimulation is still being debated. We specifically focus in this review on the role of TnI phosphorylation.

Perfused Heart Studies
A strong correlation between cTnI phosphorylation and enhanced contractile function on the application of β-adrenergic agonists has been demonstrated in the perfused, beating heart preparation.21,24 This suggested a direct role for cTnI phosphorylation in mediating the enhanced contractile function in β-adrenergic–stimulated cardiac muscle. In similar studies, it was shown that both cTnI and phospholamban can be readily phosphorylated on β-adrenergic agonist stimulation in the perfused heart preparation.25 Hartzell26 demonstrated a positive correlation between C-protein phosphorylation and increases in the relaxation rate in amphibian hearts. However, the assignment of altered function to a particular cardiac phosphoprotein has proven difficult to work out unambiguously. It has been reported that the rates of tension rise and fall are better correlated to increases in the phosphorylation of cTnI than to phospholamban in the perfused heart.27 In contrast, Garvey et al.28 have shown that on the withdrawal of β-adrenergic agonists, the return to baseline contractile function was more tightly correlated with de-
creases in phosphate content of phospholamban as compared with that of cTnI or C-protein. Insights into the basis of this apparent controversy comes in part from studies that have examined contractile function and phosphoproteins during cardiac development. Park et al. showed that contractile relaxation rates were accelerated in a dose-dependent manner by β-adrenergic stimulation in the adult heart; however, no change in relaxation rate was evident on β-adrenergic stimulation in the neonatal heart. Interestingly, in neonatal hearts, phospholamban and C-protein are expressed, but not the phosphorylatable cardiac isoform of cTnI, which is expressed exclusively in the adult myocardium. Thus, in this developmental study, alterations in relaxation rates were directly correlated to expression of phosphorylatable cTnI. Overall, these studies provide evidence that cTnI phosphorylation plays an important role in altered cardiac contractile performance during β-adrenergic stimulation. However, the relative contribution of individual cardiac phosphoproteins to altered contractile function is still unknown from these types of studies.

Transgenic Animal Studies
The targeted ablation of the phospholamban (PLB) gene has provided great insight into molecular determinants of heart performance. Under physiological conditions PLB serves to inhibit the CaATPase pump of the sarcoplasmic reticulum, and this inhibition is relieved by β-adrenergic stimulation–induced phosphorylation of PLB. The deficiency of PLB in these genetically modified animals was predicted to simulate the effects of constitutively phosphorylated PLB. In the initial characterization of these animals, there were no detected effects of β-adrenergic stimulation on indices of cardiac function as determined in the perfused working heart preparation. This appeared to indicate that phosphorylation of PLB is necessary to cause the altered inotropic and lusitropic responses of the heart during β-adrenergic stimulation. However, additional studies using isolated cardiac myocytes from these same animals showed that the contractile behavior of intact myocytes is significantly increased on application of β-adrenergic agonists, and this became more evident as load was placed on the isolated cardiac preparation. In vivo echocardiographic studies on these same animals show that isoproterenol causes significant increases in left ventricular fractional shortening, and in the velocity of circumferential shortening of the heart. Taken together, these results show that PLB plays an important role in the enhanced contractility on β-adrenergic stimulation; however, it is also clear that other cardiac phosphoproteins play a role as well. Mathematical modeling studies of Ca²⁺ transients, Ca²⁺ binding to TnC, and tension in cardiac muscle show that, although phospholamban phosphorylation causes a more rapid fall in the declining phase of the Ca²⁺ transient, the rate-limiting step in the process of muscle relaxation is more closely related to the off rate of Ca²⁺ from TnC, which, as reviewed later, is influenced by cTnI phosphorylation. Thus, this modeling study further points to the importance of the phosphorylation state of cTnI on mechanical function of cardiac muscle. Recently, a genetic cross has been established between PLB-deficient mice and transgenic mice expressing the slow skeletal isoform of TnI (ssTnI). Thus, these mice lack two key substrates for PKA:PLB and cTnI. In cardiac muscle isolated from these mice, β-adrenergic agonists had no effect on relaxation performance. Earlier, this group reported that the β-adrenergic–mediated enhancement of relaxation was blunted in ssTnI transgenic mice. Collectively, these transgenic studies indicate that PLB and cTnI contribute to the β-adrenergic stimulation–induced increase in cardiac relaxation performance.

Permeabilized Cardiac Muscle Preparations
A complete understanding of the relationship between individual cardiac phosphoproteins and contractile function requires systematic dissection of the phosphoproteins in the working cardiac myocyte. Studies on the chemically permeabilized (ie, skinned) cardiac preparations offer the first step toward the simplification of this complex system. In these experiments, the sarcolemmal and sarcoplasmic reticulum membranes are disrupted, and activation of the contractile apparatus is achieved directly by application of exogenous Ca²⁺. Thus, in these studies the only target phosphoproteins remaining are cTnI and C-protein. By using β-adrenergic agonists, or the catalytic subunit of PKA directly, both cTnI and C-protein can be readily phosphorylated in skinned cardiac muscle preparations. During steady-state conditions in skinned cardiac muscle, PKA causes a marked desensitization of Ca²⁺-activated myofibrillar ATPase activity and isometric tension generation. The molecular basis of this effect, which has been reproduced in numerous labs using cardiac muscles from a range of mammalian species, remains to be fully worked out. The observed desensitization of the contractile myofilaments to Ca²⁺ activation is hypothesized to play a central role in the increased relaxation rate evident during β-adrenergic stimulation of the heart. Briefly stated, during a twitch contraction in β-adrenergic–stimulated intact cardiac muscle, myofilaments having reduced sensitivity to Ca²⁺ activation would be expected to inactivate more rapidly as the intracellular [Ca²⁺] is falling, and thus hasten overall tension relaxation kinetics. In fact, this predicted outcome has recently been supported experimentally. The relaxation kinetics of Ca²⁺-activated skinned cardiac muscle have been investigated using the photolyzable Ca²⁺ chelator diazo-2. These experiments show increased relaxation kinetics of isometric tension in preparations pretreated with PKA as compared with nontreated controls.

Solution Biochemical Studies
Biochemical studies support a role for cTnI phosphorylation to alter the Ca²⁺ sensitivity of tension observed in intact and permeabilized cardiac muscle preparations. Results on isolated TnI-TnC and whole troponin complexes (TnI-TnC-TnT) show that TnI phosphorylation causes a reduction in the affinity of TnC for Ca²⁺ binding. The reduction in Ca²⁺ binding to TnC appears to be directly related to an increase in the off rate of Ca²⁺ from the low-affinity regulatory site of cardiac TnC. The molecular basis of this effect is not known but presumably involves both intramolecular and intermolecular modifications resulting from phosphorylation of the N-terminus of cTnI. Al Hillawi et al. reported that...
phosphorylation of cTnI markedly lessens the normal effects of Ca\(^{2+}\) to strengthen the TnI-TnC interaction. This result provides evidence that cTnI is directly involved in the desensitization of the contraction to Ca\(^{2+}\) activation in the β-adrenergic–stimulated heart. Whether these effects are present under physiological conditions in the more complex system of the 3-dimensional contractile apparatus of the working cardiac myocyte is not known.

There are four possible phosphorylation states of cTnI in response to β-adrenergic stimulation.\(^{46-48}\) Phosphate incorporation at the N-terminus of cTnI is a highly regulated process and dependent on the dynamic interaction of PKA and phosphatase 2A. A gap in current understanding is elucidating the physiological role(s) of these different phosphorylation states of cTnI in cardiac myocytes. Biochemical studies show an increase in total phosphate incorporation to about 2 moles phosphate/mole cTnI in cTnI during β-adrenergic stimulation of the heart.\(^{49}\) Swiderek et al\(^{50}\) identified two consecutive serines [positions Ser23 and Ser24 (note: numbering is from rat cardiac sequence)] located in the cardiac-specific N-terminus of cTnI as the target phospho-serines of the molecule. There is now strong evidence for an ordered phosphorylation of these adjacent serines both in vivo and in vitro.\(^{48,50,51}\) Sequence homology is evident in the immediate vicinity about these adjacent serines in most mammalian species including, mouse, rat, rabbit, bovine, and human cTnI. This highly conserved sequence is RRSS23S24... compatible with the minimal recognition motif (RRXS) for cAMP-dependent protein kinases.\(^{51,52}\) Biochemical studies provide evidence that the second phospho-serine of this pair may be preferentially phosphorylated first during adrenergic stimulation, followed by phosphorylation at the first serine (ie, Ser23).\(^{48,51}\) Modeling studies of the kinetics of phosphate incorporation into these tandem serines, which include preferential effects of both PKA and protein phosphatase 2A at these positions, show that the two monophosphorylated states of cTnI can be formed.\(^{46}\) This is supported by studies indicating monophosphorylation of Ser23 or Ser24 in heart muscle in vivo.\(^{50}\) Therefore, there are a total of four possible phosphorylation states of cTnI in vivo: (1) unphosphorylated, (2) Ser23 monophosphorylated, (3) Ser24 monophosphorylated, and (4) Ser23, Ser24 diphosphorylated.\(^{46,48,50,51}\) It is unknown which phosphorylation state of cTnI underlies the accelerated relaxation rates observed in PKA-treated cardiac muscle. Finally, the above evidence of ordered phosphorylation in cTnI can help explain the earlier puzzling results of apparent uncoupling between cTnI dephosphorylation and the return to baseline mechanical function in cardiac muscle. This is evident if the four different phosphorylation states of cTnI have different physiological effects. Whether this is true in working cardiac myocyte preparations is currently unknown.

The intramolecular and intermolecular signaling events caused by TnI phosphorylation in working cardiac myocytes are currently under investigation.\(^{53}\) A gap in present understanding centers on elucidating the molecular events that occur within the TnI molecule (intramolecular) and between TnI and other regulatory molecules of the thin filament system (intermolecular) on TnI phosphorylation. Studies on reconstituted myofibrillar preparations using an N-terminal-truncated cTnI molecule show lack of an effect of PKA to cause shifts in the myosin ATPase-Ca\(^{2+}\) relationship.\(^{54}\) However, the specificity of this extraction/readdition protocol is unclear as C-protein was also removed during the extraction procedure.\(^{55}\) Thus, the precise role of intramolecular signaling in the N-terminus of TnI remains to be determined. Intramolecular alterations in TnI due to phosphate incorporation have been reported in biochemical studies using a synthetic 14 amino acid peptide of the sequence around... RRRS23S24... .\(^{49}\) Phosphate incorporation at both serines caused specific interactions to occur between the S24 and R22 side chains. This interaction was strengthened when the two phosphates were in the diazonium form (at pH 7.0) as opposed to the monoanionic form (at pH 5.6). Thus, the addition of two negative charges brought about by phosphorylation at each of these two serines at physiological pH appears critical in initiating intramolecular alterations in the N-terminus of cardiac TnI.\(^{48}\) TnI binds tightly to actin or to TnC depending on the free [Ca\(^{2+}\)]\(^{5}\). This molecular switching mechanism of TnI is proposed to be altered by cTnI phosphorylation. Results from recent small angle neutron scattering and cross-linking studies suggest that the amino-terminus of cTnI containing Ser23/24 directly interacts with the regulatory region of TnC to stabilize Ca\(^{2+}\) binding.\(^{56,57}\) Phosphorylation of Ser23/24 causes TnI to become more compact and available evidence is consistent with the hypothesis that this conformational change involves bending of the amino-terminus of cTnI such that Ser23/24 phosphorylation destabilizes the interaction between the N-terminus of cTnI and the regulatory region of TnC.\(^{56}\) Zhang et al\(^{59}\) using the vanadate TnI extraction/reconstitution protocol with wild-type and mutant cTnI, report that mutation of the adjacent serines to alanines results in loss of the PKA-mediated desensitization of the contractile apparatus to Ca\(^{2+}\) activation. Dohet et al\(^{60}\) also used the vanadate protocol with mutant cTnI in which the serines were mutated to aspartates as a test of negative charge incorporation into cTnI. They reported that the skinned preparations reconstituted with the Asp in place of Ser mimicked the desensitization of Ca\(^{2+}\)-activated tension observed in control preparations treated with PKA. Whereas these results are intriguing, their interpretation is complicated by the known nonspecific effects of the vanadate procedure on the composition of the myofilament. For example, it has been demonstrated that in control reconstitution experiments that using wild-type cTnI alone shifts Ca\(^{2+}\) sensitivity.\(^{59}\) This indicates alterations in myofilament structure that are not fully restored by this protocol, and impacts studies focusing on relaxation with mutant cTnIs because of irreversible nonspecific effects of the extraction/reconstitution protocol.\(^{56}\) Accordingly, the analysis of cTnIs in the context of intact cardiac myocytes in which there are no other alterations in myofilament structure would be of value. Such an approach could unambiguously determine the intramolecular and intermolecular signaling events due to phosphorylation of the adjacent serines in cTnI.

The N-terminal extension of cTnI serves as a starting point for determining cTnI structure/function relationships on phosphorylation.\(^{61}\) Three consecutive arginines precede the
phosphorylatable tandem serines. Upstream from the arginines are four closely spaced proline residues, and farther upstream are four acidic amino acids. A testable working model is that in the unphosphorylated state, the upstream acidic amino acids directly interact with the arginine cluster. This model requires flexibility in the N-terminal extension that could be provided by the proline cluster that is critically positioned between the acidic amino acid and arginine clusters. By addition of PKA and the subsequent incorporation of negative charges at S23/S24 by phosphorylation, the interaction between the acidic and basic amino acid clusters is hypothesized to be weakened, and the N-terminus would then assume an extended conformation. Two main observations lend support to this working model. First, it is notable that the acidic, arginine, and proline clusters of the N-terminus of cardiac TnI are highly conserved in mammalian species from mouse to humans. The retention of these clusters across species is taken as teleological evidence of their potential key physiological significance. Second, biochemical studies on a synthetic cTnI peptide fragment suggest that R22 directly associates with S24 on phosphorylation of the tandem serines. The proposal of a conformational change in the N-terminus of cTnI, from overlapped in the unphosphorylated state to extended in the phosphorylated state, could be envisioned as the initial intramolecular switch that leads directly to alterations in cTnI binding interactions between actin and TnC depending on Ca\(^{2+}\) concentration. A testable outcome of this model is that neutralization of charge in the acidic amino acid or arginine clusters, or modification of prolines, would mimic the effects of serine phosphorylation.

**Isoforms and Chimeras of TnI**

The mechanism by which alterations in the conformation of the TnI N-terminus may signal information to the thin filament regulatory system is of great interest but has not yet been established. Sequences downstream from the cTnI N-terminus play a central role in causing intramolecular and intermolecular domain alterations. Specifically, the requirement that the inhibitory/C-terminal domain of cTnI be intact to ultimately cause intermolecular modifications of cTnI with other members of the thin filament regulatory complex has been tested. Owing to the structural dissimilarities in this domain of cTnI between the cardiac and skeletal isoforms, it was tested that the molecular switching mechanism of TnI is therefore likely TnI isoform dependent. To directly test this hypothesis, constructs were developed featuring TnI isoform chimeras for testing of function in the adult cardiac myocyte (Figure 3). Chimera N-Card/Slow-C was formed by the normal cardiac N-terminal extension together with the slow skeletal TnI inhibitory/C-terminal domain. This chimera responded with the characteristic same magnitude shift in calcium sensitivity as wild-type cTnI. This is evidence that the intramolecular signaling event(s) caused by phosphate incorporation into the cTnI N-terminus does not specifically require the cardiac inhibitory/C-terminal domain to communicate with actin and/or TnC (Figure 3).

**Covalent Modification of TnI and TnT by PKC**

**Relationship Between PKC, the Thin Filament, and Contractile Function**

Activation of protein kinase C (PKC) modifies thin filament function via phosphorylation of the thin filament proteins, TnI and TnT. The role played by each of these proteins in contraction has been more difficult to ascertain for PKC than for PKA due to the divergent contractile responses observed with different PKC agonists. Both positive and negative inotropic effects of PKC activation have been reported in the literature. These divergent responses are likely due to complexities in the PKC signaling pathway, including multiple physiological agonists, the presence of multiple phospholipase C and PKC isoforms, translocation/localization of each activated PKC isoform, and multiple end-targets. There...
are at least 12 different PKC isoforms identified that fall into three main subgroups.69 In most species, the classical PKCε isoform, plus novel subclass PKCδ and PKCe, are expressed within the myocardium.70,71 In some species, such as mouse and rabbit, the classical PKCβ isoform also is expressed in cardiac muscle.72,73 Expression of atypical class isoforms including, PKCζ, PKCa, and PKCd is evident in human and rodent myocardium.70,71 The role of each PKC isoform in acute and chronic contractile responses awaits further study.

Several agonists known to activate PKC in the myocardium also are potent vasoactive agents,74 thus it is difficult to differentiate direct myocardial effects from vasoactive effects. As a result, responses to these agonists are often investigated using cellular or multicellular cardiac preparations. Studies in these preparations have the advantage that the endogenous PKC signaling pathway is present, and the contractile response to PKC activation can be measured in myocytes independent of changes in vascular tone. Physiological agonists associated with activation of PKC and enhanced contractile function include endogenous neurohormones, such as endothelin-1, or arachidonic acid.66,68 Intra-cellular photorelease of a diacylglycerol analog also is reported to cause a positive inotropic response.76 This contractile response appears to be primarily mediated via Gq/G11-coupled receptors and phospholipase C activation.77 In contrast, activation of PKC using externally applied diacylglycerol analogs, adenosine, or κ-opioids is associated with a negative inotropic response.67,75,78 Both PKC-mediated functional responses are associated with phosphorylation of cTnI.66,79 In some cases, cTnI phosphorylation also is associated with the functional response.80–82 Troponin phosphorylation and the consequent functional response are inhibited by specific PKC antagonists.66 Phosphorylation of the thick filament proteins, myosin light chain 2 as well as myosin binding protein C (MyBP-C), also is observed with PKC activation.66,79,83 The divergence in functional effects, and phosphorylation of multiple contractile protein end-targets in conjunction with the complexity of the PKC signaling pathway, have made it difficult to determine the role played by thin filament proteins in this inotropic response. The potential for crosstalk between PKC and other signaling pathways,84 as well as possible differential activation/translocation of specific isoforms, dose- and time-dependent actions of an agonist, and species-dependent effects add additional levels of complexity.84,85 Despite these barriers, integrated analysis of information gained from transgenic and biochemical studies has provided insight into the role of individual thin filament proteins in the PKC response.

Investigating the Role of Thin Filament Proteins in the Contractile Response to PKC

One strategy for dissecting out the relationship between myocardial PKC and putative end-targets has been to develop transgenic mice with altered expression levels of a specific PKC isoform and/or a receptor for activated C kinase (RACK).85 Proteomic analysis of hearts from mice indicate a close relationship between PKCe and cTnT phosphorylation, and this relationship increased in mice expressing increased levels of PKCe.85 Posttranslational modifications to α-tropomyosin also were noted in this study. Increased expression of PKCβ, which is observed during pathophysiological states,66 is associated with elevated cTnI phosphorylation in the myocardium of transgenic mice at a time before the development of a dysfunctional cardiac phenotype.87 Transgenic mice expressing modified RACKs also have been developed, although the focus of these studies has been on cardiac hypertrophy and protection from myocardial ischemia rather than acute effects on contractile function.88 Future studies using these strategies could provide more detail about the interactions between PKC isoforms and end-targets, including thin filament proteins. Transgenic mice also have been developed in which putative phosphorylation sites by Ser/Ala substitution.82,89,90 An advantage of this transgenic approach is that a specific end target for PKC can be investigated without altering vascular function. Biochemical approaches also have provided important insights into the role played by thin filament proteins. In these studies, purified PKC or PKC from brain extracts is added to permeabilized myocytes, isolated myofilaments, and/or purified contractile proteins, which are then reconstituted with other components of the contractile apparatus.80,91,92 After activation of PKC, phosphorylation is analyzed in parallel with measurements of isometric tension in permeabilized preparations or actomyosin ATPase activity in myofibrillar or reconstituted preparations. Insights into the proteins phosphorylated by PKC, the residues phosphorylated within each protein, and changes in myofilament function that develop with PKC activation have been gained using these strategies. However, studies with membrane intact myocytes are necessary to determine whether the intact PKC pathway causes similar changes in function.

A recent strategy has been to use gene transfer as a means of replacing individual phosphorylatable thin filament proteins in intact cardiac myocytes with isoform/mutants lacking one or more PKC phosphorylation sites.93 One advantage of this approach is that the contribution of a specific contractile protein and/or the role played by individual phosphorylation sites can be evaluated at the myocyte level without interference from compensatory, load-dependent changes in function. Insights gained from this approach can be useful in the development of transgenic animals for in vivo studies.

Role of Covalently Modified Thin Filament in the Contractile Response to Activated PKC

Covalent Modification of Troponin T

Biochemical studies by Kuo and colleagues determined that PKC-mediated phosphorylation of purified cTnT decreases actomyosin ATPase activity.91 In these studies, Thr190, Ser194, Thr199, and Thr280 in the bovine cTnT sequence were the primary sites phosphorylated in response to PKC.94 All of these phosphorylation sites lie within the proposed IT arm region of the troponin core domain (Figure 2), which is postulated to play an important role in Ca2+ regulation of the troponin regulatory core domain as it undergoes hypothesized conformational changes.15 Phosphorylation sites within this domain could, therefore, be important for modulating core domain movement in response to Ca2+ activation.
PKC isoforms α, δ, ε, and ζ each phosphorylated these sites using purified cTnT, yet only phosphorylation of cTnT by PKCα was associated with diminished maximum actomyosin ATPase activity. Addition of purified PKC to permeabilized myocytes also phosphorylated TnT. Replacement of half the endogenous cTnT with nonphosphorylatable fast skeletal TnT in transgenic mice blunted the ability of a phorbol ester analog to reduce maximum tension. Moreover, replacement of endogenous cTnT with a mutant lacking two putative phosphorylation sites decreased overall cTnT phosphorylation and reduced the negative inotropic response observed with α-adrenergic activation. Taken together, these studies provide evidence that PKC-mediated phosphorylation of cTnT acts to inhibit maximum force generation in the myocardium. However, cTnT phosphorylation is considerably lower compared with cTnI phosphorylation, and acute activation of PKC by several physiological agonists does not perceptibly alter cTnT phosphorylation. It may be that cTnT phosphorylation develops during more chronic PKC activation and could play an important role during pathophysiological conditions associated with increased PKC activation of PKC and enhanced inotropy were indicated that PKC activation and enhanced inotropy were associated with diminished maximum actomyosin ATPase activity. Addition of purified PKC to permeabilized myocytes also phosphorylated TnT. Replacement of half the endogenous cTnT with nonphosphorylatable fast skeletal TnT in transgenic mice blunted the ability of a phorbol ester analog to reduce maximum tension. Moreover, replacement of endogenous cTnT with a mutant lacking two putative phosphorylation sites decreased overall cTnT phosphorylation and reduced the negative inotropic response observed with α-adrenergic activation. Taken together, these studies provide evidence that PKC-mediated phosphorylation of cTnT acts to inhibit maximum force generation in the myocardium. However, cTnT phosphorylation is considerably lower compared with cTnI phosphorylation, and acute activation of PKC by several physiological agonists does not perceptibly alter cTnT phosphorylation. It may be that cTnT phosphorylation develops during more chronic PKC activation and could play an important role during pathophysiological conditions associated with increased PKC isoform expression, such as myocardial ischemia. A recent study has provided evidence that Ser206 within rodent cTnT is the primary target for PKC phosphorylation.97 In these experiments, reduced maximum tension was observed when glutamic acid substitution of Ser206 (of mouse sequence, bovine equivalent is Ser194) was used to mimic a constitutive phosphorylation state. However, in the same study, removal of the Ser206 phosphorylation site by substitution with Ala also resulted in reduced tension. Thus, it remains unclear whether this strategy provides specific information about the PKC-mediated phosphorylation state of cTnT in the intact myofilament. In future studies, it will be desirable to determine the contribution of cTnT phosphorylation to acute and chronic PKC-mediated changes in cardiac contractile function under physiological and pathophysiological conditions.

**Covalent Modification of Troponin I by PKC**

Studies from several laboratories demonstrated a strong correlation between cTnI phosphorylation and alterations in contractile function. Rigorous biochemical studies first established a relationship between phosphorylation of specific Ser/Thr residues on cTnI and changes in reconstituted actomyosin ATPase activity. This work established that Ser23/Ser24, Ser43/Ser45, and Thr144 (Figure 4) were the primary sites phosphorylated within purified bovine cTnI by PKC. Phosphorylation of cTnI with either PKCα or PKCδ decreased maximum acto-S1 MgATPase activity and Ca2+ sensitivity, and these effects were considerably blunted in experiments with cTnIS43/45A mutants.

Experiments performed on intact rabbit hearts initially indicated that PKC activation and enhanced inotropy were not associated with increases in cTnI phosphorylation. High levels of 32P-specific activity are necessary to detect phosphorylation but are difficult to attain in whole heart preparations. More recent studies have established that changes in myocyte function observed in response to multiple PKC agonists are associated with increased cTnI phosphorylation. Overall, activation via α-adrenergic, κ-opioid, endothelin, and arachidonic acid signaling pathways resulted in significant increases in cTnI phosphorylation, whereas adenosine activation of PKC did not appreciably influence TnI phosphorylation. Arachidonic acid and endothelin enhance cardiac myocyte inotropy, and κ-opioids have significant negative inotropic actions and diacylglycerol analogs variably influence contractile function. The influence of PKC activation on contractile function appears to change under pathophysiological conditions, such as heart failure. In failing human hearts, reduced levels of cTnI phosphorylation are observed and the contractile response to agonists involved in either PKC or PKA signaling are considerably different from nonfailing myocardium. This variability has made it difficult to ascertain the role of cTnI phosphorylation in PKC-mediated changes in contractile performance based solely on associations between phosphorylation and contractile function.

Transgenic mice expressing cTnI lacking one or more of the putative phosphorylation sites have been used to investigate the role of cTnI in the myocardial response to PKC in intact hearts and myocytes. Replacement of approximately 50% endogenous cTnI with cTnI containing Ala substitutions for Ser43/45 in the heart in transgenic mice does not significantly alter cardiac morphology or function. However, activation of PKC using higher Ca2+ levels resulted in elevated pressures and a significant delay in the Ca2+ transient in transgenic versus wild-type hearts. The negative inotropic response to agonist-mediated PKC activation in transgenic mice also was significantly blunted in comparison to wild-type mice. This is evidence that cTnI phosphorylation by PKC decreases myofilament Ca2+ sensitivity and maximum tension, although the contribution of cTnT cannot be excluded due to the alterations in basal and agonist-mediated cTnT phosphorylation in this preparation. In another transgenic model in which all 5 potential cTnI phosphorylation sites (eg, Ser23/24, Ser43/45, and Thr144) were replaced with Ala on a null cTnI background, endogenous cTnI protein is completely replaced by the mutant protein. Myocytes from these mice exhibited accelerated relaxation in response to endothelin compared with a more delayed relaxation in wild-type controls; in addition, PKC agonists were reported to increase Ca2+ sensitivity of isometric force. These results provide evidence that cTnI phosphorylation by PKC delays relaxation. However, a dilated hypertrophy developed in mice expressing this mutant cTnI, and compensatory remodeling and its influence on the contractile response to PKC activation could not be ruled out. Further studies must determine if the altered functional responses to phosphorylation seen with mutant cTnIS43/45A and/or possible alterations in cTnI phosphorylation, which increases in mice expressing the cTnIS43/45A mutant, also contribute to these findings. Contractile shortening studies on myocytes expressing cTnI or TnI lacking one or more of the putative PKC phosphorylation sites have been performed to directly address the influence of cTnI phosphorylation on myofilament function. In these experiments, acute (eg, <30 minutes) activation of PKC via endothelin accelerated relaxation rate in intact myocytes. Replacement of endogenous cTnI with a
nonphosphorylatable isoform (eg, slow skeletal TnI) or chimera of TnI by gene transfer resulted in a significantly delayed relaxation rate. Prolongation of relaxation in the absence of phosphorylatable cTnI is likely due to activation of Na⁺-H⁺ exchange, which induces a cellular alkalosis. The increase in myofilament Ca²⁺ sensitivity caused by cellular alkalosis would be expected to prolong relaxation. Thus, cTnI phosphorylation acted to maintain relaxation rate during PKC activation and counteracted the effects of other subcellular events. Additional studies provide evidence that the Thr144 is not significantly phosphorylated in these acute contractile function studies despite earlier evidence that peptides containing this Thr showed high-affinity phosphorylation by purified PKC. Instead, one or both of the two sets of dual Ser residues present in the amino portion of cTnI were responsible for the accelerated relaxation response. These two sets of Ser residues lie within a highly flexible region of cTnI. Peptide solution studies provide evidence that the region around Ser43/45 serves to stabilize the Ca²⁺-insensitive interaction between TnI and TnC, and may serve to modulate the interaction between the IP region of TnI and TnC.

To fully understand the relationship between PKC, Tn phosphorylation, and contractile function, more extensive studies are needed to determine whether differential activation of PKC isoforms by individual neurohumoral pathways results in similar or unique phosphorylation patterns within cTnI. An additional area requiring attention will be to address whether crosstalk between PKC and other signaling pathways operating in parallel and/or downstream from PKC differentially influences either Tn phosphorylation and/or contractile performance. The effects of longer intervals of PKC activation, the relative contribution of cTnI compared with other PKC end-targets, and the influence of PKC activation under pathophysiological conditions also are critical areas requiring attention in future studies.

Changes in Covalent Modification of TnI and TnT in Response to Activation of Other Kinases

Additional protein kinase pathways are now recognized to influence the phosphorylation status of TnI, TnT, and troponin (Tm). Specific TnI and Tm kinase activities have been identified, although their functional significance remains unclear, as reviewed in detail by Solaro. Low molecular weight GTPases are now being recognized as kinases capable of phosphorylating TnI and/or TnT. For example, p21-activated kinase (PAK) directly phosphorylates TnI and TnT, as well as other sarcomeric proteins. Activation of PAK increases peak isometric force and myofilament Ca²⁺ sensitivity in permeabilized cardiac myocytes. However, the contribution of PAK activation to physiological contractile performance, and the contribution of phosphorylated TnI and TnT to these changes in performance remains to be determined. Rho-associated kinase also is under investigation. Other low molecular weight G proteins are thought to play important roles under pathophysiological conditions, but their influence on cardiac myocyte contractile performance is not fully known.

Another recent avenue of Tn phosphate analysis has been to determine the influence of effectors downstream of PKC on contractile function. Mitogen-activated protein kinases (MAPks) can be activated by PKC. Activation of the p38 branch of the MAPK pathway results in negative inotropy. This effect is not explained by a change in the intracellular Ca²⁺ transient nor by phosphorylation of purified troponin added to recombinant p38 MAPK. Instead, evidence is emerging that activation of protein phosphatase 2A by p38 MAPK is an important mechanism responsible for dephosphorylation of Tn, which could contribute to the observed negative inotropic effect. Adenosine appears to be one physiological agonist that may be an important activator of the p38 MAPK/PP2A pathway. The growing awareness of phosphatases in mediating end-target phosphorylation status and contractile function should give rise to the critical studies necessary for identifying receptor crosstalk. Moreover, detailing the dose- and time-dependence of protein phosphatase isoform activation by known physiological agonists and cellular signaling pathways will provide insight into mechanisms of altered cardiac contractile performance.

Noncovalent Modification in TnI

Myocardial infarction represents a major health problem in this country and throughout the Western world. During acute myocardial ischemia, left ventricular pressure falls precipitously due, at least in part, to intracellular acidification. In ischemia, heart muscle pH falls from normal values of about 7.00 to values of 6.2 or lower. Intracellular acidification causes impairment of numerous cellular properties including having direct effects on the contractile apparatus. Interestingly, the degree of contractile dysfunction caused by acidification is both striated muscle lineage-dependent and cardiac muscle development-dependent. A number of studies have taken advantage of these differences to aid understanding of the molecular basis of contractile dysfunction in acidic conditions. For example, in the heart, there are two isoforms of TnI that are developmentally regulated. In embryonic and neonatal heart of all mammalian species, the slow skeletal isoform of TnI (ssTnI) is expressed, and soon after birth, there is an irreversible transition to the adult isoform cardiac TnI (cTnI). Solaro and colleagues first proposed that isoform transitions in troponin could account for the differing sensitivity of neonatal and adult myocardium to acidification. Using acute genetic engineering techniques featuring viral-mediated gene transfer into adult cardiac myocytes it was directly demonstrated that TnI isoforms directly affect cardiac contractile dysfunction caused by acidic pH. Thus, ssTnI expression renders the cardiac myocyte more resistant to acidic pH-mediated decreases in the calcium sensitivity of tension development (quantitated by the magnitude of the rightward shift in the tension-Ca²⁺ relationship going from pH 7.00 to 6.20). These findings have been confirmed in transgenic mouse studies in which native cTnI was replaced by a ssTnI transgene. This is direct evidence that TnI isoforms play a role in pH-mediated contractile dysfunction and also indicates that isoform-dependent domains within ssTnI/cTnI could account for differing pH sensitivities. Recent gene
transfer studies using TnI chimeras constructed from the cTnI/ssTnI isoforms, show that the pH-sensitive domain is resident in the C-terminal region of the molecule.\textsuperscript{65,118,119} These TnI chimeras also provided evidence for at least two domains responsible for normal calcium activated contractile behavior in cardiac myocytes (Figure 4).

The basis for this TnI isoform dependence could relate to charge differences in the C-terminal portion of TnI. Examination of amino acid differences between ssTnI and cTnI (in the inhibitory and more C-terminal region), in species ranging from rat, to bovine, to human, reveals the preponderance of basic amino acids in this domain with a net positive charge of +5 for cTnI and +8 for ssTnI, meaning an isoform charge difference of +3 for ssTnI. Because these charge differences occur in a region of TnI known for important calcium-dependent interactions with actin and TnC,\textsuperscript{5,120} and because chimeras of TnI give markedly different results in contractile assays,\textsuperscript{46} this strengthens the proposal that isoform-dependent charge differences confer calcium and pH sensitivity to TnI in the context of the adult cardiac myocyte.

It was recently reported that a single amino acid confers both calcium and acidic pH sensitivity of tension to ssTnI.\textsuperscript{121} Using targeted amino acid substitutions in ssTnI in conjunction with recombinant adenovirus gene transfer to otherwise normal adult cardiac myocytes, a single amino acid has been shown to fully covert the pH sensitivity observed with ssTnI to the cTnI phenotype. Initial experiments showed that stoichiometric replacement of native cTnI with a ssTnI mutant [ssTnI\textsuperscript{IQAE R125Q, H132A, V134E}] converted ssTnI to the cTnI phenotype when measuring Ca\textsuperscript{2+}-activated tension at pH 7.0 and pH 6.2 in permeabilized myocytes. The single ssTnI\textsuperscript{H132A} replacement fully converted ssTnI to the cTnI phenotype. A ssTnI\textsuperscript{R125Q} single mutant retained the ssTnI phenotype. These results demonstrate, using single adult cardiac myocyte recordings of isometric tension, that the histidine residue at codon 132 uniquely confers ssTnI isoform-specific alterations in mechanical function in adult cardiac myocytes. In subsequent studies, Smillie and colleagues\textsuperscript{122} have recently reported results from the converse experiment, that is placing a mutation at codon 162 in human cTnI from Ala to His. This caused a conversion of the cTnI acidic pH phenotype to that of the ssTnI phenotype. Collectively these studies highlight that TnI isoform differences in calcium-activated function can be traced to unique amino acids differences in these proteins. A further understanding of these isoform-specific outcomes could lead to development of novel TnI molecules to tune myocardial performance in under pathophysiological conditions.

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References


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