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Structural and Kinetic Effects of PAK3 phosphorylation mimic of cTnI(S151E) on the cTnC-cTnI Interaction in the Cardiac Thin Filament

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Abstract

Residue Ser151 of cTnI is known to be phosphorylated by p21-activated kinase 3 (PAK3). It has been found that PAK3-mediated phosphorylation of cTnI induces an increase in myofilament Ca^{2+} sensitivity, but the detailed mechanism is unknown. We investigated how the structural and kinetic effects mediated by pseudo-phosphorylation of cTnI (S151E) modulates Ca^{2+} -induced activation of cardiac thin filaments. Using steady-state, time-resolved Förster Resonance Energy Transfer (FRET) and stopped-flow kinetic measurements, we monitored Ca^{2+} -induced changes in cTnI-cTnC interactions. Measurements were done using reconstituted thin filaments, which contained the pseudo-phosphorylated cTnI(S151E). We hypothesized that the thin filament regulation is modulated by altered cTnC-cTnI interactions due to charge modification caused by the phosphorylation of Ser151 in cTnI. Our results showed that the pseudo-phosphorylation of cTnI (S151E) sensitizes structural changes to Ca^{2+} by shortening the intersite distances between cTnC and cTnI. Furthermore, kinetic rates of Ca^{2+} dissociation-induced structural change in the regulatory region of cTnI were significantly reduced by cTnI (S151E). The aforementioned effects of pseudo-phosphorylation of cTnI were similar to the effects of strong crossbridges on structural changes in cTnI. Our results provide novel information on how cardiac thin filament regulation is modulated by PAK3 phosphorylation of cTnI.

Keywords

Cardiac troponin I; p21 activated kinase; Phosphorylation; Förster Resonance Energy Transfer (FRET); stopped-flow kinetics

A main feature of Ca^{2+} activation of cardiac muscle contraction and relaxation is the dynamic interactions among the thin filament contractile regulatory proteins, resulting in multiple structural transitions in the thin filament. Among the thin filament regulatory

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proteins, troponin I (cTnI) is central to these structural transitions through its interactions with troponin T (cTnT), troponin C (cTnC), tropomyosin (Tm) and actin. cTnI is the inhibitory unit of the troponin complex, which also consists of cTnC, a Ca^{2+} -binding protein, and cTnT, a Tm-binding protein. At diastolic levels of intracellular Ca^{2+} , cTnI inhibits strong actomyosin interactions by interacting with actin. Binding of Ca^{2+} to cTnC releases the inhibitory action of cTnI on actin by increasing the association between cTnC and cTnI. Such structural changes in cTnC-cTnI are further propagated to affect the movement of Tm on actin filaments, which is linked to the activation of thin filaments by Ca^{2+} .

In addition to Ca^{2+} activation, thin filament activation is further modulated by strong crossbridge formation and also by the phosphorylation of thin filament regulatory proteins [3, 4]. Phosphorylation of specific serine and threonine residues in cTnI represents a major physiological mechanism for alteration of thin filament activation [3]. A recent study has identified Ser151 of cTnI as a substrate for phosphorylation by p21-activated kinase (PAK), of which there are at least three cardiac isoforms (PAK1, PAK2, and PAK3) [5]. Experimental evidence suggests that PAK1 promotes dephosphorylation of cTnI through type 2A phosphatase in heart [6], while PAK3 increases Ca^{2+} sensitivity of myofilament through phosphorylation of Ser151 of cTnI [5]. However, the structural and functional significance of phosphorylation of Ser151 in cTnI, mediated by PAK3, is not well understood.

Experiments reported here were aimed at testing how Ca^{2+} -induced thin filament activation is altered by PAK3 phosphorylation of Ser151 in cTnI. Ser151 of cTnI is strategically located between two important functional regions of cTnI: the inhibitory region and the regulatory region. The inhibitory region (residues 138-149) plays a switching role in the Ca^{2+} -induced activation of thin filaments by interacting alternatively with actin and cTnC. The regulatory region (residues 150-167) plays a role in the *on/off* process by either interacting with or dissociating from cTnC, depending on whether Ca^{2+} is present or absent. Our hypothesis is that the surface charge modification at residue Ser151 of cTnI alters cTnC-cTnI interactions to affect kinetics of cTnC-cTnI interactions. To test our hypothesis, we examined structural and kinetic changes in cTnC-cTnI interactions by measuring FRET distances between the central helix of cTnC and the regulatory region of cTnI in a fully-reconstituted cardiac thin filament preparation. Data from our study demonstrate that pseudo-phosphorylation of cTnI (S151E) affects Ca^{2+} -induced structural transition between cTnI and cTnC. Such altered interactions between cTnC and cTnI lead to an increase in thin filament Ca^{2+} sensitivity and a decrease in the kinetics of the Ca^{2+} dissociation-induced structural change of the regulatory region of cTnI. Our data suggest that the charge modification, as assessed by pseudo-phosphorylation cTnI(S151E), promotes the Ca^{2+} -induced interaction between cTnC and cTnI, and therefore favors the transition of thin filaments from the “off” to “on” states.

RESULTS

Steady-State Measurements of the cTnC-cTnI Interactions within Cardiac Thin Filament

The Ca^{2+} -induced interaction between cTnC and the regulatory region of cTnI plays a critical role in opening the cTnC N-domain and regulating the interaction between the inhibitory region of cTnI and actin, therefore, force development of cardiac muscle. In this study, the interaction between cTnC and the regulatory region of cTnI was investigated by monitoring changes in FRET distance from residue 89 located at the central helix of cTnC to either residue 160 or residue 167 of cTnI within the cardiac thin filament. The residues 160 and 167 are chosen because they are strategically located at the middle and the C-terminus of the regulatory region, respectively. The distances from these two residues to the central helix of cTnC are sensitive to Ca^{2+} binding to cTnC. Upon Ca^{2+} binding to the N-domain of

cTnC, it is expected that the two residues within the regulatory region of cTnI would move close to cTnC. Figure 1 shows Ca^{2+} -induced changes of the steady-state fluorescence of donor AEDANS (iodoacetamidoethylaminonaphthalene-1-sulfonic acid) attached to residue 160 of cTnI in the absence and the presence of acceptor DDPM (N-(4-dimethylamino-3,5-dinitrophenyl) maleimide) attached to residue 89 of cTnC. The corresponding donor fluorescence intensity decays are also shown in Figure 1. Steady-state measurements showed that within the thin filament, the fluorescence intensity of AEDANS at 480 nm in the donor-only sample is not sensitive to Ca^{2+} binding to the N-domain of cTnC in the thin filament preparation, but it decreased by 10% in the presence of strongly-bound S1 (comparing black and dark red curves in Figure 1A and Figure 1B). When the acceptor DDPM attached to residue 89 of cTnC was present, the donor fluorescence intensity was quenched by 28% in the absence of Ca^{2+} (red curve in Figure 1A) due to FRET. Upon Ca^{2+} binding to the regulatory site of cTnC, the donor fluorescence was further quenched by 35% (pink curve in Figure 1A), indicating an increase in energy transfer and a decrease in distance between residue 89 of cTnC and residue 160 of cTnI. Similar changes were observed when S1 and MgADP, which are known to produce strong crossbridge between S1 and actin, were present (Figure 1B).

The FRET measurements were also performed in the presence of pseudo-phosphorylated cTnI(S151E) that mimics PAK3-mediated phosphorylation of Ser151. cTnI(S151E) had no effect on the donor-only fluorescence (data not shown), but increased the FRET between the donor and acceptor in the thin filament samples (light blue and dark blue curves in Figure 1A). The increased FRET observed in either the presence or absence of Ca^{2+} suggests that pseudo-phosphorylation of cTnI (S151E) may have structural effects on cTnC-cTnI interaction by decreasing the distance between cTnC and the regulatory region of cTnI. However, the observed structural effects of cTnI (S151E) were masked when the strongly-bound S1 was present (Figure 1B).

The observed Ca^{2+} -induced changes in FRET were quantified by time-resolved fluorescence measurements of the intensity decay of the donor. Figure 1C and Figure 1D show the intensity decays of donor AEDANS in the sample preparations used in Figure 1A and Figure 1B. These decays were analyzed using global analysis software (GlobeCurve) as previously described [7] to yield a distribution of inter-site distances (Figure 2). The distance at the peak of the distribution was taken as the mean distance between donor and acceptor sites. Table 1 summarizes all mean distances and half-widths (HW) of distributions of the distances between cTnC and cTnI under different conditions. The mean distance between residue 160 of cTnI and residue 89 of cTnC in the thin filament was 33.4 Å without bound Ca^{2+} and decreased by 6.9 Å to 26.7 Å in the presence of bound Ca^{2+} at the regulatory site. In the presence of strongly-bound S1, both the distances in the Ca^{2+} -saturated state and in apo-state were decreased because of the feedback effect of strong crossbridges [1, 8]. Similar changes were also observed in the distances between residue 89 of cTnC and residue 167 of cTnI (Table 1). These changes were significant ($P < 0.05$). These FRET distance changes were also determined in the presence of pseudo-phosphorylation of cTnI (S151E). As shown in Figure 2B, Ca^{2+} binding and the strongly-bound S1 induced changes in the mean distances similar to those observed in the sample preparations without pseudo-phosphorylation cTnI. The presence of pseudo-phosphorylation of cTnI (S151E) caused significant decreases in the mean distances ($P < 0.05$) at all conditions (Table 1). These results suggested that phosphorylation of cTnI at residue 151 modified the cTnC-cTnI interactions within the thin filament regardless of the presence of strong crossbridges, and whether or not the regulatory site in cTnC was saturated with Ca^{2+} . Another feature of the FRET distance measurements is that HW of distributions of all FRET distances is sensitive to Ca^{2+} . Binding of Ca^{2+} to the regulatory site of cTnC significantly decreased HW by 23 ~

43% (Table 1). The decreased distribution of distance between cTnC-cTnI suggests a reduction in structural flexibility of cTnC or cTnI at the interface between the two proteins.

In our calculations of FRET distances, a value of $2/3$ was used for the orientation factor κ^2 based on the assumption of isotropic and rapid tumbling of the fluorophores. If probe mobility was substantially modified under different conditions, a different value would be needed to calculate distance parameters. However, the acceptor was non-fluorescent and it was not feasible to evaluate the possible range of κ^2 for our calculation. We measured the anisotropy decay of the donor probe attached to residues 160 and 167 of cTnI and determined the anisotropies associated with the attached probe. The calculated anisotropies showed very similar donor mobility under all conditions (data not shown), suggesting that ligand binding to the thin filaments did not appreciably affect the mobility of the donor in the cTnI sites. The observed FRET changes reflected a conformational effect and not changes in donor mobility.

Steady-State Ca^{2+} -Titration of the cTnC-cTnI Interactions within Cardiac Thin Filament

FRET measurement between the fluorescence donor-modified cTnI and acceptor-modified cTnC permit us to investigate how cTnC-cTnI interactions are affected by different $[\text{Ca}^{2+}]$. The normalized changes in the FRET distance between cTnI(160C)_{AEDANS} and cTnC(89C)_{DDPM} in the thin filament in the presence/absence of strongly-bound S1 are depicted in Figure 3. Ca^{2+} -titration of the FRET between cTnI(167C)_{AEDANS} and cTnC(89C)_{DDPM} in the thin filament in the presence/absence of strongly-bound S1 were also performed (data not shown). As Ca^{2+} levels increased, the FRET between the donor and acceptor increased due to the Ca^{2+} -mediated decrease in the distance between the regulatory region of cTnI and cTnC. These Ca^{2+} titration curves were fitted to the Hill equation, and the recovered pCa_{50} and the Hill coefficients are given in Table 2. Our data showed that Ca^{2+} sensitivity of interactions between the regulatory region of cTnI and cTnC increased when the strongly-bound S1 was present (from pCa_{50} of 5.88~5.93 to pCa_{50} of 6.22~6.29). Pseudo-phosphorylation of cTnI(S151E) increased the Ca^{2+} sensitivity by 0.17~0.30 pCa units regardless of whether the strongly-bound S1 was present or not. The Hill coefficients, and thus the cooperativity of the interactions between the regulatory region of cTnI and cTnC, were not significantly affected either by the presence of strongly-bound S1 or by the charge change induced by the phosphorylation mimic (S151E) of cTnI.

Kinetics of Ca^{2+} Dissociation-Induced Structural Changes in the Thin Filament

Steady-state FRET measurements between the regulatory region of cTnI and the regulatory domain of cTnC provided a basis to use FRET to determine Ca^{2+} dissociation-induced effects on cTnC-cTnI interactions. These experiments were performed by mixing a buffer containing BABTA, a strong Ca^{2+} chelating agent, with a reconstituted thin filament sample containing donor- and acceptor-labeled proteins at pCa 3.8. Under this condition, the Ca^{2+} -specific site and the two $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites were saturated with Ca^{2+} . The stopped-flow experiments were monitored with the fluorescence intensity of the donor AEDANS in different sample preparations. Shown in Figure 4 are examples of two sets of kinetic tracings of FRET between AEDANS-modified cTnI(160C) and DDPM-modified cTnC(89C) within the reconstituted thin filament in the absence and presence of pseudo-phosphorylation at residue 151 of cTnI. Each kinetic tracing was obtained by averaging 8-10 stopped-flow tracings. Stopped-flow FRET kinetic measurements were also performed to monitor structural kinetics of interactions between AEDANS-modified cTnI(167C) and DDPM-modified cTnC(89C) in fully-reconstituted thin filaments containing normal or phosphorylation mimic of cTnI (data not shown). All acquired kinetic tracings can be described with two transition phases, one fast and the other slow. Generally the fast transient accounted for more than two thirds of the total FRET changes in each monitored structural

transition, while the slow phase accounted for the rest of the total FRET change. The recovered parameters from all FRET kinetic tracing fittings were summarized in Table 2.

Consistent with our previous observations⁹, the Ca^{2+} dissociation-induced movement of the regulatory region of cTnI was fast at the interface between actin and troponin within the thin filament. The fast component of the movement was $109 \sim 119 \text{ s}^{-1}$ in the absence of strongly-bound S1 (Table 2). When strong crossbridges were formed in the presence of S1-ADP, the fast kinetics of the structural transition of the regulatory region of cTnI were significantly reduced to $43 \sim 79 \text{ s}^{-1}$ (Table 2), suggesting significant feedback kinetic effects of the strong crossbridges on the Ca^{2+} -induced thin filament regulation. When measurements were performed in the presence of pseudo-phosphorylation of cTnI (S151E), the kinetics of the Ca^{2+} -induced structural transition of the regulatory region of cTnI were reduced by 42% $\sim$ 58% within the thin filament (Table 2). However, this phosphorylation effect on the structural kinetics of the cTnI regulatory region was blunted in the presence of the strongly-bound crossbridges. These observations may provide some insights into the mechanism underlying the role of PAK3 phosphorylation of cTnI in modulating the thin filament regulation.

Simultaneous Measurement of Force and ATPase Activity of Cardiac Muscle Fibers Reconstituted with Pseudo-Phosphorylation of cTnI (S151E)

To examine the functional effects of pseudo-phosphorylation of cTnI, we exchanged cTnI mutants into cardiac muscle fiber bundles from rats and measured force and ATPase activity simultaneously. Compared to results from the muscle fibers exchanged with wild-type cTnI, L160C and S167C mutants of cTnI had no significant effect on maximum force, ATPase activity and Ca^{2+} sensitivity of tension development. However, when compared to the wild-type cTnI, S167C mutant cTnI decreased the Hill coefficient by $\sim 14\%$. To evaluate whether fluorophore labeling of cTnI affected protein function, we exchanged AEDANS-labeled cTnI (L160C) and cTnI (S167C) into detergent-skinned muscle fiber bundles. Since AEDANS interferes with ATPase assay, we performed only force measurements (data not shown), which gave similar maximum forces and force-pCa relationships as shown in Table 3. These results suggest that both mutations and fluorophore labeling have negligible detrimental effect on cTnI function. When pseudo-phosphorylation of cTnI (S151E) was present, Ca^{2+} sensitivity of force development was significantly decreased, while the Hill coefficients were not affected. cTnI (S151E) also slightly decreased maximum tension and ATPase activity of the reconstituted muscle fibers. However, the statistical analysis of the tension cost (ATPase/tension) did not provide significant information on the relationship between the pseudo-phosphorylation of cTnI (S151E) and crossbridge cycling kinetics.

DISCUSSION

To understand the structural and functional significance of PAK3-mediated phosphorylation of cTnI, we used FRET approaches to investigate the effects of cTnI (S151E) on the Ca^{2+} -induced structural transitions in cardiac thin filaments. Pseudo-phosphorylation of cTnI (S151E) had three major effects: 1) enhanced the interaction between the regulatory region of cTnI and cTnC, 2) increased the sensitivity of cardiac thin filaments to Ca^{2+} , and 3) slowed kinetics of structural changes between cTnI-cTnC upon Ca^{2+} dissociation from troponin. Interestingly, these observed changes are similar to strong crossbridge mediated effects on thin filament.

The strengthened cTnI-cTnC interaction, caused by pseudo-phosphorylation of cTnI (S151E), was manifested by decreases in the distances between cTnI and cTnC. Results from the steady-state and time-resolved FRET measurements (Figure 1, Figure 2, and Table 1) showed that the distances between the central helix of cTnC and the regulatory region of

cTnI were significantly decreased in the presence of pseudo-phosphorylation of cTnI, regardless of the presence or absence of strongly-bound S1. Previous studies have shown that strong crossbridges formed between myosin heads and actin significantly decrease the distance between the inhibitory region of cTnI and the central helix of cTnC, leading to the enhancement cTnC-cTnI interaction¹. The enhanced cTnC-cTnI interaction is believed to be the result of feedback structural effect of strongly-bound S1 on the thin filament. The mechanism underlying the feedback effect is that the strongly-bound S1 effectively shifts Tm on the actin surface from the “closed” to the “open” position. The shift of Tm on actin surface weakens cTnI-actin interactions and favors Ca²⁺-mediated interactions between cTnC and cTnI. This hypothesis is supported by the decrease in FRET distances between cTnC and cTnI when strongly-bound S1 is present (Figure 1 and Table 1). We believe that the structural effect induced by pseudo-phosphorylation of cTnI (S151E) is due to the phosphorylation-induced charge modification at the interface between cTnC and cTnI. It is known that the inhibitory region of cTnI is highly positively charged, especially at the junction between the inhibitory and the regulatory regions. In particular, there are three positively-charged arginine residues next to the phosphorylatable residue, Ser15110. PAK3 phosphorylation-induced negative charges at Ser151 may potentially neutralize the overall positive surface charges at the junction between the regulatory and the inhibitory regions of cTnI. Such charge neutralization favors strong hydrophobic interaction between the regulatory region of cTnI and the regulatory domain of cTnC, thus decreasing FRET distances between cTnC and cTnI (Figure 1 and Table 1). This phosphorylation-induced enhancement of the cTnC-cTnI interaction may play a role in regulating the thin filament from the “blocked” to “closed” states. Since these structural changes in thin filaments are similar to those induced by strong crossbridges, our data also suggests that the “closed” to “open” states may also be enhanced by pseudo-phosphorylation of cTnI by relieving the inhibition of Tm movement on actin surface.

A consequence of the enhanced cTnC-cTnI interaction, caused by pseudo-phosphorylation of cTnI(S151E), is an increased sensitivity of cardiac thin filaments to Ca²⁺. For example, S151E mutation increased Ca²⁺ sensitivity of structural transition between cTnI and cTnC (Table 2) as monitored by FRET distances between the central helix of cTnC and residues 160 and 167 of cTnI. Further additional increase in Ca²⁺ sensitivity was observed when strongly-bound S1 was present (Table 2). These additive effects of pseudo-phosphorylation of cTnI and strongly-bound S1 on Ca²⁺ sensitivity suggest a complex nature of Ca²⁺ binding to cardiac thin filaments. It is possible that Ca²⁺ sensitivity of thin filaments may be determined by structural dynamics associated with the whole C-domain of cTnI rather the regulatory region alone. It is likely that the phosphorylation-induced change is attributed to the enhanced interaction between the regulatory region of cTnI and cTnC caused by the charge modification. The additional structural change caused by strongly-bound S1, however, may be contributed to by other functional regions of cTnI, such as the inhibitory region and the mobile domain. These two regions are known as actin-binding regions, and their structural dynamics are sensitive to the presence of strong crossbridges.

In our Ca²⁺ titration experiments, we noticed that the values of pCa₅₀ and the Hill coefficient obtained from FRET titrations of reconstituted thin filament samples (Table 2) were different from the values obtained from pCa-force measurements with the reconstituted muscle fiber bundles (Table 3). These discrepancies between the two systems could be caused by different reasons. First, the force measurements of muscle fiber bundles were performed under isometric conditions at room temperature (20°C). FRET measurements were carried out in reconstituted thin filaments at 10°C. Second, FRET titrations were performed in reconstituted thin filaments that lack an ordered protein lattice structure present in sarcomeres of muscle fibers. The lattice structure in intact preparations provides optimal geometric and mechanical constraints on protein-protein interactions, which are

likely to have an impact on the overall structural dynamics. The absence of protein lattice structure may also affect the cooperativity (the Hill coefficient) of the reconstituted system. For example, low values of the Hill coefficient are typically observed in the reconstituted samples^{1, 9, 11}. However, in a recent FRET measurement in muscle fiber bundles, we found that Ca^{2+} sensitivity and the Hill coefficient associated with the opening of the N-domain in cTnC were comparable to values obtained from simultaneous measurement of force and ATPase activity in normal muscle fibers (results will be presented in a new manuscript). Therefore, our results provide evidence for the importance of temperature and protein lattice structure in determining Ca^{2+} sensitivity and cooperativity of the thin filament.

In previous study with reconstituted samples, we observed that the presence of strongly-bound S1 decreased the Hill coefficients¹². This decrease is likely due to the disengagement of the inhibitory region of cTnI from actin when Tm shifts its position from the “closed” state to the “open” state. However, in this study the Hill coefficient was not significantly affected by the presence of either the strongly-bound S1 or the phosphorylation mimic of cTnI. It is unlikely that the FRET probes used in this study are limiting the cooperativity in the cTnC-cTnI interaction since these labeled proteins have no detrimental effect on maximal force, ATPase activity and Ca^{2+} sensitivity of skinned muscle fibers. It is possible that the cooperativity of the system monitored by FRET distances between the central helix of cTnC and the regulatory region of cTnI is insensitive to the presence of the strongly-bound S1 and phosphorylation mimic of cTnI. However, this speculation requires further investigation.

Ca^{2+} -mediated effects on cTnC-cTnI interactions play central roles in activating and deactivating muscle contraction by coupling Ca^{2+} -induced changes in cTnC to the attachment and detachment of strong crossbridges to and from actin. Our previous study suggested that Ca^{2+} -induced interactions between cTnC and the regulatory region of cTnI could be kinetically coupled with the processes that regulate cTnI-actin interactions⁹. Consistent with our previous report⁹, our stopped-flow measurements showed fast kinetics of structural changes associated with the separation of the regulatory region of cTnI from cTnC upon Ca^{2+} removal. Thus, it is likely that this fast structural transition is kinetically coupled with the Ca^{2+} -induced closing of the regulatory N-domain of cTnC and the dynamic interaction between the mobile domain of cTnI and actin to initiate the movement of Tm on the surface of actin filament that regulates crossbridge detachment. However, our data showed that the presence of strong crossbridges had significant feedback impact on these structural transitions. For example, the kinetics of the Ca^{2+} dissociation-induced interaction between cTnC and the regulatory region of cTnI decreased by 28% – 64% in the presence of strongly-bound S1 (Table 2). This is because the strong crossbridges effectively hinder the movement of Tm from the “open” to the “closed” position on the actin surface and slow the kinetics of structural changes that switch cTnI from interacting with cTnC to interacting with actin upon Ca^{2+} removal. When the pseudo-phosphorylation of cTnI (S151E) was present, Ca^{2+} dissociation-induced kinetics decreased to the level observed in the presence of strongly-bound S1, even though no strongly-bound S1 was present. No significant additional reduction in the structural kinetics was observed when strongly-bound S1 was introduced. These results suggest that both the pseudo-phosphorylation cTnI (S151E) and strongly-bound S1 have similar effects on Ca^{2+} dissociation-induced structural interactions between the regulatory region of cTnI and cTnC, but with different mechanism.

To further understand the mechanism of PAK3 phosphorylation of cTnI on thin filament regulation, we measured Ca^{2+} -activated maximal tension and ATPase activity of cardiac muscle fiber bundles (Table 3) reconstituted with the PAK3 phosphorylation mimic of cTnI(S151E). cTnI(S151E) induced a statistically significant decrease in maximum ATPase

activity, but only a marginal decrease in the maximum tension (Table 3). These changes in maximal tension and ATPase activity accounted for a marginal change in the tension cost, which did not provide any meaningful linkage between the pseudo-phosphorylation-induced slow kinetics observed in the Ca^{2+} dissociation-induced thin filament relaxation and crossbridge detachment kinetics.

In summary, we present novel information regarding structural and kinetic effects of PAK3 phosphorylation of cTnI at residue Ser151 on the Ca^{2+} -induced thin filament regulation of cardiac muscle function. The enhanced cTnC-cTnI interaction caused by charge modification within the regulatory region of cTnI provides a molecular basis for the PAK3 phosphorylation-induced changes in structure, Ca^{2+} sensitivity, and relaxation kinetics of the thin filament. Our new findings shed light on the molecular basis of how the Ca^{2+} -activated cardiac thin filament regulation is modulated by strongly-bound crossbridges and PAK3 phosphorylation of cTnI.

MATERIALS AND METHODS

Sample Preparations and Characterizations

To implement FRET in this study, the following single-cysteine mutants from rat clones were generated: cTnC(S89C), cTnI(L160C), cTnI(S167C), cTnI(S151E/L160C) and cTnI(S151E/S157C) mutants. The last two mutants contain pseudo-phosphorylation at Ser151 to mimic PAK3 phosphorylation. These mutants were generated and purified from wild-type rat cTnC and cTnI clones using similar approaches previously described in the literature 1; 8; 13. The recombinant wild-type cTnT was purified as previously reported 14. The other proteins that comprise the thin filament, namely cTm 15, actin 16, and myosin subfragment 1 (S1) from chymotryptic digestion of myosin 17, were obtained from bovine cardiac tissue.

For the FRET measurements, the single cysteine residue of the cTnI(160C) and cTnI(167C) mutants was modified with IAEDANS as FRET donor according to previously described procedures 13; 14. The single cysteine residue of cTnC(89C) was modified with DDPM as FRET acceptor by following a previously described procedure 1. The label ratio was determined using $\epsilon_{325\text{nm}} = 6,000 \text{ cm}^{-1}\text{M}^{-1}$ for AEDANS and $\epsilon_{442\text{nm}} = 2930 \text{ cm}^{-1}\text{M}^{-1}$ for DDPM, respectively. The identities of all cTnI mutants and the labeled proteins were verified using electrospray mass spectrometric analysis. Label ratios for all protein modification were >95%.

The troponin complexes and the thin filament containing different modified proteins were reconstituted using previously described procedures 12. SDS-PAGE and native gel analysis and Ca^{2+} regulation of the actin-activated S1-ATPase activity assay were performed to examine stability and functional effects of cTnI mutations and probe modifications as previously described 9. The results (data not shown) show no evidence of protein degradation and suggest that the functional effects of the mutations and modifications of proteins on the Ca^{2+} regulatory activity were negligible.

Fluorescence Measurements

The steady-state measurements were carried out at 10 ± 0.1 °C on an ISS PCI photon-counting spectrofluorometer equipped with a micro titrator 14. FRET was used in titration experiments to monitor Ca^{2+} -induced changes in each distance. The procedures previously described were used to convert titration data to FRET efficiency 7. In a typical titration experiment, the fluorescence intensity of the donor (AEDANS) excited at 343 nm was monitored at 480 nm in a Ca^{2+} -EGTA-NTA buffer containing 50 mM Mops, pH 7.0, 1 mM

DTT, 2 mM EGTA, 5 mM NTA (nitrilotriacetic acid), 5 mM MgCl₂, and 0.15 M KCl. The free [Ca²⁺] was calculated using the pCa Calculator program provided by Dweck, et al. 18.

Determination of Intersite Distances

FRET from donor AEDANS to the acceptor DDPM was determined from the intensity decays of the donor determined with the donor-alone and donor-acceptor samples. As previously described 19, these decays were measured in the time-domain with an IBH 5000 photon-counting lifetime system equipped with a 347nm of LED light source. The distance-dependent donor decay of a donor-acceptor pair separated by a given distance r is given by

$$I_{DA}(r, t) = \sum_{i=1} \alpha_{D_i} \exp \left[-\frac{t}{\tau_{D_i}} - \frac{t}{\tau_{D_i}} \left(\frac{R_0}{r} \right)^6 \right] \quad (1)$$

where α_{D_i} are the fractional amplitudes associated with the decay times τ_{D_i} for the donor. R_0 is the Förster critical distance at which the transfer efficiency is 0.5. The observed decay of an ensemble of donor-acceptor pairs is given by

$$I_{DA}(t) = \int_0^{\infty} P(r) I_{DA}(r, t) dr \quad (2)$$

$P(r)$ is the probability distribution of distances and is assumed to be a Gaussian function

$$P(r) = \frac{1}{Z} \frac{1}{\sigma \sqrt{2\pi}} \exp \left[-\frac{1}{2} \left(\frac{r - \bar{r}}{\sigma} \right)^2 \right] dr \quad (3)$$

where $\bar{r}$ is the mean distance and σ is the standard deviation of the distribution. The half-width (hw) of the distribution is given by $hw = 2.3544\sigma$. $P(r)$ is normalized by the area, and Z is the normalization factor.

Stopped-Flow Measurements

The kinetic measurements were carried out at 10.0 °C in a KinTek F2004 spectrometer with a 1.5-ms dead time. In the Ca²⁺-dissociation experiments monitored by FRET, a protein sample saturated with Ca²⁺ in a buffer of 50 mM Mops, pH 7.0 containing 1 mM DTT, 5 mM MgCl₂, 0.15 M KCl, and 0.16 mM Ca²⁺ (pCa 3.8) was mixed with an equal volume of the same buffer in which Ca²⁺ was replaced with 2 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid). After mixing, [protein] = 2 μM and [BAPTA] = 1000 μM. The kinetic tracings of donor AEDANS fluorescence intensity ($F_D(t)$) were first determined from a donor-only sample, followed by determination of the kinetic tracing ($F_{DA}(t)$) for the corresponding donor-acceptor sample. Eight-to-ten kinetic tracings were collected for each set of donor only and donor-acceptor samples, and the averages of each set of samples were used to calculate the time-dependent FRET efficiency, $E(t)$:

$$E(t) = 1 - \frac{F_{DA}(t)}{F_D(t)} \quad (4)$$

The resultant FRET efficiency kinetic decays were fitted to a sum of exponentials by a nonlinear least squares method 20.

Preparation of detergent-skinned cardiac muscle fiber bundles and reconstitution of troponin into skinned fiber bundles

Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt), and hearts were rapidly excised and placed into ice-cold high relaxing (HR) solution (mM: 20 BDM, 50 BES, 20 EGTA, 6.29 MgCl₂, 6.05 Na₂ATP, 30 potassium propionate, 10 NaN₃, pH 7.0). Fresh cocktail of protease inhibitors (4 mM benzamidine-HCl, 5 μM bestatin, 2 μM E-64, 10 μM leupeptin, 1 μM pepstatin, and 200 μM PMSF) was added to buffered solutions. Papillary muscle bundles were excised from the left ventricles of rat hearts. Very thin muscle fiber bundles (~150 μm in width and 1.5–2.0 mm in length) were dissected and detergent skinned, as described previously 21. Reconstitution of endogenous troponin in cardiac muscle fibers was based on the method described previously 22. Endogenous troponin in detergent-skinned rat cardiac muscle fibers was removed by first treating fibers with the extraction solution containing cTnT and wild-type cTnI or cTnI mutants for 3–4 h. The extraction solution contained 50 mM *N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES) (pH 7.0 at 20°C), 200 mM KCl, 10 mM 2,3-butane-dione monoxime, 5 mM EGTA, 6.27 mM MgCl₂, 1.0 mM DTT, 0.01% NaN₃, and 6.13 mM MgATP and a fresh cocktail of protease inhibitors. Fibers were washed with the extraction solution followed by washing in 4 ml of HR for 10 minutes with constant stirring. Complete reconstitution of endogenous Tn subunits was achieved by exposing the cTnT-cTnI treated fibers to cTnC (4mg/ml in HR, pH 7.0) for 90–120 minutes at room temperature under constant stirring.

Simultaneous measurement of steady-state isometric force and ATPase activity in detergent-skinned cardiac muscle fiber bundles

The muscle fiber was attached to a motor and a force transducer by aluminum clips. Muscle fiber sarcomere length was measured as previously described 23. The resting sarcomere length was readjusted to 2.2 μm (after 2 or 3 cycles of full activation and relaxation) and monitored by a He-Ne laser diffraction system. The muscle fiber was immersed in a 15-μl bath containing activating solutions with different pCa. Activating solution in the bath was continuously stirred by means of motor-driven vibration of a membrane positioned at the base of the bath. Maximum activating solution (pCa 4.3) contained 31 mM potassium propionate, 5.95 mM Na₂ATP, 6.61 mM MgCl₂, 10 mM EGTA, 10.11 mM CaCl₂, 50 mM BES (pH 7.0), 10 mM NaN₃, 0.9 mM NADH, and 10 mM phosphoenolpyruvate, as well as 4 mg/ml pyruvate kinase (500 U/mg), 0.24 mg/ml lactate dehydrogenase (870 U/mg), and 20 μM A2P5, and a cocktail of protease inhibitors. During steady-state activation, force and ATPase activity (20°C) were measured simultaneously as described before 23; 24.

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The abbreviations used are

cTnC	cardiac troponin C
cTnI	cardiac troponin I
cTnT	cardiac troponin T
FRET	Förster resonance energy transfer
NTA	nitrilotriacetic acid
Mops	3-(<i>N</i> -morpholino)propanesulfonic acid

EGTA	ethylene glycol-bis-(β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
BAPTA	1,2-bis(2-aminophenoxy)ethane- <i>N,N,N',N'</i> -tetracetic acid
IAEDANS	5-(iodoacetamidoethyl)aminonaphthelene-1-sulfonic acid
DDPM	N-(4-dimethylamino-3,5-dinitrophenyl)maleimide

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Figure 1.

Donor (AEDANS) fluorescence spectra and fluorescence decays of FRET in the cardiac thin filament reconstituted with cTnI(160C)AEDANS and cTnC(89C)DDPM. **A:** Steady-state fluorescence spectra measured at different conditions with the reconstituted thin filament preparations without the presence of strongly bound crossbridges; **B:** Steady-state spectral measurements of the samples used in the panel A, but with the presence of strongly bound crossbridges; **C:** Fluorescence intensity decays of the samples used in the Panel A; and **D:** Fluorescence intensity decays of the samples used in the Panel B. Color codes: black – donor-only sample without Ca^{2+} ; dark red – donor-only sample plus Ca^{2+} , red – donor-acceptor sample without Ca^{2+} , pink - donor-acceptor sample plus Ca^{2+} , light blue - donor-acceptor sample containing mimic phosphorylated cTnI without Ca^{2+} , blue - donor-acceptor sample containing mimic phosphorylated cTnI plus Ca^{2+} , and dark blue in Panel C and Panel D – profile of the excitation. Excitation wavelength was 343 nm, and the samples were in 50 mM Mops at pH 7.0, 1mM DTT, 1 mM EGTA, 5 mM MgCl_2 , 0.15 M KCl. When Ca^{2+} was present, it was at 2 mM CaCl_2 .

Figure 2.

Distance analysis of the time-resolved FRET data shown in panel B of Figure 1. **A:** Area normalized distribution of distance between residue Cys160 of cTnI and residue Cys89 of cTnC within the thin filament without the presence of the pseudo-phosphorylation of cTnI(S151E). **B:** Area normalized distribution of distance between residue Cys160 of cTnI and residue Cys89 of cTnC within the thin filament containing the pseudo-phosphorylation of cTnI(S151E). A value of $\kappa^2 = 2/3$ was used in these calculations. Circle – thin filament samples, triangle – thin filament in the presence of strongly bound crossbridges, solid circle and triangle – samples in the absence of Ca^{2+} and the open circle and triangle – samples in the presence of Ca^{2+} . All distributions are plotted using equation 3.

Figure 3.

FRET-based Ca^{2+} titration of the changes of FRET between cTnI(160C)_{AEDANS} and cTnC(89C)_{DDPM} within the thin filament at different conditions. Black: from thin filament containing non-phosphorylated cTnI without strongly bound S1, red: from thin filament containing the pseudo-phosphorylation of cTnI(S151E) without the presence of strongly bound S1; blue: from thin filament containing non-phosphorylated cTnI in the presence of strongly bound S1, pink: from thin filament containing the pseudo-phosphorylation of cTnI(S151E) in the presence of strongly bound S1. The curves were fitted with the Hill equation to obtain values of pCa_{50} and the Hill coefficient. These parameters are listed in Table 2.

Figure 4.

FRET-based kinetic tracings of normalized FRET changes between cTnI(160C)_{AEDANS} and cTnC(89C)_{DDPM} triggered by Ca^{2+} dissociation from cTnC in the reconstituted thin filaments containing non-phosphorylated cTnI (black dots) and the pseudo-cTnI(S151E) (black triangles). These two sets of kinetic tracings were obtained by averaging 7 or 8 stopped-flow tracings. The kinetic tracings were fitted with a two-exponential function (solid smooth lines), and recovered rate constants and amplitudes are given in Table 2.

FRET Distances between cTnC(S89C)_{DDPM} and AEDANS-labeled cTnI at the residues 160 and 167, respectively, within the reconstituted thin filament containing non-phosphorylated cTnI and PAK3 mimic phosphorylated cTnI(S151E) under different conditions

Table 1

Thin Filament Reconstitution	Mg ²⁺			Ca ²⁺		
	Mean Distance(Å)	HW(Å)	χ ²	Mean Distance(Å)	HW(Å)	χ ²
cTnI(L160C) _{AEDANS} -cTnC(S89C) _{DDPM}	33.4	8.6	1.17	26.7	5.3	1.21
cTnI(L160C) _{AEDANS} -cTnC(S89C) _{DDPM} + S1-ADP	30.9	7.1	1.22	25.5	4.3	1.20
cTnI(S151E/L160C) _{AEDANS} -cTnC(S89C) _{DDPM}	31.1	7.8	1.12	24.7	5.7	1.19
cTnI(S151E/L160C) _{AEDANS} -cTnC(S89C) _{DDPM} + S1-ADP	29.9	7.3	1.20	24.5	5.2	1.14
cTnI(S167C) _{AEDANS} -cTnC(S89C) _{DDPM}	40.2	9.4	1.25	33.6	5.8	1.19
cTnI(S167C) _{AEDANS} -cTnC(S89C) _{DDPM} + S1-ADP	39.0	7.6	0.95	32.0	4.3	1.12
cTnI(S151E/S167C) _{AEDANS} -cTnC(S89C) _{DDPM}	39.1	7.5	1.11	34.2	6.3	1.24
cTnI(S151E/S167C) _{AEDANS} -cTnC(S89C) _{DDPM} + S1-ADP	38.3	8.1	1.23	33.7	5.2	1.08

Table 2

Kinetic and Equilibrium Parameters sensed by Ca^{2+} -induced changes in FRET between cTnC(89C)_{DDPM} and AEDANS-labeled cTnI at residues 160 and 167, respectively, within the reconstituted thin filament containing non-phosphorylated cTnI and PAK3 mimic phosphorylated cTnI(S151E) under different conditions *

FRET transition within thin filament	Thin Filament			Thin Filament + S1-ADP		
	Transition rates, s^{-1}	pCa ₅₀	Hill coefficient	Transition rates, s^{-1}	pCa ₅₀	Hill coefficient
cTnI(L160C) _{AEDANS} -cTnC(S89C) _{DDPM}	119.1±17 (0.55) 31.1±5 (0.45)	5.93±0.06	1.03±0.11	43.2±5.2 (0.84) 13.3±2 (0.14)	6.22±0.12	1.07±0.06
cTnI(S151E/L160C) _{AEDANS} -cTnC(S89C) _{DDPM}	60.3±10 (0.69) 24.7±6 (0.31)	6.12±0.06	1.24±0.09	50.8 (0.61) 17.4±2 (0.39)	6.39±0.09	1.09±0.08
cTnI(L167C) _{AEDANS} -cTnC(S89C) _{DDPM}	108.9±12 (0.60) 23.9±3 (0.40)	5.88±0.05	1.15±0.08	78.8±2 (0.47) 21.6±2 (0.53)	6.29±0.08	1.02±0.09
cTnI(S151E/L167C) _{AEDANS} -cTnC(S89C) _{DDPM}	62.6±7 (0.81) 21.9±3 (0.19)	6.18±0.09	1.18±0.08	66.1±7 (0.67) 14.2±5 (0.33)	6.49±0.09	1.04±0.05

* Kinetic parameters for conformational transitions were derived from Ca^{2+} dissociation stopped-flow experiments in which the initial pCa was 3.8. Equilibrium parameters were derived from Ca^{2+} titration experiments in which pCa varied from 9.2 to 3.5. In equilibrium experiments, pCa₅₀ was determined from Ca^{2+} titration curves. These equilibrium titration curves were analyzed by the Hill equation to obtain pCa₅₀ and the Hill coefficient.

Measurements of maximum force and tension-pCa relationship of cardiac muscle fiber bundle reconstituted with wild-type cTnI and cTnI mutants containing PAK3 mimic phosphorylation (S151E)

Table 3

cTnI Reconstitutions	Maximum Force (mN/ mm ²)	Maximum ATPase (p mol/mm ³ /s)	pCa50-force	n-force	Tension Cost
Wild-type cTnI	47.46±1.68	221.64±6.73	5.49±0.02	5.09±0.22	4.67±0.21
cTnI(L160C)	45.78±1.37	216.69±5.94	5.39±0.03	5.21±0.43	4.69±0.19
cTnI(S151E/L160C)	44.23±1.24	198.55±5.61	5.61±0.02	5.46±0.58	4.51±0.17
cTnI(S167C)	49.23±2.21	229.78±6.23	5.43±0.02	4.37±0.39	4.67±0.20
cTnI(S151E/S167C)	47.02±2.14	200.94±5.91	5.68±0.03	4.08±0.34	4.43±0.21