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Structural basis for the *in situ* Ca^{2+} sensitization of cardiac troponin C by positive feedback from force-generating myosin cross-bridges

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ABSTRACT

The *in situ* structural coupling between the cardiac troponin (cTn) Ca^{2+}-sensitive regulatory switch (CRS) and strong myosin cross-bridges was investigated using Förster resonance energy transfer (FRET). The double cysteine mutant cTnC(T13C/N51C) was fluorescently labeled with the FRET pair 5-(iodoacetamidoethyl)aminonaphthelene-1-sulfonic acid (IAEDENS) and N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM) and then incorporated into detergent skinned left ventricular papillary fiber bundles. Ca^{2+} titrations of $cTnC(T13C/N51C)_{AEDENS/DDPM}$-reconstituted fibers showed that the Ca^{2+}-dependence of the opening of the N-domain of cTnC (N-cTnC) statistically matched the force–Ca^{2+} relationship. N-cTnC opening still occurred steeply during Ca^{2+} titrations in the presence of 1 mM vanadate, but the maximal extent of ensemble-averaged N-cTnC opening and the Ca^{2+}-sensitivity of the CRS were significantly reduced. At nanomolar, resting Ca^{2+} levels, treatment with ADP·Mg in the absence of ATP caused a partial opening of N-cTnC. During subsequent Ca^{2+} titrations in the presence of ADP·Mg and absence of ATP, further N-cTnC opening was stimulated as the CRS responded to Ca^{2+} with increased Ca^{2+}-sensitivity and reduced steepness. These findings supported our hypothesis here that strong cross-bridge interactions with the cardiac thin filament exert a Ca^{2+}-sensitizing effect on the CRS by stabilizing the interaction between the exposed hydrophobic patch of N-cTnC and the switch region of cTnI.

Introduction

Phenomenological evidence has long pointed toward the ability of force-generating, strong myosin cross-bridges (XBs)[1] to exerting a positive, activating feedback on myocardial contractile regulation [1–6]. This positive feedback mechanism is uniquely important to the physiological function of cardiac muscle [6,7], and understanding its molecular basis is thus a goal of considerable therapeutic potential [8]. Myocardial contractile regulation is traced to cardiac troponin (cTn), which is the protein complex that responds to sarcomeric Ca^{2+} signals in regulating the ability of XBs to interact with the cardiac thin filament and generate force. The cTn complex is a ternary heteromer [9,10] whose subunits include: cTnC, a calmodulin-like Ca^{2+}-sensing protein; cTnI, an adaptor protein whose interactions with other thin filament proteins are Ca^{2+} dependent, and which works in concert with tropomyosin to inhibit the XB cycle; and cTnT, another adaptor protein that integrates the cTn complex into the ultrastructure of the thin filament. Therefore, cTn may be thought of as a binary, "activate/deactivate" Ca^{2+}-sensitive regulatory switch (CRS) [11,12] whose function essentially involves a conformational signaling process driven principally by a Ca^{2+} chelation reaction. The terminal, critical structural outcome of this conformational signaling is a change in the steric accessibility of myosin binding sites on actin to XBs [13,14].

The conformational changes by which the activating CRS signal leads to interactions between strong cycling XBs and the thin filament have been well characterized by *in vitro* biophysical studies. In the absence of Ca^{2+}, the C-terminal cTnI inhibitory region (cTnI-Ir; residues 130–150) and mobile domain (cTnI-Md; residues 168–210) are both bound to actin [15]. This confines the azimuthal position of tropomyosin on actin over myosin binding sites [16] in what is known as the blocked state of the three-state model of thin filament regulation [17,18]. Activation is initiated through a drag-and-release mechanism [8,11,19], wherein N-cTnC binds to the

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[1] *Abbreviations used:* BDM, 2,3-butanedione monoxime; CRS, Ca^{2+}-sensitive regulatory switch; cTn, cardiac troponin; DDPM, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; $cTnC_{FRET}$, $cTnC(T13C/N51C)_{AEDENS/DDPM}$; FRET, Förster resonance energy transfer; IAEDENS, 5-(iodoacetamidoethyl)aminonaphthelene-1-sulfonic acid; HR, highly relaxing; Ir, inhibitory region; Md, mobile domain; N-cTnC, N-domain of cTnC; PI, protease inhibitors; Sr, switch region; Vi, orthovanadate; XB, cross-bridge.

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cTnI switch region (cTnI-Sr; residues 151–167) and consequently "drags" on cTnI to "release" the actomyosin-ATPase inhibitory interactions between cTnI and actin. The N-cTnC–cTnI-Sr interaction that is central to this mechanism is triggered by the binding of Ca^{2+} to N-cTnC. This stimulates N-cTnC to sample an open conformation [20–22] wherein its buried hydrophobic core becomes a surface exposed hydrophobic patch ready for interaction with cTnI-Sr. Open N-cTnC conformers are then conformationally selected by the binding of cTnI-Sr to the exposed hydrophobic patch [22]. The N-cTnC–cTnI-Sr interaction thus simultaneously stabilizes the Ca^{2+}-bound, open N-cTnC conformation [5,22] and destabilizes the blocked-state actin–cTnI-Ir and actin−cTnI-Md interactions [19,23]. Repositioning of cTnI on the thin filament enables tropomyosin to move to a "closed-state" position [17,18] wherein the myosin binding sites of actin are partially uncovered. It has been suggested that Ca^{2+} binding alone may not completely stabilize these structural changes, such that the thin filament could be left in a conformational equilibrium between its blocked and closed states [11,12]. In any case, the structural changes stimulated by Ca^{2+} binding enable myosin heads to thereafter bind to actin and interact as strong XBs that induce additional azimuthal movement of tropomyosin to an "open-state" position [17,18]. Since myosin binding sites are completely uncovered and Ca^{2+} binding is significantly enhanced in the open state, it is this myosin-induced movement of tropomyosin from the closed- to open-state position that is implicated as the source of positive feedback from strong XBs [5,8].

Numerous *in vitro* structural studies conducted in reconstituted thin filaments have also shown that strong XBs exert structural effects on cTn that are consistent with the positive feedback mechanism. It was shown through Förster resonance energy transfer (FRET) that, when averaging over the observed molecular ensemble, the presence of S1·ADP during Ca^{2+} titrations shifts cTnI further away from actin [23] and closer toward N-cTnC [11]. Based on electron microscopy [13] and fluorescence polarization [19] studies, this is caused by S1-mediated disruption of the closed-state interactions between actin and cTnI-Md that are associated with the fly casting mechanism [24]. The consequently increased capacity for localization between N-cTnC and cTnI-Sr thus stabilizes (*i.e.* makes more stable) the Ca^{2+}-sensitizing N-cTnC–cTnI-Sr interaction [11,19,25,26]. Such S1-induced stabilization of the N-cTnC–cTnI-Sr interaction is also evidenced by an increased Ca^{2+}-sensitivity of CRS signaling [27,28], slowed kinetics of the deactivating CRS signal [19,25,27], and reduced exposure of cTnI-Sr to solvent [19]. *In vitro* biophysical studies thus implicate strong XB induced stabilization of the N-cTnC–cTnI-Sr interaction as the source of the positive feedback mechanism [7,19] and the hysteresis [29,30] observed in the N-cTnC−Ca^{2+} chelation reaction. However, it is not yet clear how structural interpretations based on *in vitro* data would hold in the myofilament lattice (which we refer to as *in situ*) [31].

Though some *in situ* biophysical studies have indicated that the positive feedback mechanism affects cTnC structure in the molecular environment of the sarcomere, the structural information provided has been less mechanistically clear due to the nature of the techniques used. Smith et al. tested for changes induced by orthovanadate (Vi) in the *in situ* dichroism of fluorescently labeled cTnC(C35S) [32]. They determined that ~87% of the structural change signal associated with maximal thin filament activation was due to Ca^{2+} binding, with the remaining ~13% attributable to the positive feedback mechanism; due to the nature of dichroism, it was difficult to connect these changes in signal to specific changes in thin filament activation states. In a different study by another laboratory, *in situ* fluorescence polarization measurements were conducted by Sun et al. to determine the effects of strong XB interactions on N-cTnC structure [31]. They labeled the C and E helices each with bifunctional rhodamine, and found through the use of blebbistatin and rigor that strong XBs stimulate additional changes in C and E helix orientation beyond what is induced by Ca^{2+} binding alone. Interestingly, their data challenged the long hypothesized role of strong XBs in the cooperative activation of the cardiac thin filament [4,33,34]. However, it was unclear how the orientational changes they observed relate to the *in vitro* conformational behavior of N-cTnC, and they recommended future studies accordingly. Therefore, it is still unknown how the geometric and mechanical constraints of the myofilament lattice may affect the N-cTnC–cTnI-Sr interaction, or whether *in situ* evidence will affirm strong XB induced stabilization of the N-cTnC–cTnI-Sr interaction as the structural basis for the positive feedback mechanism in cardiac muscle.

In this biophysical study, the *in situ* structural coupling between the cTn CRS and strong XBs was examined using FRET spectroscopy. We hypothesized that strong XB interactions with the cardiac thin filament exert a Ca^{2+}-sensitizing effect on the CRS by stabilizing the interaction between the exposed hydrophobic patch of N-cTnC and cTnI-Sr. This hypothesis was formed based on reasoning that strong XB attachment could stabilize the N-cTnC–cTnI-Sr interaction in two possible ways. Based on observations of the conformational behavior of the CRS, some biophysical evidence has suggested that the Ca^{2+} saturated thin filament could be in a conformational equilibrium between its blocked and closed states in the absence of strong XB attachment [11,12,22,35]. Therefore, one form of stabilization could be that strong XBs indirectly stabilize the open conformation of N-cTnC by moving tropomyosin to the open-state position. Such tropomyosin movement would exert a disrupting effect on the cTnI–actin interactions that counterbalance the N-cTnC–cTnI-Sr interaction, thereby increasing the availability of cTnI-Sr to interact with the hydrophobic patch of N-cTnC. A second form of stabilization is also possible, wherein strong XBs would induce a tightening of the N-cTnC–cTnI-Sr interaction itself by disrupting the counterbalancing constraints on cTnI-Sr [20,32]. Strong XBs could thus indirectly increase the "openness" of N-cTnC molecules bound to cTnI-Sr. Increased ensemble-averaged hydrophobic patch exposure and enhanced CRS Ca^{2+}-sensitivity [11,12] should result from either possible form of the hypothesized strong XB induced stabilization of the N-cTnC–cTnI-Sr interaction.

In order to test our hypothesis, a double cysteine mutant cTnC(T13C/N51C) was labeled with the FRET pair 5-((((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDENS) and (4-dimethyl-amino-3,5-dinitrophenyl)-maleimide (DDPM) [28] (Fig. 1a) and incorporated into dissected, permeabilized left ventricular papillary myocardium from *Rattus norvegicus*. As used in study, this FRET labeled N-cTnC mutant simultaneously provides information on two aspects of N-cTnC structure: (1) the fraction of molecules in the N-cTnC ensemble that are in the open conformation, and (2) the extent of separation between Cys-13 and Cys-51 that is associated with the open conformation of N-cTnC [11,12]. In our experiments, the effect of Ca^{2+}-binding on ensemble-averaged N-cTnC opening was isolated from the effect of strong XB interactions with the thin filament by conducting Ca^{2+} titrations in the absence or presence of either Vi or ADP·Mg. Ca^{2+}-titration experiments were controlled with regard to potential effects from cTnC variants, labeling and reconstitution into myocardial fibers. Our *in situ* FRET results supported our hypothesis that the positive feedback mechanism is mediated by the stabilizing effect of strong XBs on the N-cTnC–cTnI-Sr interaction. The ensemble-averaged extent of N-cTnC opening was both highly sensitive to strong XB interactions and also positively correlated with the Ca^{2+}-sensitivity of CRS signaling. Interestingly, our results also indicated that strong XB interactions were not needed for the cardiac thin filament to activate cooperatively, and the positive feedback mechanism was confined to the A-band in the myofilament lattice.