MODULATION OF THE CA$^{2+}$ SENSITIVITY OF CARDIAC TROPONIN C BY PKC PHOSPHORYLATED CARDIAC TROPONIN T AND CA$^{2+}$ SENSITIZERS

By

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Cardiac troponin (cTn) exerts temporal control over the cyclic processes of contraction and relaxation in the heart. It does this by binding with calcium which initiates an allosteric chain of protein structural transitions culminating in force generation. Regulation over the sensitivity with which cTn binds calcium is thus of great importance in maintaining proper cardiac output. Unsurprisingly many diseases of the heart are caused by, or associated with, chronic elevation or depression in cTn's calcium sensitivity. Therapies which seek to reverse these chronic alterations in calcium sensitivity may have tremendous value, however insufficient understanding of the mechanisms by which diseases, or the inotropes intended to combat them, actually alter cTn's calcium sensitivity have limited their success. The work contained in this dissertation seeks to contribute to the collective understanding of these mechanisms by looking at two basic types of sensitivity modulators. The first are post translational modifications, specifically protein kinase C (PKC) phosphorylations of the tropomyosin binding subunit of cTn (cTnT) which have been shown to depress calcium sensitivity. The second, calcium sensitizers, are a class of inotrope specifically designed to increase the sensitivity of the calcium binding subunit of cTn (cTnC) for calcium. The spectroscopic technique of fluorescence resonance energy transfer (FRET) was used to monitor regulatory switching, while fluorescence homodimerization induced self
quenching was used to study opening of cTnC's hydrophobic cleft. Additionally, a newly
developed absorbance based technique was used to measure the dynamic equilibrium between
open and closed conformations in the hydrophobic cleft of cTnC. Using these techniques, the
impact on the structure/function of cTn by these two types of modulators was determined.
Briefly the findings of this work are 1) Most calcium sensitizers work by binding to the
hydrophobic cleft and shifting its dynamic equilibrium towards the open state but have varying
effects on cTn's relaxation kinetics 2) Altering the dynamic equilibrium of cTnC's hydrophobic
cleft will generally result in a change in cTnC's calcium sensitivity 3) Alterations in
position/flexibility of the switch region relative to the hydrophobic cleft are sufficient to explain
the calcium sensitivity effects of cTnT PKC pseudo-phosphorylation mutants.
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Chapter 1: The troponin complex and Ca\(^{2+}\) sensitivity

1.1 Motivation for research

According to the 2017 release of "Heart Disease and Stroke Statistics" published by the American Heart Association (AHA) cardiovascular disease (CVD) remains the single most significant health burden in the United States. Approximately 30% of U.S. citizens live with at least one form of CVD with this number expected to climb to 43.9% by 2030. One of every three deaths in the U.S. this year will be due to CVD, which equates to one person dying of CVD every 40 seconds. The toll of heart disease on both life expectancy and the economy are immense, projections say that by 2030 the total direct costs of CVD will be $818 billion with an additional $276 billion in indirect costs, and it is estimated that if all deaths related to CVD were prevented life expectancy in the U.S. would increase 7 years [1]. These grim statistics motivate spending at the national level, primarily through the National Institutes of Health (NIH), to conduct research aimed at all levels of the heart and its pathology. According to the NIH's research portfolio, $2.07 billion was dedicated to cardiovascular research in 2017 with a total of $3.83 billion being spent on all CVD related research, underscoring the importance and scale of cardiovascular research conducted in this country. The work presented in this dissertation focuses on understanding the heart at the most basic structural level, the protein level, with the goal of contributing to our understanding of the molecular processes which underlie the Ca\(^{2+}\) mediated regulation of contraction. Specifically, this dissertation looks at the way troponin's Ca\(^{2+}\) sensitivity, kinetics, structure and dynamic equilibria are modulated by disease related post translational modifications (PTMs) and inotropic agents, with the ultimate goal of facilitating creation of treatments which therapeutically modulate troponin's Ca\(^{2+}\) sensitivity.
1.2 Cardiac troponin, the Ca$^{2+}$ activated force regulator in the heart

The job of the heart is to perfuse the body with blood, and it does this by cyclically contracting and relaxing through the "beating" of its muscular tissue known as myocardium. The myocardium consists of cells called cardiomyocytes, inside of which long fiber like structures, the myofibrils, are responsible for generating the force which drives contraction. The basic repeating structure within the myofibril is the sarcomere, which consists of interdigitated thick and thin filaments. The thick filament consists primarily of myosin, the ATPase motor protein, which is able to bind with the thin filament and produce force. The thin filament is composed of double helix of polymerized actin which extends perpendicularly from its attachment point known as the Z-disk. As myosin binds to actin, producing force, the thick and thin filaments slide past each other pulling neighboring z-disks closer together, thus producing contraction in the myocardium. Temporal control over the process of contraction is enacted by tropomyosin and the cardiac troponin complex which lie along the thin filament. The basic repeating unit of the thin filament consists of seven monomeric units of actin, one tropomyosin dimer, and one heterotrimeric troponin complex. The tropomyosin dimer consists of two tropomyosin molecules in a coiled coil that winds along actin’s surface, with the troponin complex tethered to tropomyosin. The troponin complex is an obligate heterotrimeric protein consisting of three distinct subunits, each with a specific function. Cardiac troponin I (cTnI), the inhibitory subunit, is able to bind to actin and, along with tropomyosin, inhibits strong myosin binding to actin. Cardiac troponin C, the Ca$^{2+}$ binding subunit, undergoes a structural transition upon Ca$^{2+}$ binding to its regulatory site (site II) which results in increased exposure of a hydrophobic cleft within its N-terminal domain to which cTnI can bind, thus removing cTnI's inhibition of strong actin-myosin binding. Cardiac troponin T (cTnT), the tropomyosin binding subunit, tethers the
troponin complex to tropomyosin at its N-terminal half while its C-terminal half interacts with, and fixes the relative orientations of, cTnI and cTnC creating what is known as the "core domain" of troponin [2, 3]. Canonically the process of Ca$^{2+}$ induced activation of contraction in the sarcomere is thought of as a tightly coupled cascade of allosteric protein structural transitions. The process begins with Ca$^{2+}$ binding to the single functional Ca$^{2+}$ binding site in cTnC's N-terminal domain (site II also referred to as the regulatory site). This induces a modest structural rearrangement of cTnC helices B and C relative to helices N, A, and D which partially exposes hydrophobic residues in what is intuitively referred to as the "hydrophobic cleft/patch/pocket". The switch region of cTnI is then able to bind with the hydrophobic cleft of Ca$^{2+}$ bound cTnC in a process known as Ca$^{2+}$ switching. Because the switch region of cTnI is sandwiched between the inhibitory region and mobile domain of cTnI, both of which associate with actin, binding of the inhibitory region of cTnI to cTnC's hydrophobic cleft pulls these two flanking regions of cTnI off of actin thus relinquishing their inhibitory effects on actin-myosin binding. These structural transitions in cTnC and cTnI are conducted via cTnT to tropomyosin, causing tropomyosin to move azimuthally relative to the axis of the thin filament away from myosin binding sites on actin's surface. With the steric inhibition of both cTnI and tropomyosin on actin-myosin binding removed force generating strong crossbridges can now be formed resulting in force production. The process of relaxation is essentially the reverse of contraction, initiated by Ca$^{2+}$ dissociation from the regulatory site of cTnC which causes both cTnI and tropomyosin to return to their myosin blocking positions on actin's surface. Thus in both contraction and relaxation the affinity with which cTnC binds with Ca$^{2+}$, known as its Ca$^{2+}$ sensitivity, is of central importance. For in depth reviews on these topics please see [4-9].
Maintaining the proper timing and degree of force production required to efficiently pump blood requires that the heart carefully tune sarcomeric Ca\(^{2+}\) levels and/or cTnC's Ca\(^{2+}\) sensitivity. This is because the higher cTnC's Ca\(^{2+}\) sensitivity generally the greater the active force produced at a given Ca\(^{2+}\) concentration and the slower the rate of cardiac relaxation. Perhaps the most often used example which illustrates the interrelationship between cTnC's Ca\(^{2+}\) sensitivity, relaxation rate and cardiac output is that of beta-adrenergic stimulation. When the body’s demand for oxygen increases during times of stress or exercise additional cardiac output is required, this is achieved by a process known as beta-adrenergic stimulation. Briefly, beta-adrenergic stimulation occurs when epinephrine or norepinephrine bind to beta receptors on cardiac myocytes, this initiates a pathway which up-regulates PKA and results in increased phosphorylation of cTnI ser23 and ser24. These residues occur in the cardiac specific 31 residue N-terminal region of cTnI known to be essential in modulating the Ca\(^{2+}\) sensitivity of the troponin complex. This N-terminal region of cTnI is believed to electrostatically interact with the regulatory domain of cTnC (on the side opposite the hydrophobic cleft) and properly align it with cTnI's switch region facilitating binding [9]. Phosphorylation of ser23 and ser24 reduces the strength of this electrostatic interaction resulting in misalignment and reduced binding probability between cTnI's switch region and the hydrophobic cleft of cTnC thereby reducing Ca\(^{2+}\) sensitivity [9]. This reduced Ca\(^{2+}\) sensitivity is compensated for by increased peak Ca\(^{2+}\) levels during systole, another result of the beta-adrenergic pathway. The reduced cTnC Ca\(^{2+}\) sensitivity results in an increased relaxation rate within the thin filament and the sarcomere, which is necessary to support the increased heart rate associated with the beta-adrenergic response. Thus the heart is able to beat faster and pump more blood to the body without impairing relaxation or decreasing the stroke volume. The phenomena of beta-adrenergic stimulation is a relatively well
characterized process illustrating the essential regulatory role that the Ca\textsuperscript{2+} sensitivity of cTnC has in the heart. The remainder of this chapter and dissertation will delve into our studies on some of the less well understood areas of cTnC Ca\textsuperscript{2+} sensitivity modulation. In section 1.3 motivation for studying the role of PKC phosphorylated cTnT in altering the Ca\textsuperscript{2+} sensitivity of the cTnI-cTnC switching event will be given as well as a summary of our study on this topic. The full study is presented in chapter 2. In section 1.4 and chapter 3 Ca\textsuperscript{2+} sensitizer induced changes in the Ca\textsuperscript{2+} sensitivity and relaxation kinetics of cTnC will be examined by monitoring hydrophobic cleft opening. Section 1.5 and chapter 4 will then take a dynamic equilibrium perspective on cTnC function, considering cTnC and its Ca\textsuperscript{2+} sensitivity in terms of dynamic equilibrium. Finally

1.3 PKC phosphorylation of cTnT

The example of PKA phosphorylation of cTnI given in section 1.2 is just one of many PTMs of myofilament proteins thought to play an important role in tuning the properties of the sarcomere. Several PKC isoforms are known to be upregulated during heart failure [10] which are able to phosphorylate four residues within the C-terminal half of cTnT (the T2 region), these residues being Thr-195, Ser-199, Thr-204 and Thr-285 in rat myocardium [11-13]. Although the precise physiological role that PKC phosphorylation of cTnT plays in heart failure is not agreed upon, the general reduction in contractility observed in the presence of PKC phosphorylated cTnT [14, 15] have caused many to believe that increased PKC phosphorylation of cTnT may contribute to the pathophysiology of heart failure [13, 16, 17]. The region of cTnT in which these phosphorylations occur, the T2 region, serves to scaffold cTnI and cTnC, fixing their positions relative to one another. This means the T2 region could play a role similar to the cardiac specific
N-terminal extension of cTnI and tune the alignment/local concentration of cTnI's switch region "seen" by cTnC's hydrophobic cleft. A very clever article discusses how the local concentration of cTnI's switch region in the vicinity of cTnC's hydrophobic cleft can explain the effects on Ca\(^{2+}\) sensitivity observed in a wide range of mutations and PTMs [18]. Some very important work on the effect of PKC phosphorylated cTnT on the sarcomere has been done which showed that depending on the specific sites phosphorylated or the myosin isoform context significantly different results could be observed [13, 19]. These studies however looked at cardiac muscle fiber, and thus did not provide information on the effect these phosphorylations have on the structure/function of the cTn core domain or how these changes may explain the observed changes in Ca\(^{2+}\) sensitivity. In order to help fill this hole in our knowledge we performed a fluorescence resonance energy transfer (FRET) based study which examined the effect of PKC pseudo-phosphorylation mutants of cTnT on the cTnI-cTnC interaction. This study is presented in chapter 2. Briefly, it was observed that the Ca\(^{2+}\) sensitivity of the cTnI-cTnC switching event was significantly blunted by the presence of the cTnT(T204E) in the context of the thin filament. Pseudo-phosphorylations at additional sites increased the Ca\(^{2+}\) sensitivity, suggesting PKC phosphorylation can either increase or decrease Ca\(^{2+}\) sensitivity depending on which site or combination of sites is phosphorylated. Structural and kinetic results consistently pointed towards a shift from the inactivated to the activated state of the thin filament underlying the Ca\(^{2+}\) sensitization observed for most of the pseudo-phosphorylations. This trend was also present for cTnT(T204E) which is inconsistent with the Ca\(^{2+}\) desensitization induced by this mutant. We propose that cTnT(T204E) subtly changes the alignment between cTnI's switch region and cTnC's hydrophobic cleft, reducing the effective collisional probability of these two regions without actually changing their average separations.
1.4 Ca\textsuperscript{2+} sensitizers

Most diseases of the heart are associated with altered Ca\textsuperscript{2+} sensitivity, and chronic changes in Ca\textsuperscript{2+} sensitivity are considered sufficient by some to induce cardiomyopathy [20, 21]. Either the Ca\textsuperscript{2+} sensitivity is chronically elevated as in hypertrophic cardiomyopathy (HCM), or depressed as is often the case in dilated cardiomyopathy (DCM) [5, 22, 23]. Naturally this has lead to the design and implementation of various pharmacological agents which seek to remedy these diseases by altering Ca\textsuperscript{2+} sensitivity. One of the newest classes of compounds, called Ca\textsuperscript{2+} sensitizers, aim to mitigate the progression of heart failure by increasing contractility of damaged heart by directly binding to, and altering the Ca\textsuperscript{2+} sensitivity of, cTnC [24, 25]. Unfortunately the efficacy of these compounds has been lack luster, with most studies showing no improvement in mortality [26, 27]. The reasons underlying this poor performance may come from several causes, prime among which is impaired relaxation rate, but a general lack of molecular level insight into the mechanisms of action at the troponin and thin filament level make it difficult to know when use of a given Ca\textsuperscript{2+} sensitizer is appropriate given the context of the disease being treated. Additionally, a refinement in our understanding of how Ca\textsuperscript{2+} sensitizers alter troponin behavior will facilitate creation of improved drugs with specifically tailored characteristics.

Chapter 3 presents work done to characterize the structural/functional impact of four common Ca\textsuperscript{2+} sensitizers (EMD 57033, levosimendan, bepridil, and pimobendan) on cTnC's hydrophobic cleft using a novel self-quenching fluorescent technique. The results from this study showed a Ca\textsuperscript{2+} sensitizers generally lived up to their name and increased Ca\textsuperscript{2+} sensitivity for the event of hydrophobic cleft opening. Bepridil and pimobendan had a very pronounced impact on cTnC's Ca\textsuperscript{2+} sensitivity, with levosimendan and EMD 57033 inducing relatively minor or no sensitization. Surprisingly the rate of relaxation, which would generally be expected to decrease when cTnC's Ca\textsuperscript{2+} affinity is higher, was only reduced for pimobendan, while bepridil actually increased the rate of relaxation and the both levosimendan and EMD 57033 had no discernable effect. The somewhat surprising result that bepridil enhanced relaxation rate is explained by the fact that cTnI's switch region and bepridil are known to sterically clash with each other
when bound to cTnC’s hydrophobic cleft, which results in destabilized cTnI binding and faster transition away from this state [25, 28].

1.5 Dynamic Equilibria in cTnC

In recent years a shift in the way we think about proteins has occurred. As imaging techniques have improved the significant role that transiently accessed structural states play in protein function has become clear. Indeed, proteins are now thought of as existing in "dynamic equilibrium" wherein the protein can access various distinct conformations under a single set of environmental conditions, and that often the reactive or important structures for protein function are only a small subset of those available [29-31]. Indeed it appears that nature often uses this dynamic equilibrium to achieve the proper reactivity of its proteins or tunes this equilibrium through various PTMs or ligand binding events for purposes of regulation [32-34]. Thus to fully understand how a protein functions it is necessary to characterize the dynamic equilibrium of that protein, the structure and role of each accessible structure within that equilibrium and how ligand binding alters this equilibrium. An increasing body of evidence suggests cTnC’s hydrophobic cleft exists in dynamic structural equilibrium and that Ca$^{2+}$ binding to cTnC shifts this equilibrium towards the open conformation [28, 35, 36]. Because the open conformation of the hydrophobic cleft is known to have significantly higher affinity for Ca$^{2+}$ [36] it is reasonable to hypothesize that mutations/drugs/PTMs which modulate the Ca$^{2+}$ sensitivity of cTnC may do so by altering the equilibrium between the open and closed conformations in the hydrophobic cleft. Direct observation or quantification of cTnC’s conformational equilibria are lacking however, and our current understanding of cTnC’s dynamic equilibrium are based on fitting of data with models crafted from generous applications of assumptions. What is needed is more direct data showing empirically the presence of both open and closed conformations in cTnC’s hydrophobic
cleft. Such data would help conclusively prove the hydrophobic cleft does in fact exist in a conformational equilibrium, and would also allow for the impact on these equilibria by Ca$^{2+}$ sensitivity modulators to be tested. The study presented in chapter 4 was designed with these aims in mind. By employing a novel fluorescent dimerization approach, developed in this lab, it was possible to quantify the equilibrium between closed and open conformations of the hydrophobic cleft in cTnC, as well as to observe the impact on this equilibrium by Ca$^{2+}$, cTnI, cTnT, Ca$^{2+}$ sensitizers and the Ca$^{2+}$ sensitivity blunting PKC phosphorylation mimic cTnT(T204E). The results show that the hydrophobic cleft of isolated cTnC does in fact exist in an equilibrium between open and closed conformations in both the presence and absence of Ca$^{2+}$. This equilibrium was modulated by Ca$^{2+}$ binding which served to increase the open population of cTnC's hydrophobic cleft from approximately 14.5% to 33.9%. Amongst other things, this suggests conformational selection as the most likely mechanism by which cTnI binds to the hydrophobic cleft. As might be expected, inclusion of cTnI significantly increased the population of cTnC with an open hydrophobic cleft in the presence of Ca$^{2+}$ in line with the idea that cTnI binding to the hydrophobic cleft stabilizes this conformation and thus increases its population. An anomalous increase in observed hydrophobic cleft opening in the absence of Ca$^{2+}$ induced by cTnI was attributed to steric clash between cTnI's switch region and our reporter dye, as opposed to a true hydrophobic cleft opening. Inclusion of cTnT, reduced the amount of cTnI induced dimer disruption in the absence of Ca$^{2+}$, most likely by decreasing the mobility of cTnI via the stabilizing effect of the IT arm. The Ca$^{2+}$ desensitizing phosphorylation mimic, cTnT(T204E), appears to further reduce the cTnI-dimer clash, suggesting reduced collisional rate between cTnI’s regulatory region and cTnC may explain how cTnT(T204E), which has minimal direct contact with cTnC, is able to reduce Ca$^{2+}$ sensitivity of the troponin complex. Addition of Ca$^{2+}$
sensitizing agents increased the TAMRA monomer/open population in cTnC in both the presence and absence of Ca\(^{2+}\), consistent with the view that these drugs stabilize and bind to the open conformation of the hydrophobic cleft. All sensitizers showed an enhanced impact on cTnC’s dynamic equilibrium in the presence of Ca\(^{2+}\) or cTnI, indicating that increased opening of the hydrophobic cleft induced by Ca\(^{2+}\) or cTnI facilitates increased sensitizer binding, which in turn further increases the probability of hydrophobic cleft opening. Taken together these results show that cTnC's hydrophobic cleft exists in a dynamic equilibrium between at least open and closed conformations, and that modulation of this equilibrium likely represents a central pathway by which mutations/drugs/PTMs impact Ca\(^{2+}\) sensitivity. Additionally, the local effective concentration of cTnI's switch region seen by cTnC's hydrophobic cleft appears important in regulating Ca\(^{2+}\) sensitivity, and a reduction in this parameter may underlie the desensitization seen in cTnT(T204E) containing samples.
1.6 References


Chapter 2: FRET Study of the Structural and Kinetic Effects of PKC Phosphomimetic Cardiac Troponin T Mutants on Thin Filament Regulation

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2.1 Abbreviations

cTnT(3M): Cardiac troponin tropomyosin binding subunit with residues T195, S199 and T204 replaced by glutamic acid.

cTnT(4M): The same as cTnT(3M) but with residue T285 also replaced by glutamic acid.

Ir: Inhibitory region.

Md: Mobile domain.

$r̄$: The average FRET distance between donor and acceptor determined from lifetime decay analysis.

Sr: Switch region

2.2 Abstract
FRET was used to investigate the structural and kinetic effects that PKC phosphorylations exert on Ca$^{2+}$ and myosin subfragment-1 dependent conformational transitions of the cardiac thin filament. PKC phosphorylations of cTnT were mimicked by glutamate substitution. Ca$^{2+}$ and S1-induced distance changes between the central linker of cTnC and the switch region of cTnI (cTnI-Sr) were monitored in reconstituted thin filaments using steady state and time resolved FRET, while kinetics of structural transitions were determined using stopped flow. Thin filament Ca$^{2+}$ sensitivity was found to be significantly blunted by the presence of the cTnT(T204E) mutant, whereas pseudo-phosphorylation at additional sites increased the Ca$^{2+}$-sensitivity. The rate of Ca$^{2+}$-dissociation induced structural changes was decreased in the C-terminal end of cTnI-Sr in the presence of pseudo-phosphorylations while remaining unchanged at the N-terminal end of this region. Additionally, the distance between cTnI-Sr and cTnC was decreased significantly for the triple and quadruple phosphomimetic mutants cTnT(T195E/S199E/T204E) and cTnT(T195E/S199E/T204E/T285E), which correlated with the Ca$^{2+}$-sensitivity increase seen in these same mutants. We conclude that significant changes in thin filament Ca$^{2+}$-sensitivity, structure and kinetics are brought about through PKC phosphorylation of cTnT. These changes can either decrease or increase Ca$^{2+}$-sensitivity and likely play an important role in cardiac regulation.

2.3 Highlights

- cTnT phosphorylation by PKC was mimicked in *in vitro* thin filaments.
- I-C switching was monitored by FRET for these phospho-mimics.
- Phospho-mimics altered I-C switching Ca$^{2+}$-affinity, structure, and kinetics.
- cTnT(T204E) significantly reduced Ca$^{2+}$-affinity of I-C switching.
• cTnT(3M) and (4M) increased Ca\(^{2+}\)-affinity, reduced I-C separation and kinetics.

2.4 Introduction

In cardiac muscle, Ca\(^{2+}\)-activation of myofilaments is linked to the thin filament, a structure composed of the heterotrimeric troponin (cTn) complex and tropomyosin (Tm) bound to the double helical actin filament [1, 2]. cTn is formed from subunits cTnC, cTnI, and cTnT, wherein cTnC binds Ca\(^{2+}\), cTnI binds actin and inhibits actomyosin ATPase activity in relaxed muscle, and cTnT anchors the cTn complex on the actin filament [3]. Tm forms elongated dimeric coiled-coil \(\alpha\)-helices that wind around the actin filament and form two continuous strands on actin's surface through head-to-tail contacts occurring in a short overlap region. Each cTn is located at the overlap between adjacent Tm’s. These arrangements give rise to a stoichiometry of cTn•Tm•actin\(_7\); this is the structural regulatory unit, which not only serves as the basic repeating structure from which the thin filament is built, but also governs Ca\(^{2+}\)-induced generation of force.

In relaxed muscle and at a resting concentration of Ca\(^{2+}\), cTnI-Tm acts as a regulatory switch that prevents the attachment of cross-bridges to actin through steric blocking of myosin head (S1)-binding sites on actin [4-6]. To activate actomyosin ATPase, and thereby generate force, the intracellular [Ca\(^{2+}\)] rises to saturate the regulatory sites in cTn. This induces a series of intramolecular and intermolecular structural changes in the thin filament beginning with opening of the N-domain of cTnC (N-cTnC) and subsequent interaction between N-cTnC and the switch region of cTnI (cTnI-Sr) [7, 8]. This changes the conformation for both the inhibitory region (cTnI-Ir) and cTnI-Sr [9-11] switching cTnI-Ir from interacting with actin to interacting with cTnC [12]. Furthermore, the interaction between the mobile domain of cTnI (cTnI-Md) and actin becomes disrupted  [13, 14]. These structural changes in the core domain of cTn are propagated through cTnT to Tm leading to azimuthal movement of Tm on the actin surface [15] which
permits strong cross-bridge binding. These Ca\textsuperscript{2+}-induced protein structural transitions are thus the molecular basis of the cardiac thin filament regulation mechanism in which cTnT is crucial.

Because of its many interactions within the thin filament, cTnT has several important roles in sarcomeric regulation. It is necessary for full Ca\textsuperscript{2+}-mediated activation/deactivation of the thin filament [16, 17] and for regulating cross-bridge recruitment dynamics and turnover kinetics [18, 19]. cTnT is composed of two distinct regions, TnT1 (residues 1-181) and TnT2 (residues 181-288), which can be produced from cleavage through mild chymotryptic digestion [20, 21]. TnT1 contains a highly acidic hypervariable N-terminal region, which has no biochemically or structurally verified binding partner, followed by a conserved central region, which interacts with the head-to-tail junction of axially joined Tm dimers through an N-terminal 39 residue Tm binding region [22]. The TnT2 fragment contains a short Tm binding region at its N-terminus, which anchors cTn onto Tm [22] and has been shown to bind Tm in a Ca\textsuperscript{2+}-specific manner which is likely important for Ca\textsuperscript{2+}-activation [23]. The C-terminal region of cTnT2 interacts with the N-domain of cTnI and the C-domain of cTnC to form the globular core domain of the cTn complex [24]. The core domain of cTn alone is able to regulate the interaction between S1 and actin–Tm [25]. Furthermore, the T2 region of cTnT is thought to modulate the Ca\textsuperscript{2+}-sensitivity of thin filament activation since it directly interacts with cTnI and cTnC. In addition to acting as a Ca\textsuperscript{2+}-sensitivity tuner, TnT2 is believed to be essential in maintaining the proper blocked state conformation of the thin filament as well as in conducting structural transitions initiated by cTnI-Sr switching to the T1 region and thereby to Tm allowing full ATPase activity. Highlighting the importance of TnT2 region is the fact that several mutations in this region are known to be causal in familial hypertrophic cardiomyopathy [26, 27]. Moreover, it is within the TnT2 region that all of the established protein kinase C (PKC) phosphorylation sites lie, these sites occurring at Thr-
195, Ser-199, Thr-204 and Thr-285 in rat myocardium [28-30]. Phosphorylation at these sites modulates Ca\(^{2+}\)-activated thin filament regulation [31]. For example, PKC phosphorylation of cTnT has been reported to induce a 50% reduction in maximum actomyosin Mg-ATPase activity \textit{in vitro} [32] and a 30% reduction of maximum force of mouse cardiac fibers [33]. These effects reduce the heart’s ability to maintain normal function, suggesting that changes in the level of PKC phosphorylation of cTnT in the failing heart may contribute to its pathophysiology [30, 34, 35]. Hence, deeper insight into the mechanism by which PKC phosphorylation of TnT2 modulates thin filament regulation is urgently needed.

By incorporating recombinant Tn into skinned myocardium, Sumandea et al. demonstrated that among the four PKC phosphorylation sites of cTnT, Thr-204 is the functionally critical site for reduction in Ca\(^{2+}\)-sensitivity, cooperativity, Mg-ATPase activity and maximum force generation [30]. However, the detailed molecular mechanism underlying the effects of PKC phosphorylation of Thr-204, as well as each of the other three residues and their combinations, is still elusive. In order to investigate how PKC phosphorylation of cTnT structurally and kinetically affects the conformational transitions in cTn that transduce Ca\(^{2+}\)-signals into tropomyosin movement, we mimicked PKC phosphorylations via glutamate substitution mutants cTnT(S199E), cTnT(T204E), cTnT(S199E/T204E), cTnT(T195E/S199E/T204E) referred to as cTnT(3M), and cTnT(T195E/S199E/T204E/T285E) referred to as cTnT(4M). The distance of the cTnI-Sr to cTnC was monitored via steady-state and time-resolved Förster Resonance Energy Transfer (FRET) between either cTnl(S151C\textsubscript{AEDANS})-cTnC(S89C\textsubscript{DDPM}) or cTnl(S167C\textsubscript{AEDANS})-cTnC(S89C\textsubscript{DDPM}). We hypothesized that PKC phosphomimetic mutants of cTnT alter Ca\(^{2+}\)-dependent binding of the cTnI-Sr to the hydrophobic patch of N-cTnC and concomitant release of cTnI-Ir and cTnI-Md from actin (collectively termed I-C switching) by changing cTnT-cTnI
and/or cTnT-cTnC interactions. We reasoned that such structural changes should arise from either directly altered charge-charge interactions or propagated structural changes initiated at the TnT2-Tm binding region and conducted through the IT-arm of the cTn core domain [36]. By using FRET to measure the Ca\(^{2+}\)-sensitivity and deactivation kinetics of I-C switching and the Ca\(^{2+}\)- or Mg\(^{2+}\)-state cTnI-cTnC distances, we tested our hypothesis by characterizing how each phosphomimetic mutant impacts the cTnI-Sr–N-cTnC, cTnI-Ir–actin, and the cTnI-Md–actin interactions.

Our results showed that the T204E mutant significantly decreased the Ca\(^{2+}\)-sensitivity of I-C switching and slowed Ca\(^{2+}\)-dissociation-induced structural transition kinetics, but no change in the proximity between cTnC and cTnI was apparent for this mutation. This result seems to indicate that T204E is able to greatly desensitize I-C switching to Ca\(^{2+}\) without altering average I-C separation. Additional pseudo-phosphorylation at residues Thr-195, Ser-199, and Thr-285 not only decreased the desensitizing effect of T204E, but even led to hypersensitization with cTnT(3M) and cTnT(4M) in the absence of S1. This Ca\(^{2+}\)-sensitizing effect of cTnT(3M) and cTnT(4M) in the Mg\(^{2+}\) state was correlated with a significant decrease in Mg\(^{2+}\)-state cTnI-cTnC distances as well as a reduction in "switching distance" (total Ca\(^{2+}\)-induced change in distance between N-cTnC and cTnI-Sr). Finally, significant effects of cTnT pseudo-phosphorylations on the kinetics of Ca\(^{2+}\)-dissociation-induced changes in N-cTnC–C-cTnI distances were confined to the structural changes sensed by cTnC(89C\(_{DDPM}\))–cTnI(167C\(_{AEDANS}\)), suggesting a specific kinetic effect of cTnT on the C-terminal portion of the C-domain, particularly the mobile domain of cTnI (cTnI-Md), in response to thin filament deactivation, perhaps indicating a change in the orientation but not the distance between cTnC and cTnI along with a concomitant disruption of the fly casting mechanism [13, 14]. Taken together, these results indicate that T204E reduces the
Ca$^{2+}$-sensitivity of cTnI-cTnC switching through subtle structural changes not apparent from cTnI-cTnC distance measurements, whereas additional pseudo-phosphorylations compensated for this Ca$^{2+}$-sensitizing effect in the absence of S1 by promoting increased, Ca$^{2+}$-sensitizing interaction between cTnI-Sr and N-cTnC.

2.5 Materials and Methods

Protein sample preparation and characterization

To implement FRET in this study, a series of recombinant single cysteine mutants were generated from wild type rat protein clones using approaches similar to those previously reported [10, 13, 37, 38]. Briefly, using the GeneTailor$^\text{TM}$ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA), rat cDNA clones of wild-type cTnC, cTnI and cTnT sub-cloned into the plasmid pSBETa was used as a template DNA to generate single-cysteine cTnC and cTnI mutants and phosphomimetic mutants of cTnT. The mutants generated included: cTnI(S151C), cTnI(S167C), cTnC(S89C), cTnT(S199E), cTnT(T204E), cTnT(S199E/T204E), cTnT(T195E/S199E/T204E) and cTnT(T195E/S199E/T204E/T285E). Note that the cTnC mutant, endogenous cysteine residues Cys-35 and Cys-84 have each been substituted with serine; similarly, in the cTnI mutants, Cys-81 and Cys-98 have been substituted with serine and isoleucine, respectively. Recombinant cTnC, cTnI and cTnT clones were over expressed in E. coli strain BL21(DE3) cells and purified as previously described [7, 9, 12, 39]. All cTnI proteins were modified with 5-(((2-iodoacetyle)amino)ethyl)amino)naphthalene-1-sulfonic acid (AEDANS) as FRET donor according to previously described procedures [13]. cTnC(S89C) was purified and labeled with N-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) as FRET acceptor by following a previously described procedure to produce cTnC(S89C-DDPM) [13]. The labeling ratio was determined spectroscopically using $\varepsilon_{325}= 6000$ cm$^{-1}$M$^{-1}$ for AEDANS and
ε_{442}=2930 \text{ cm}^{-1}\text{M}^{-1} \text{ for DDPM. Labeling ratios for all protein modification were } >95\%. \text{ Cardiac Tm was purified from bovine heart [40]; actin [41] and myosin subfragment-1 (S1) from the chymotryptic digestion of myosin [42] were obtained from rabbit skeletal muscle. AEDANS-labeled cTnI mutants and DDPM labeled cTnC(S89C) were reconstituted into thin filament samples with other thin filament proteins using a cTn:Tm:actin molar ratio of 1:1:7.5 and checked by native gel electrophoresis as previously reported [12, 13, 43]. The regulatory function of the labeled cTnI and cTnC mutants, used before in our laboratory, was also verified by testing their ability to participate in the Ca^{2+}-dependent regulation of acto-S1 ATPase activity (data not shown). Multiple experiments were performed on prepared samples within 5 days, and no protein degradation was observed by electrophoretic analysis over this time period.}

\textbf{Steady-state fluorescence measurements}

Steady-state FRET measurements were performed at 15 ± 0.1 °C on an ISS PC1 photon-counting spectrofluorometer equipped with a micro-titrator using a band pass of 3 nm on both the excitation and emission monochromators. Samples containing 1 μM protein dissolved in working buffer, which consisted of 30 mM MOPS pH 7.0, 5 mM MgCl\textsubscript{2}, 0.15 M KCl, 1 mM DTT, and 1 mM EGTA, were tested in the presence and absence of 3 mM CaCl\textsubscript{2} both with and without ADP-Mg\textsuperscript{2+}-S1 present. Samples were excited with 343 nm light and emission spectra of donor AEDANS of either donor-only or donor-acceptor samples at different conditions were recorded from 420 nm to 600 nm using a scanning emission monochromator. FRET between AEDANS and DDPM occurring in reconstituted samples was assessed by comparing the AEDANS fluorescence from AEDANS-DDPM samples with that of matching control, AEDANS-only samples.
Steady-state fluorescence Ca$^{2+}$ titrations

To examine the Ca$^{2+}$ sensitivity of changes in cTnC–cTnI distances within reconstituted thin filament samples, steady-state FRET was measured during Ca$^{2+}$-titrations performed using the micro-titrator as described previously [13]. cTnC–cTnI distances were investigated by monitoring the changes in FRET efficiency between either (1) Cys-89$_{DDPM}$ of cTnC and Cys-151$_{AEDANS}$ of cTnI, or (2) Cys-89$_{DDPM}$ of cTnC and Cys-167$_{AEDANS}$ of CTnI. In a typical titration experiment, up to 100 data points were acquired after successively injecting aliquots of 3 µL of a 14 mM Ca$^{2+}$ solution to 1.2 mL samples in a titration buffer containing 50 mM MOPS, pH 7.10, 2 mM EGTA, 5 mM nitrilotriacetic acid (NTA), 150 mM KCl, 5 mM MgCl$_2$, and 1 mM DTT. Changes in FRET vs. Ca$^{2+}$ concentrations were acquired by monitoring donor fluorescence intensity of samples at 490 nm with excitation at 343 nm. Data from Ca$^{2+}$ titrations were fit using the Hill equation:

$$I = (I_{\text{max}} - I_{\text{min}}) \cdot \frac{[Ca^{2+}]^{n_H}}{[Ca^{2+}]^{n_H} + (10^{-pCa_{50}})} + I_{\text{min}}$$  \hspace{1cm} (1)

Where $I$ represents steady state AEDANS intensity that is correlated to FRET efficiency since donor only fluorescence intensity is insensitive to Ca$^{2+}$ concentrations, $I_{\text{max}}$ AEDANS intensity under Ca$^{2+}$ free conditions, $I_{\text{min}}$ AEDANS intensity under Ca$^{2+}$ saturated, $pCa_{50}$ the $-Log_{10}(\{Ca^{2+}\})$ at which apparent half occupancy of N-cTnC Ca$^{2+}$-binding sites occurs, and $n_H$ the Hill coefficient/slope that represents the steepness of the Ca$^{2+}$-dependent reduction in AEDANS fluorescence.
Time-resolved fluorescence measurements

To quantify the FRET observed in the steady-state measurements, fluorescence intensity decays of AEDANS of the reconstituted samples in the absence/presence of the acceptor under the same biochemical conditions as used for steady-state measurements were recorded with a FluoreCube (Horiba Jobin Yvon) lifetime system equipped with TBX pico-second photon detection modules. The decays of the donor-only samples were fitted with the multi-exponential function [44]:

\[ f(t) = \alpha_i e^{-t/\tau_i^{-1}} \]  

(2)

where the \( \alpha_i \) represents the fractional amplitude associated with each lifetime \( \tau_i \). In the presence of the acceptor, the intensity decays of donor–acceptor samples were fit to Eq. 3:

\[ I_{DA}(r,t) = \sum_{i=1}^{\infty} \alpha_{Di} \exp \left[ -\frac{t}{\tau_{Di}} - \frac{t}{\tau_{Di}} \left( \frac{R_o}{r} \right)^6 \right] \]  

(3)

Where \( r \) is a unique distance separating the donor and acceptor and \( R_o \) 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

\[ I_{DA}(t) = \int_0^\infty P(r)I_{DA}(r,t)dr \]  

(4)

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

\[ P(r) = \frac{1}{Z} \frac{1}{\sigma\sqrt{2\pi}} \exp \left[ -\frac{1}{2} \left( \frac{r-r_0}{\sigma} \right)^2 \right] dr \]  

(5)
where $\bar{r}$ is the mean distance and $\sigma$ is the standard deviation of the distribution. The half width at half maximum (hw) of the distribution is given by $hw = 1.1772\sigma$.

**FRET stopped-flow measurements**

FRET stopped-flow experiments were performed to examine the kinetics describing changes in the cTnC-cTnI proximity induced by Ca$^{2+}$ dissociation using a T format KinTek stopped-flow spectrometer with a 1.8 ms dead time. Ca$^{2+}$-saturated reconstituted thin filament samples were rapidly mixed with an equal volume of buffer containing an excess amount of the Ca$^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid (BAPTA) [45]. As in equilibrium FRET experiments, the time dependent change in AEDANS emission intensity was first determined for a donor only sample, followed by determination of the time dependent emission intensity for the corresponding donor-acceptor sample. A total of 8–20 kinetic traces were collected for each set of donor-only and donor-acceptor samples. The set of kinetic traces for each sample was averaged and then nonlinear regression was used to fit the averaged trace with an exponential function and determine the rate of conformation change.

**Statistical analysis**

$t$-tests were performed as a standard way to establish statistical significance between parameters of control (wild type cTnT) and mutations. Tabulated parameter values are given as averages over $n$ trials as indicated in each table, and the standard errors or confidence intervals obtained from fitting are shown next to each value. Unless otherwise mentioned, parameters such as kinetic rates and $pCa_{50}$ values were obtained through non-linear regression using the least squares method.
2.6 Results

2.6.1 Steady state fluorescence intensity measurements of the cTnC-cTnI interaction in cardiac thin filament

The objective of this study was to examine the structural and kinetic effects of the pseudo-phosphorylation of cTnT on thin filament regulation, specifically on the interaction between cTnC and cTnI and Ca\(^{2+}\)-induced cTnC–cTnI switching, within reconstituted thin filaments. The proximity between cTnC and cTnI was monitored by measuring FRET between AEDANS attached to residue 151 or residue 167 of cTnI as fluorescence donor and DDPM attached to residue 89 in the central linker of cTnC as an acceptor. Fig. 1 shows typical steady-state fluorescence intensity measurements of the cTnI(S167C)\(_{\text{AEDANS}}\)-cTnC(S89C)\(_{\text{DDPM}}\) pair under different biochemical conditions. Peak fluorescence of cTnI(S167C)\(_{\text{AEDANS}}\) was quenched when the acceptor, DDPM, was present in the reconstituted thin filament (comparing black curve to red curve), indicating significant FRET between the donor and acceptor. Upon Ca\(^{2+}\) binding to cTnC, the fluorescence was further quenched (blue trace), indicating increased FRET due to Ca\(^{2+}\)-induced opening of the hydrophobic pocket in the N-domain of cTnC and subsequent association of this pocket with the cTnI-Sr. This brings cTnI(S167CAEDANS) and cTnI(S151CAEDANS), which are located in the cTnI-Sr, closer to cTnC(S89CDDPM), thus increasing the FRET efficiency and decreasing the fluorescence intensity of AEDANS. In the absence of Ca\(^{2+}\), addition of S1-ADP significantly increased FRET efficiency, indicating a decrease in separation between cTnI and cTnC (comparing red and green traces). It is known that S1 strongly binds to actin cross-bridge in the presence of Mg\(^{2+}\)-ADP and that such strong-binding cross-bridges move Tm from the blocked position to the open position on actin’s surface, which pushes cTnI off of actin and allows it to partially interact with cTnC [12, 46]. These
structural changes are reflected by changes in fluorescence intensity (red and green curves) shown in Fig. 1. For S1-ADP containing samples, the thin filament became fully activated when saturating levels of Ca$^{2+}$ were present, which is reflected by maximum quenching of donor fluorescence.

![Image of fluorescence intensity](image)

**Figure 1.** Comparison of AEDANS fluorescence intensity as a function of emission wavelength for either donor-acceptor samples (DA) or donor only samples (D) under various physiological conditions. These representative traces of cTnI(S167C$_\text{AEDANS}$)-cTnC(S89C$_\text{DDPM}$) show substantial FRET quenching compared to donor only control in low Ca$^{2+}$ conditions, with increased quenching upon addition of S1-ADP or Ca$^{2+}$.

### 2.6.2 Steady state Ca$^{2+}$-titrations of the cTnC-cTnI interactions within cardiac thin filament

Through controlled addition of Ca$^{2+}$ to thin filament samples, the change in AEDANS fluorescence intensity was monitored via spectrofluorometer as a function of [Ca$^{2+}$]. Fitting these Ca$^{2+}$ titrations curves with the Hill equation (Eq. 1) provided the Ca$^{2+}$ sensitivity and cooperativity of the samples in the form of $pCa_{50}$ and $n_H$ respectively. This allowed for comparison of the Ca$^{2+}$-sensitivities and cooperativities of the various pseudo-phosphorylations of cTnT. Fig. 2 shows typical Ca$^{2+}$ titration curves displayed as FRET efficiency vs. $pCa$ (where
$pCa = -\log_{10}[Ca^{2+}]$. These parameters are summarized in Table 1. It was important to establish that wild type cTnT containing thin filaments provided pCa$_{50}$’s and Hill slopes in reasonable accordance with prior studies to validate the physiological relevance of our control samples and experimental conditions. Wild type cTnT containing samples yielded expected values of pCa$_{50}$ and Hill slope for the Ca$^{2+}$-induced cTnC-cTnI interaction both for cTnI(167) and cTnI(151) containing thin filaments (See Table 1). These same samples showed a significant increase in pCa$_{50}$ upon addition of S1-ADP, consistent with previous observations [13, 38].
Figure 2. Ca$^{2+}$ titration curves for cTnI(167C<sub>AEDANS</sub>)-cTnC(89C<sub>DDPM</sub>) or cTnI(151C<sub>AEDANS</sub>)-cTnC(89C<sub>DDPM</sub>) labeled thin filaments containing either wild type or mutant cTnT. The normalized FRET efficiency increases as AEDANS and DDPM are brought into closer proximity upon addition of gradually increasing levels of Ca$^{2+}$.

Pseudo-phosphorylations of cTnT showed diverse site-specific effects on the Ca$^{2+}$ sensitivity of the cTnC–cTnI interaction. For cTnT(S199E), little overall effect on the Ca$^{2+}$ sensitivity of I-C switching was seen compared to control, with the exception of the cTnI(S167C)<sub>AEDANS</sub>-cTnC(S89C)<sub>DDPM</sub> pair in the Mg$^{2+}$-state where desensitization was observed. In contrast,
cTnT(T204E) consistently showed the greatest degree of Ca\(^{2+}\) desensitization both with and without S1-ADP for the structural transitions sensed either by cTnI(S151C\textsubscript{AEDANS}) or cTnI(S167C\textsubscript{AEDANS}). These observations are consistent with a prior study by Sumandea et al. in demembranated cardiac muscle fibers which identified Thr-204 of cTnT as a critical site for phosphorylation-induced depression of Ca\(^{2+}\)-sensitivity and force [30]. Interestingly, in our study we observed that pseudo-phosphorylation of Ser-199 appeared to mitigate T204E-induced Ca\(^{2+}\)-desensitization when both phosphomimetic mutations were present in cTnT. In the absence of S1-ADP, pseudo-phosphorylation of Thr-195 and Thr-285 actually elevated pCa\(_{50}\) values above those of control. These Ca\(^{2+}\)-sensitizing effects were absent when strongly-bound S1-ADP was present, with Ca\(^{2+}\) sensitivity levels returning to those seen in control. This implied that pseudo-phosphorylation of Thr-195 and Thr-285 counteract the Ca\(^{2+}\)-desensitizing effect of Thr-204 by promoting increased Ca\(^{2+}\)-sensitizing interaction between cTnI-Sr and N-cTnC, as this is the same mechanism by which strong-binding cross-bridges exert an activating, positive feedback on thin filament regulation [47]. It was also observed in the study by Sumandea et al., which most closely resembles this study in the current literature, that pseudo-phosphorylations at Thr-195 and/or Thr-285 significantly reduced the desensitization caused by the T204E mutation alone. Our differing observation of a hypersensitization in the Mg\(^{2+}\) and Ca\(^{2+}\) states, an effect not observed by Sumandea et al., is explained by the fact that cross-bridge cycling, and therefore positive feedback, is was present during activation of their demembranated fibers. Thus our results with S1-ADP present would be expected to more closely resemble the findings of Sumandea et al. wherein T195E and T285E mitigated Ca\(^{2+}\)-desensitization by T204E, which is the case.
The cooperativity of Ca\textsuperscript{2+}-dependent changes in the cTnC–cTnI distances as given by \( n_H \), reflect decreased allostery for samples with S1-ADP present, as would be expected since S1-ADP is able to bind to actin in the absence of Ca\textsuperscript{2+} thus displacing cTnI from actin and moving Tm to the open position [48]. Therefore, pre-incubation with S1-ADP removes the longitudinal cooperativity usually brought about through Tm as this mechanism of cooperativity is already saturated at the start of the titration by the structural effects of S1-ADP binding [47]. Comparing the cooperativity of the PKC phosphomimetic cTnT mutants with control showed that for cTnT(T204E) containing thin filaments an increased cooperativity was seen, which was blunted both by the presence of S1-ADP as well as by pseudo-phosphorylations at additional sites. Since the presence of S1-ADP mitigated the increased cooperativity seen in cTnT(T204E) containing thin filaments, this suggests cTnT(T204E) impacts the Ca\textsuperscript{2+}-binding cooperativity through the steric conduction of the structural changes occurring in the core of cTn to Tm, which would no longer be a factor upon addition of S1-ADP as discussed earlier. Meanwhile, though S1-ADP exerted a Ca\textsuperscript{2+}-sensitizing effect on samples containing cTnT(T204E), the Ca\textsuperscript{2+}-sensitivity of cTnT(T204E) samples was nevertheless decreased compared to control in the presence of S1-ADP, implying that this Ca\textsuperscript{2+}-desensitization mechanism is not directly related to cTnI-Sr–N-cTnC interaction but is more likely related to the IT-arm, which alters the orientation of cTnI and cTnC relative to each other. For reasons we will discuss in coming sections, a more general stabilization of the blocked state conformation of cTn by cTnT(T204E) is unlikely to be involved in the mechanism underlying Ca\textsuperscript{2+}-desensitization. The sites responsible for Ca\textsuperscript{2+} sensitization (Thr-195 and Thr-285) did not significantly alter the cooperativity of the cTnC-cTnI interaction.
Table 1. Numerical results from fitting of the Hill equation (Eq. 1) to Ca\(^{2+}\) titration curves for each pseudo-phosphorylation mutant and control. Parameter standard error is given to the right of each parameter value.

<table>
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<tr>
<th>cTnI:cTnT Mutants (n = 5-10)</th>
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<th>Thin filament + S1-ADP</th>
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<tr>
<td></td>
<td>pCa(_{50})</td>
<td>n(_{H})</td>
</tr>
<tr>
<td>S151C(_{AEDANS}):wt</td>
<td>6.27 ± 0.01</td>
<td>0.786 ± 0.014</td>
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<tr>
<td>S199E</td>
<td>6.29 ± 0.02</td>
<td>0.789 ± 0.019</td>
</tr>
<tr>
<td>T204E</td>
<td>6.09 ± 0.02</td>
<td>0.853 ± 0.01</td>
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<tr>
<td>S199E/T204E</td>
<td>6.18 ± 0.02</td>
<td>0.731 ± 0.012</td>
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<td>0.831 ± 0.017</td>
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<td>T195E/S199E/T204E/T285E</td>
<td>6.47 ± 0.03</td>
<td>0.711 ± 0.019</td>
</tr>
<tr>
<td>S167C(_{AEDANS}):wt</td>
<td>6.11 ± 0.02</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>S199E</td>
<td>6.03 ± 0.01</td>
<td>0.881 ± 0.024</td>
</tr>
<tr>
<td>T204E</td>
<td>5.98 ± 0.02</td>
<td>1.14 ± 0.04</td>
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<td>S199E/T204E</td>
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<td>1.13 ± 0.04</td>
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<tr>
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<td>6.17 ± 0.01</td>
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<td>T195E/S199E/T204E/T285E</td>
<td>6.21 ± 0.01</td>
<td>0.924 ± 0.013</td>
</tr>
</tbody>
</table>

2.6.3 Distance distribution analysis for the cTnC-cTnI interaction in cardiac thin filament

We next examined the structural effects of PKC pseudo-phosphorylations of cTnT on cTnC–cTnI distances by fitting FRET distance distributions to time-resolved fluorescence intensity decays. This was done to better understand how structural changes specific to our phosphomimetic cTnT mutations pseudo- might correlate with the observed changes in Ca\(^{2+}\)-sensitivity. Fig. 3 shows a typical set of AEDANS fluorescence intensity decays observed in reconstituted thin filament samples in the absence or presence of acceptor under different biochemical conditions. The decays underwent FRET distance distribution analysis to derive \(\bar{r}\) and \(h_w\) values using GlobalCurve (Eq. 5) [12]. A representative distance distribution is shown in Fig. 4 for a thin filament sample containing cTnI(S151C\(_{AEDANS}\))-cTnC(S89C\(_{DDPM}\))-cTnT(wt). All other sets of time-resolved intensity decays acquired for the different PKC pseudo-
phosphorylations of cTnT were analyzed, and the recovered \( \bar{r} \) and \( hw \) parameter values are summarized in Tables 2 and 3.

**Figure 3.** Representative fluorescence decays for donor only and cTnI(S167C_AEDANS)-cTnC(S89C_DDPM) labeled thin filaments. Donor-acceptor decays are given for four physiological conditions; with \( \text{Ca}^{2+} \) or without \( \text{Ca}^{2+} \), and with or without S1-ADP. The donor only decay gives the longest lifetime followed by donor-acceptor (DA) without \( \text{Ca}^{2+} \). Addition of S1-ADP decreases the lifetime in the \( \text{Ca}^{2+} \) free state but not \( \text{Ca}^{2+} \) saturated conditions and addition of \( \text{Ca}^{2+} \) decreased the lifetime for all DA samples with or without S1-ADP. Donor only samples were unchanged for all conditions as was expected.
Figure 4. Representative distance distributions derived by GlobalCurve fitting of fluorescence intensity decay data. Traces are for cTnI(S151C<sub>AEDANS</sub>)-cTnC(S89C<sub>DDPM</sub>)-cTnT(WT) samples in low Ca<sup>2+</sup> without S1-ADP (solid blue trace), in low Ca<sup>2+</sup> with S1-ADP present (solid red trace) and both with and without S1-ADP present under saturating levels of Ca<sup>2+</sup> (solid red and blue traces respectively).

Thin filament samples containing cTnT(wt) showed the greatest Mg<sup>2+</sup>-state cTnI-cTnC separation both for cTnI(S151C<sub>AEDANS</sub>) and cTnI(S167C<sub>AEDANS</sub>). Upon addition of saturating levels of Ca<sup>2+</sup> the mean cTnI(S151C<sub>AEDANS</sub>)–cTnC(S89C<sub>DDPM</sub>) and cTnI(S167C<sub>AEDANS</sub>)–cTnC(S89C<sub>DDPM</sub>) distances decreased by 6.0 and 8.3 Å respectively. As noted above, we call this Ca<sup>2+</sup>-induced change in cTnI-cTnC separation the switching distance, and will denote it as \( r_{sw} \) henceforth (values of \( r_{sw} \) are displayed graphically in Fig. 5). Accompanying the Ca<sup>2+</sup>-induced reduction in cTnI-cTnC separation was a decrease in \( h_w \). Because \( h_w \) is inversely related to the extent of the range of inter-probe distances experienced as cTnC and cTnI undergo conformational sampling, such that a larger \( h_w \) corresponds to greater inter-probe mobility.
reflective of underlying protein structural flexibility, the smaller $hw$ values observed in the Ca$^{2+}$-state suggest that N-cTnC and the cTnI-Ir/cTnI-Sr intraprotein interface adopt a more rigid conformation relative to each other upon Ca$^{2+}$-activation, which is consistent with previous protein dynamics studies of the C-domain of cTnI [13, 49]. Addition of strongly-bound S1-ADP to cTnT(wt) containing thin filaments reduced $r_{sw}$ as well as the Mg$^{2+}$-state cTnI-cTnC separation and both the Mg$^{2+}$- and Ca$^{2+}$-state $hw$ values, again indicating that S1-ADP disrupted cTnI-actin interactions resulting in partial association of cTnI with cTnC and subsequent reduction in inter-probe flexibility.

**Figure 5.** Column graph showing switching distances for cTnT mutants with or without S1-ADP, shown in red and black respectively. The graph at left is labeled at cTnI(151) with AEDANS while the graph at right is labeled at cTnI(167), acceptor labeling at cTnC(89C) for both. This graph shows Ca$^{2+}$ induced proximity changes between cTnI(151) and cTnC(89) or cTnI(167) and cTnC(89). As can be seen switching distance is greater for cTnI(167) than cTnI(151) and samples with S1-ADP all show decreased switching distances compared to the same samples without S1-ADP. The impact of cTnT pseudo-phosphorylation is seen as a decrease in switching distances, which is most clearly pronounced in cTnT(3M) and cTnT(4M). Interestingly cTnT pseudo-phosphorylation's impact is lost upon addition of S1-ADP for the cTnI(151) labeled samples.
Results in Tables 2 and 3 show that cTnI-cTnC distances decrease in thin filaments containing PKC phosphomimetic cTnT mutants compared with wild type cTnT under some conditions. The degree of distance reduction varied with pseudo-phosphorylation site and their combinations, and generally became statistically significant at the 95% confidence level only with samples containing cTnI(S167C\textsubscript{AEDANS}) and either cTnT(3M) or cTnT(4M). The reduction in distance was more significant in the Mg\textsuperscript{2+} state than in the Ca\textsuperscript{2+} state, suggesting that cTnT(3M) and cTnT(4M) destabilize especially the cTnI-Md–actin interaction associated with the blocked state of thin filament regulation, which allows for increased interaction between cTnI-Sr and N-cTnC. A decreased $h_w$ was also observed for these samples; this concurrent observation of reduced I-C separation and decreased apparent inter-probe flexibility likely derives from an increased sub-population of thin filament regulatory units in which cTnI-Sr is interacting with N-cTnC. With a larger sub-population of cTn molecules wherein cTnI-Sr is already interacting with cTnC at a given moment, the total apparent shift in ensemble-averaged cTnI-cTnC distance upon Ca\textsuperscript{2+} saturation will be decreased, explaining why samples containing cTnT(3M) and cTnT(4M) also exhibited the smallest values of $r_{sw}$. Relating these structural results with the results from the Ca\textsuperscript{2+}-titrations, we see that cTnT(3M) and cTnT(4M) have the greatest Ca\textsuperscript{2+}-sensitivity as well as the smallest Mg\textsuperscript{2+}-state I-C separation. This result is not surprising as we see the same relationship with the Mg\textsuperscript{2+}-S1-ADP state; a partial association of cTnI with cTnC and a subsequent increase in Ca\textsuperscript{2+}-affinity. These results differed from the case of the Ca\textsuperscript{2+}-desensitizing cTnT(T204E), wherein no statistically significant change in Mg\textsuperscript{2+}-state cTnI-cTnC separation was observed. This implies that the mechanism of action for the T204E mutation is completely different than that of T195E and T285E in not significantly impacting the distance between cTnI-Sr and N-cTnC; a plausible T204E structural change that might go undetected by
our FRET scheme is a change in C-cTnI–N-cTnC orientation that affects the kinetics associated with I-C switching without disrupting the average position of a functional regions per se, thereby reducing Ca\(^{2+}\)-sensitivity. Observation of such a kinetic effect was previously made in the case of the effects of PKA-mediated phosphorylation of cTnI on N-cTnC opening [37]. When S1-ADP was present, all measured distances between donor and acceptor in the Mg\(^{2+}\) state were decreased because of destabilization of the cTnI-Ir and cTnI-Md interactions with actin caused by the Ca\(^{2+}\)-independent movement of Tm by strongly-bound S1, with cTnT(3M) and cTnT(4M) still showing significantly smaller Mg\(^{2+}\) state cTnI-cTnC distances compared to control. However, the measured distances in the Ca\(^{2+}\) state were not significantly affected by the presence of S1-ADP binding. It is noticed that no significant cTnT PKC pseudo-phosphorylation induced change in \(r_{sw}\) was sensed by cTnI(151C\_AEDANS) samples, while decreased \(r_{sw}\) values were observed for samples containing cTnI(167C\_AEDANS) with cTnT(3M) and cTnT(4M) inducing the most significant reduction (Fig. 5). These results suggest that pseudo-phosphorylation of cTnT has a different structural effect on the cTnI-Ir/cTnI-Sr compared to the cTnI-Sr/cTnI-Md, which becomes apparent in the presence of strongly-bound S1.
Table 2. Ca\(^{2+}\) and S1-dependent changes in distance between cTnI(S151C\textsubscript{AEDANS}) and cTnC(S89C\textsubscript{DDPM}) in reconstituted cardiac thin filaments. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>cTnI:cTnT Mutants (n=4-6)</th>
<th>Thin filament + Mg(^{2+})</th>
<th>Thin filament + S1-ADP + Mg(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\bar{r})</td>
<td>(h_w)</td>
</tr>
<tr>
<td>S151C\textsubscript{AEDANS}:WT</td>
<td>41.2 ± 2.0</td>
<td>15.0 ± 2.2</td>
</tr>
<tr>
<td>S199E</td>
<td>40.7 ± 2.0</td>
<td>14.7 ± 2.2</td>
</tr>
<tr>
<td>T204E</td>
<td>40.3 ± 2.4</td>
<td>14.2 ± 2.4</td>
</tr>
<tr>
<td>S199E/T204E</td>
<td>39.9 ± 2.6</td>
<td>13.7 ± 2.9</td>
</tr>
<tr>
<td>T195E/S199E/T204E</td>
<td>38.8 ± 2.1*</td>
<td>12.7 ± 2.5*</td>
</tr>
<tr>
<td>T195E/S199E/T204E/T285E</td>
<td>38.2 ± 2.8*</td>
<td>12.0 ± 3.1*</td>
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</table>

<table>
<thead>
<tr>
<th>cTnI:cTnT Mutants (n=4-6)</th>
<th>Thin filament + Ca(^{2+})</th>
<th>Thin filament + S1-ADP + Ca(^{2+})</th>
</tr>
</thead>
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<tr>
<td></td>
<td>(\bar{r})</td>
<td>(h_w)</td>
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<tr>
<td>S151C\textsubscript{AEDANS}:WT</td>
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<td>10.3 ± 3.4</td>
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<td>9.67 ± 3.1</td>
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<tr>
<td>T195E/S199E/T204E</td>
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</tr>
<tr>
<td>T195E/S199E/T204E/T285E</td>
<td>34.1 ± 2.1*</td>
<td>7.70 ± 2.3</td>
</tr>
</tbody>
</table>
Table 3. Ca\(^{2+}\) and S1-dependent changes in distance between cTnI(S167C\textsubscript{AEDANS}) and cTnC(S89C\textsubscript{DDPM}) in reconstituted cardiac thin filaments. *Indicates a significant difference from wild type at the 90% confidence level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>cTnI:cTnT Mutants (n=3-6)</th>
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<th>Thin filament + S1-ADP + Mg(^{2+})</th>
<th>Thin filament + Ca(^{2+})</th>
<th>Thin filament + S1-ADP + Ca(^{2+})</th>
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<tr>
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<td>hw</td>
<td>̄r</td>
<td>hw</td>
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<tr>
<td>S167C\textsubscript{AEDANS}:WT</td>
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<td>S199E</td>
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<td>42.3 ± 0.8</td>
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<td>15.6 ± 1.5</td>
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<td>13.5 ± 2.9</td>
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<td>40.5 ± 1.1**</td>
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<td>11.6 ± 3.1*</td>
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<td>40.5 ± 1.6**</td>
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<td>39.0 ± 2.7**</td>
<td>11.2 ± 3.0*</td>
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<td>̄r</td>
<td>hw</td>
</tr>
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<td>7.71 ± 1.2</td>
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<td>7.15 ± 1.3**</td>
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<td>7.2 ± 1.7*</td>
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2.6.4 Kinetics of Ca\(^{2+}\) dissociation-induced structural changes of cTnC-cTnI interaction in cardiac thin filament

The kinetic effects of PKC phosphomimetic mutations of cTnT on thin filament regulation were characterized through FRET stopped-flow experiments. Specifically, we measured the kinetics of Ca\(^{2+}\)-dissociation-induced structural transitions involving changes in cTnI-cTnC distances by rapidly removing Ca\(^{2+}\) from samples using BAPTA, a Ca\(^{2+}\)-chelator, in a stopped flow device. Fig. 6 shows typical FRET transients generated in this manner. Fitting these transients to an exponential function enabled us to recover kinetic rates associated with the structural transitions which are given in Table 4. When samples were reconstituted with cTnT(wt), recovered kinetic rate constants were 34.1 s\(^{-1}\) for structural transition involving cTnI(S151C\textsubscript{AEDANS}) and a faster
46.0 s$^{-1}$ for the structural transition involving cTnI(S167CAEDANS), and both rate constants were reduced in the presence of strongly-bound S1. These results are consistent with our previous observations that cTnI-Md and cTnI-Sr show faster kinetics than cTnI-Ir, and that deactivation kinetics become universally slower in the presence of strongly bound S1 [13].

**Figure 6.** Representative BAPTA chelation traces for cTnT mutants S199E or 4M and control. The graph on the left shows samples using cTnI(S151CAEDANS)-cTnC(S89CDDPM) FRET pairs, while the graph on the right shows samples using cTnI(S167CAEDANS)-cTnC(S89CDDPM) FRET pairs. Deactivation kinetics at cTnI(151) are not significantly impacted by pseudo-phosphorylation mutants while at cTnI(167) deactivation kinetics are decreased.

Kinetic rate constants of Ca$^{2+}$-dissociation-induced structural transitions involving cTnI(S151CAEDANS) showed negligible change between control and samples containing PKC phosphomimetic cTnT mutants, while rate constants involving cTnI(S167CAEDANS) were decreased significantly for all pseudo-phosphorylations. This trend of reduced deactivation kinetics at cTnI(S167CAEDANS) was diminished in the presence of S1-ADP. Similar to the Ca$^{2+}$-titration and FRET structural measurements, these results suggest that PKC phosphorylations of cTnT may modulate thin filament regulation via changes to the Mg$^{2+}$-state structure of the cTn-Tm-actin complex which manifest themselves as an altered orientation between cTnI and cTnC.
that particularly affects the C-terminal portion of cTnI-Sr and, by implication, cTnI-Md. These structural changes in turn alter the Ca$^{2+}$-sensitivity and deactivation kinetics of I-C switching without appreciably changing the cTnC-cTnI distance; as mentioned above, similar effects were previously observed to be exerted by PKA phosphorylation of cTnI on N-cTnC opening [37]. Furthermore, because it was observed that cTnT(T204E) induces a marked decrease in the Ca$^{2+}$-sensitivity of I-C switching along with reduced Ca$^{2+}$-dissociation induced kinetics, we can reason that Ca$^{2+}$-association-induced structural transition kinetics are also slowed by cTnT(T204E). Our kinetics data were thus also consistent with the interpretation that cTnT(T204E) through a unique propagated structural change modifies the orientation of cTnI and cTnC relative to one another such that structural transition kinetics are generally slowed without affecting the relative distance between cTnC and cTnI. This alteration in I-C switching likely propagates from cTnT(204) through the IT-arm as this is the most direct structural contact between cTnT(204) and cTnI-cTnC [36].

Similar to the case of cTnT(T204E), it is likely that cTnT(S199E) also imposed a change in cTnC-cTnI orientation that caused the slowed kinetics it exerted. However, an apparent decrease, significant at the 90% confidence level, in the cTnI(S167C$_{AEDANS}$)-cTnC(S89C$_{DDPM}$) distance (Table 3) may help explain why this orientational change does not lead to Ca$^{2+}$-sensitization as in the case of cTnT(T204E). In the case of cTnT(S199E/T204E), cTnT(3M) and cTnT(4M), which contain the T204E mutation along with additional pseudo-phosphorylations, promotion of the Ca$^{2+}$-sensitizing cTnI-Sr–N-cTnC interaction helps to compensate for the Ca$^{2+}$-desensitizing hindrance on the I-C switching structural transition induced by T204E. This is especially noticeable in the 3M and 4M mutations, which showed significantly reduced I-C distances in the Mg$^{2+}$-state and resensitized or hyper sensitized Ca$^{2+}$-affinities accordingly. Interestingly, it can
also be seen from the kinetics data that the pseudo-phosphorylation induced effects more strongly impacted the region of cTnI(S167C<sub>AEDANS</sub>) than cTnI(S151C<sub>AEDANS</sub>), which again implies that as far as cTnI is concerned, the cTnI-Md–actin interaction is most impacted by cTnT phosphorylation. Although we cannot ascertain the specific reason for this from the present FRET experiments, a likely explanation is that because cTnI-Md is the furthest away from the IT-arm, where we believe cTnI-cTnC orientational changes are arising, cTnI-Md is the most affected by the structural changes induced by PKC phosphomimetic mutations.

Table 4. Deactivation rate parameters obtained from exponential fitting of FRET kinetic transients ± 90% confidence interval. Rate constants for thin filament samples with S1-ADP are given in the right column. The top half of the table shows results obtained from cTnI(151C<sub>AEDANS</sub>)-cTnC(89C<sub>DDPM</sub>) FRET pairs, while the bottom half show results obtained from cTnI(167C<sub>AEDANS</sub>)-cTnC(89C<sub>DDPM</sub>) pairs. *Indicates a significant difference from wild type at the 90% confidence level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>cTnI:cTnT mutants (n = 20-60)</th>
<th>Kinetic rate (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Thin filament</td>
</tr>
<tr>
<td>S151&lt;sub&gt;C_AEDANS&lt;/sub&gt;;wt</td>
<td>34.1 ± 1.9</td>
</tr>
<tr>
<td>S199E</td>
<td>32.5 ± 2.0</td>
</tr>
<tr>
<td>T204E</td>
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</tr>
<tr>
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<td>35.1 ± 2.2</td>
</tr>
<tr>
<td>T195E/S199E/T204E</td>
<td>32.8 ± 2.9</td>
</tr>
<tr>
<td>T195E/S199E/T204E/T285E</td>
<td>32.2 ± 2.2</td>
</tr>
<tr>
<td>S167&lt;sub&gt;C_AEDANS&lt;/sub&gt;;wt</td>
<td>46.3 ± 3.3</td>
</tr>
<tr>
<td>S199E</td>
<td>37.9 ± 2.7**</td>
</tr>
<tr>
<td>T204E</td>
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<tr>
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<td>38.8 ± 4.1**</td>
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<tr>
<td>T195E/S199E/T204E/T285E</td>
<td>35.1 ± 2.6**</td>
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2.7 Discussion

In a seminal chemically-skinned cardiac muscle fiber study on the functional effects of PKC phosphorylation on thin filament regulation, Sumandea, et al. identified Thr-204 (rat sequence...
as a functionally critical PKC phosphorylation site of cTnT by showing significant reductions in maximum tension, actomyosin Mg-ATPase activity, myofilament Ca\(^{2+}\)-sensitivity, and cooperativity caused by pseudo-phosphorylation at this residue [30], which are the effects expected from studies of the role of PKC phosphorylation in cardiac physiology [50]. The same study also suggested that pseudo-phosphorylation at cTnT Thr-195 and Thr-285 partially reverse the observed Ca\(^{2+}\)-blunting effects caused by cTnT(T204E). In the present biophysical study, the primary findings were as follows. (1) Similar to Sumandea et al.’s findings [30], cTnT(T204E) was the critical phosphorylation site to desensitize the cTnC-cTnI interaction to Ca\(^{2+}\). This desensitization was either blunted or replaced by sensitization in the presence of other phosphorylations. (2) Multiple PKC site pseudo-phosphorylation of cTnT structurally affected the cTnI-Md-actin and cTnI-Sr-N-cTnC interactions primarily in the Mg\(^{2+}\) state, this being most prominent in thin filaments containing cTnT(3M) or cTnT(4M) wherein the cTnI-cTnC separation was most significantly reduced. Promotion of increased cTnI-Sr-N-cTnC interaction in the Mg\(^{2+}\)-state is most likely the underlying cause of the increased Ca\(^{2+}\)-sensitivities observed for cTnT(3M) and cTnT(4M) samples. (3) The kinetics of deactivation for thin filaments with pseudo-phosphorylations were significantly slowed compared to control at cTnI(167) but not at cTnI(151), which implied a differential impact of pseudo-phosphorylation at these two positions that is most likely due to changes in the orientation between C-cTnI and N-cTnC and destabilization of the cTnI-Md-actin interaction. Taken together these findings paint a picture of cTnT in which the Ca\(^{2+}\)-sensitivity and kinetics of the I-C switching can be significantly modulated depending on which sites on cTnT are phosphorylated by PKC. At the reconstituted thin filament level, cTnT(T204E) is able to significantly reduce the Ca\(^{2+}\)-sensitivity of I-C switching, whereas cTnT(T195E) and cTnT(T285E) are able to mitigate Ca\(^{2+}\)-desensitization by
T204E, and can even hypersensitize the thin filament to Ca\(^{2+}\) in the absence of strongly bound S1. The following paragraphs discuss the likely molecular mechanisms underlying these primary findings by correlating Ca\(^{2+}\)-titration, structural, and kinetics results for each mutation.

Sumandea’s Ca\(^{2+}\)-desensitization mechanism for T204E suggests that a charge change at Thr-204 extends an \(\alpha\)-helix which normally terminates at this residue, changing the position of the "fulcrum" through which structural changes associated with I-C switching propagate to Tm near the N-terminus of cTnT. This mechanism should not alter average I-C proximity, consistent with our structural results that I-C separation in the Mg\(^{2+}\)- or Ca\(^{2+}\)-state in the presence or absence of S1 has only a slight but statistically insignificant reduction compared to control. An important distinction between Sumandea’s study and ours is that we directly measure the distance between cTnI and cTnC that is related to the interaction between cTnI-Sr and the hydrophobic pocket of N-cTnC, and this interaction lies upstream of the force generation and Mg-ATPase measurements in Sumandea’s study. Thus T204E may interfere with conduction of structural events initiated by I-C switching, as pointed out by Sumandea, as well as the I-C interaction itself as implied by our observation of decreased Ca\(^{2+}\)-sensitivity and Ca\(^{2+}\)-dissociation-induced kinetics of the I-C switching structural transition. The direct effect of T204E on the functionally crucial I-C switching event appears to arise from a subtle, propagated structural change which reorients cTnI and cTnC relative to one another without significantly altering average I-C separation, and/or alters cTn dynamics. Both of these mechanisms are consistent with Sumandea's notion of an \(\alpha\)-helix extension which could alter I-C orientation (likely through the IT-arm) and cTn dynamics by changing cTnT’s flexibility. Kinetics data suggests T204E slows deactivation at the switch region/mobile domain of cTnI, a likely result of a change in I-C orientation since such a change may be expected to significantly alter the usual approach of the
mobile domain of cTnI toward its binding site on actin when transitioning tropomyosin from the closed to the blocked state and thus hinder the fly casting mechanism [13]. However, we note that FRET as used in this study is able to present this argument for a change in I-C orientation resulting from an α-helix extension in cTnT only by implication; though it is beyond the scope of this study, additional structural information from a technique that can monitor orientational changes between cTnI and cTnC is needed to verify that this mechanism truly occurs.

If indeed charge modification at cTnT(204) extends an α-helix in this region, then it would seem reasonable that additional charge modifications at Serine-195 and 199 could interfere with the stability of this α-helix, even though no such interference was found by computational modeling of α-helix stability [30]. A disruption of this α-helix extension by pseudo-phosphorylation at cTnT 195 or 199 could be partially responsible for the reduced Ca$^{2+}$-desensitization seen in cTnT(S199E/T204E) or the Ca$^{2+}$-sensitization seen in cTnT(3M) and cTnT(4M). It has also been shown that chicken fast skeletal TnT(158-191), which corresponds to rat cTnT(188-227) has actin binding properties and plays a role in thin filament activation [20], and that in the region containing cTnT T195E, S199E and T204E there lies a Tm binding site [22]. Thus charge changes in this region could have important effects on the activation state of cTn through changes to cTnT-Tm or cTnT-actin interactions. If the actin and Tm binding properties of this region are important for the movement of Tm from the blocked to the closed state [50], or vice versa, then changes in cTnT charge in this region could significantly hinder structural transitions in such a way that Ca$^{2+}$-sensitivity could be decreased or increased while Ca$^{2+}$-dissociation-induced structural transition kinetics are slowed, presumably because Ca$^{2+}$-association-induced structural transition kinetics are also modified.
Finally, the importance of phosphorylation at cTnT(285) should not be understated. This phosphorylation site is cardiac specific and occurs in a region of cTnT which was shown to be important in deactivation by experiments where the C-terminal 28 residues of cTnT were removed resulting in increased Ca$^{2+}$-sensitivity, which was attributed to a disruption blocked state of thin filament regulation [51]. Our results are consistent with a disruption of the cTnI-Md–actin interaction that is associated with the blocked state, as the mutants containing the cTnT(T285E) mutation showed the greatest Ca$^{2+}$-sensitivity, the smallest I-C separation in the Mg$^{2+}$-state as well as significantly reduced deactivation kinetics at cTnI(167). The individual impact of PKC pseudo-phosphorylation at this site is difficult to determine since this study only examined this site in conjunction with pseudo-phosphorylation at the other three sites. The proximity of this site to both cTnI and the C-domain of cTnC suggest direct influence on I-C interaction likely underlies the observed blocked state destabilization, as opposed to cTnT(T195E) and cTnT(S199E) which likely work through local disruption of the cTnT-Tm or cTnT-actin interactions. Thus taken together, the above findings highlight the functional importance of the C-terminus of cTnT in modulating Ca$^{2+}$-sensitivity, maintaining the stability of the blocked state of thin filament regulation, and facilitating proper transitions between the blocked and closed states.

The question should arise as to what these *in vitro* findings may imply for cardiac function. Previous studies have shown that, in general, PKC phosphorylation of cTnT reduces maximal force and Mg-ATPase activity [50], as well as the affinity of cTnT for Tm binding affinity [28, 52]. This decreased cTnT-Tm binding would likely uncouple the I-C switching event from force generation as well as reduce cooperativity, which reconciles our observation that cTnT(3M) and cTnT(4M) both appear to sensitize the I-C switching structural transition to Ca$^{2+}$ in spite of the
fact that previous studies which have shown that these mutations decrease force and Mg-ATPase activity. If the underlying mechanism for Ca\(^{2+}\)-sensitization seen in cTnT(3M) and cTnT(4M) (and to a lesser extent cTnT(S199E/T204E)) is a reduced cTnT-Tm interaction, which we believe to exert predominantly kinetic effects, coupled with a destabilized cTnI-Md–actin interaction that allows for increased Ca\(^{2+}\)-sensitizing cTnI-Sr–N-cTnC interaction, then it is likely the Ca\(^{2+}\)-sensitizations seen in our study would be of secondary importance to the uncoupling of I-C switching from Tm movement due to the fact that positive feedback from strong-binding cross-bridges also involves increased cTnI-Sr–N-cTnC interaction [47]. Thus our study is consistent with the effects seen in demembranated of a variable level of Ca\(^{2+}\)-desensitization sarcomeric force generation and decreased maximum tension, the latter of which is most likely due to the uncoupling of I-C switching from movement of Tm from the blocked to closed position that exposure of myosin binding sites on actin is reduced. One expectation based on the findings of the present study would be a decrease in diastolic function in hearts expressing cTnT(3M) or cTnT(4M). Because of the likely disruption of the cTnI-Md–actin interaction induced by these mutations, it is possible that they may result in a higher basal force and a reduced ability of the thin filament to inhibit Mg-ATPase in vivo at low levels of Ca\(^{2+}\); however, we note that this effect has not yet been reported [30, 50]. Regardless, our results contribute to the case that cTnT represents another powerful avenue by which in vivo thin filament regulation can be modulated.

2.8 Acknowledgements

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2.9 References


Chapter 3: Fluorescence Based Characterization of Calcium Sensitizer Action on the Troponin Complex

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3.1 Abstract

A fluorescence based approach was used to monitor the opening and closing of cTnC's hydrophobic pocket in the presence and absence of four common Ca\textsuperscript{2+}-sensitizers, EMD 57033, levosimendan, bepridil and pimobendan. Ca\textsuperscript{2+}-titration experiments were employed to determine the effect on Ca\textsuperscript{2+}-sensitivity and cooperativity of cTnC opening, while stopped flow experiments were used to investigate the impact on cTnC relaxation kinetics. By examining the behavior of each Ca\textsuperscript{2+}-sensitizer on cTnC at different levels of reconstitution (cTnI-cTnC, full troponin, or full troponin in thin filament) the importance of these proteins on sensitizer efficacy was evaluated, lending insight into the mechanism of action behind each drug. This study shows EMD 57033 is unable to sensitize cTnC to Ca\textsuperscript{2+}, and likely requires the presence of myosin to illicit a response. Levosimendan, bepridil, and pimobendan were all able to increase the sensitivity of cTnC for Ca\textsuperscript{2+} to varying degrees, with levosimendan and pimobendan reducing the rate of cTnC closing while bepridil increased this rate. Additionally, the same experiments were run on thin filament samples containing cTnT(T204E), a known Ca\textsuperscript{2+}-blunting phosphorylation
mimic. Levosimendan, bepridil, and pimobendan were found to elevate the Ca^{2+}-sensitivity of cTnT(T204E) containing thin filaments to within range of the wild type thin filaments.

3.2 Introduction

Within the heart Ca^{2+}-induced contraction is regulated by the thin filament (TF) which consists of actin (Act), tropomyosin (Tm) and troponin. Cardiac troponin (cTn) in turn consists of cardiac troponin I the actomyosin inhibitory unit (cTnI), C the Ca^{2+}-binding unit (cTnC), and T the tropomyosin binding unit (cTnT). Ca^{2+}-binding to cTnC shifts its structural equilibrium towards one in which its hydrophobic cleft is exposed allowing cTnI to bind cTnC thus shifting cTnI away from actin’s surface allowing myosin to bind actin and generate force (for thin filament reviews see [1-5]). Drugs which directly target and enhance this conversion of the Ca^{2+}-signal into force show potential in the fight against heart failure. These agents are collectively known as Ca^{2+}-sensitizers since common to all is an increase in the Ca^{2+}-sensitivity of force generation (for reviews on Ca^{2+}-sensitizers see [6-10]). Although centered around the TF, the mechanisms of action for Ca^{2+}-sensitizers appear to be quite varied [7]. Attempts to understand each Ca^{2+}-sensitizer's mechanism of action has proceeded via two main approaches, structural and tissue. Structural studies use techniques such as NMR, MS or x-ray crystallography with the goal of identifying the region of drug-protein interaction and any significant changes in protein structure induced by drug binding [10-20], while tissue studies focus on the Ca^{2+}-force relationship, kinetics of contraction, and the energetics associated with contraction [21-27]. Structural studies give detailed information on drug-protein interaction and structure with resolution down to the level of individual amino acids, however they tend to lack functional data on Ca^{2+}-sensitivity, cooperativity, kinetics, and energy consumption. On the other hand, tissue studies yield
functional data on $Ca^{2+}$-sensitivity, relaxation rate etc. without clearly linking it with its most basic underlying causes, the molecular level changes induced by drug binding.

A model in which to test $Ca^{2+}$-sensitizers which bridges the gap between the molecular and tissue level would do much to relate the underlying protein-drug interactions with their tissue and organ level effects. In the present study we demonstrate a fluorescence based approach to characterize the behavior of $Ca^{2+}$-sensitizers, using reconstituted TF or simpler units of cTn as model systems. This testing method possesses the essential features important in evaluating the effects of a $Ca^{2+}$-sensitizer on contraction, namely $Ca^{2+}$-sensitivity and relaxation kinetics, while utilizing only the most basic proteins involved in $Ca^{2+}$-induced regulation of force. We demonstrate the efficacy of this new method by testing four common $Ca^{2+}$-sensitizers (EMD 57033, levosimendan, bepridil, and pimobendan) for their impact on $Ca^{2+}$-sensitivity and relaxation kinetics for cTnI-cTnC (the basic functional unit of $Ca^{2+}$-regulation in the heart), whole cTn, and full TF. We also look at the effect of these four $Ca^{2+}$-sensitizers on TF samples containing cTnT(T204E), which has been shown to significantly reduce cooperativity and $Ca^{2+}$-sensitivity in thin filaments and cardiac fiber studies [28, 29]. This study provides an example of an effective intermediate model for drug screening amenable to high throughput methods while also adding to our collective understanding of the effects of $Ca^{2+}$-sensitizers on $Ca^{2+}$-mediated TF regulation.

3.3 Material and Methods

Mutant generation, protein expression, and labeling

Creation of a self-quenching labeled protein in this study was achieved by generating a recombinant double cysteine mutant of cTnC, cTnC(T13C/N51C), which was subsequently
double labeled with tetramethylrhodamine-5-maleimide (TAMRA-5-maleimide or simply TAMRA, Setareh Biotech). The cTnC(T13C/N51C), wild type cTnI and cTnT (cTnI(WT) and cTnT(WT)) as well as cTnT(T204E) were generated from wild type rat protein clones using approaches similar to those previously reported [30-33]. Briefly, using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA), rat cDNA clones of wild-type cTnI, cTnC, and cTnT sub-cloned into the plasmid pSBEta were used as template DNA to generate cTnI(WT), cTnC(T13C/N51C), cTnT(WT) and cTnT(T204E). Note that the cTnC mutant's endogenous cysteine residues Cys-35 and Cys-84 have each been substituted with serine. Recombinant cTnC, cTnI and cTnT clones were over expressed in E. coli strain BL21(DE3) cells and purified as previously described [34-37].

Labeling of cTnC(T13C/N51C) with TAMRA-5-maleimide was accomplished by eliminating the presence of reducing agent, in this case DTT, to facilitate exposure of the sulfhydryl groups in the cysteine residues. Subsequent incubation with 4-5 molar excess TAMRA-5-maleimide overnight produced a mixture of unlabeled, singly labeled and doubly labeled cTnC(T13C/N51C). Purification of the doubly labeled cTnC was carried out by chromatographic separation using a DEAE column. The labeling ratio was determined spectroscopically using $\varepsilon_{542} = 97,970 \text{ cm}^{-1}\text{M}^{-1}$. Labeling ratios for all protein modification were >95%. Cardiac Tm was purified from bovine heart [38] while actin was purified from rabbit skeletal muscle [39]. Doubly labeled cTnC(T13C/N51C) was reconstituted into troponin in a 1:1:1 ratio and into thin filaments using a cTn:Tm:actin molar ratio of 1:1:7.0 and checked by native gel electrophoresis as previously reported [33, 37, 40]. The regulatory function of the double cysteine cTnC mutant, used before in our laboratory, was also verified by testing its ability to participate in the Ca$^{2+}$-dependent regulation of acto-S1 ATPase activity (data not shown). Multiple experiments were
performed on prepared samples within 5 days, and no protein degradation was observed by electrophoretic analysis over this time period.

Ca\(^{2+}\)-sensitizer preparation

The four Ca\(^{2+}\)-sensitizers used in this study, EMD 57033 (Merck), Levosimendan (Sigma-Aldrich), Bepridil (Sigma-Aldrich) and Pimobendan (Merck) were dissolved in enough DMSO to make 6 mM drug stock solutions. Stock solutions were kept at 4°C to prevent degradation and were remade every 2 weeks.

Steady-state fluorescence measurements

Steady-state fluorescence measurements were performed at 15 ± 0.1 °C on an ISS PC1 photon-counting spectrofluorometer equipped with a micro-titrator using a band pass of 3 nm on both the excitation and emission monochromators. Samples containing 1 \(\mu\)M protein dissolved in working buffer, which consisted of 30 mM MOPS pH 7.0, 5 mM MgCl\(_2\), 0.20 M KCl, 1 mM DTT, and 1 mM EGTA, were tested in the presence and absence of 3 mM CaCl\(_2\). Samples were excited with 495 nm light and the emission spectra of cTnC(T13C/N51C) either singly or doubly labeled with TAMRA were collected from 500-600 nm using a scanning emission monochromator. Validation of distance dependant self-quenching between residue 13 and 51 in cTnC was achieved by measuring the relative intensity change at 578 nm of the doubly labeled sample upon addition of saturating levels of Ca\(^{2+}\).

Steady-state fluorescence Ca\(^{2+}\) titrations

In order to characterize the effect of each drug on the Ca\(^{2+}\)-sensitivity of the N-domain opening of cTnC, steady-state fluorescence was measured during Ca\(^{2+}\)-titrations performed using the micro-titrator as described previously [33]. Because of the position of residues 13 and 51 in
cTnC the distance between these two residues increases significantly as Ca$^{2+}$ binds to site II in cTnC and induces opening of the hydrophobic patch which initiates the structural changes necessary for generation of force [41]. Since both residues are labeled with TAMRA the fluorescence intensity at 578 nm is relatively low while the N-domain of cTnC is closed because both TAMRA molecules are in close proximity allowing for significant self-quenching, as the N-domain of cTnC binds to Ca$^{2+}$ and opens the two TAMRA molecules become further apart reducing the amount of quenching, resulting in a higher fluorescence intensity at 578 nm. In a typical titration experiment, up to 100 data points were acquired after successively injecting aliquots of 3 µL of a 14 mM Ca$^{2+}$ solution into 1.2 mL of 1 µM cTnI-cTnC, cTn or TF in a titration buffer containing 50 mM MOPS, 1.6 mM EGTA, 4 mM nitrilotriacetic acid (NTA), .20 M KCl, 5 mM MgCl$_2$, and 1 mM DTT at pH 7.10 with 3.3% DMSO by volume and 100 µM drug (or no drug in the same vehicle solution). Fluorescence vs. free Ca$^{2+}$ concentrations data were acquired by monitoring total fluorescence intensity of samples at 578 nm with excitation at 545 nm. Data from Ca$^{2+}$-titrations were fit using the Hill equation:

$$I = (I_{max} - I_{min}) \frac{[Ca^{2+}]^{n_H}}{[Ca^{2+}]^{n_H} + (10^{-pcCa_{50}})} + I_{min}$$

(1)

Where $I$ represents steady state TAMRA intensity, $I_{min}$ is TAMRA intensity under Ca$^{2+}$ free conditions, $I_{max}$ is TAMRA intensity under Ca$^{2+}$ saturated conditions, $pcCa_{50}$ the $-\log_{10}(\{Ca^{2+}\})$ at which apparent half occupancy of N-cTnC Ca$^{2+}$-binding sites occurs, and $n_H$ the Hill coefficient which represents the steepness of the Ca$^{2+}$-dependent increase in TAMRA fluorescence.

Stopped-flow measurements
Stopped-flow experiments were performed to examine the kinetics of N-cTnC closing by Ca\textsuperscript{2+} dissociation using a T format KinTek stopped-flow spectrometer with a 1.8 ms dead time. Ca\textsuperscript{2+}-saturated cTnI-cTnC, cTn, or TF \textsuperscript{s} samples were rapidly mixed with an equal volume of buffer containing an 10 mM of the Ca\textsuperscript{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid (BAPTA) \cite{42}. The change in TAMRA emission intensity as a function of time after mixing was recorded for each sample. For each experiment 15–20 of these kinetic traces were collected for every Ca\textsuperscript{2+}-sensitizer, for each level of reconstitution (cTnI-cTnC, cTn, TF). The set of kinetic traces for each sample was averaged and then nonlinear regression was used to fit the averaged trace with a mono-exponential function and determine the rate of conformation change.

Statistical analysis

In order to establish statistical significance between control and Ca\textsuperscript{2+}-sensitizer treated samples one tailed paired \textit{t}-tests were performed on the titration parameters of \textit{pCa}_{50} while two tailed paired \textit{t}-tests were performed on all other parameters reported. The justification for use of a one tailed \textit{t}-test for the parameter of \textit{pCa}_{50} is that we are only interested in identifying sensitization as would be the case in an industrial drug screening aimed at identifying effective Ca\textsuperscript{2+}-sensitizers, agents that are unable to prove significant by this test would be eliminated as potential inotropes. Tabulated parameter values are given as averages over \textit{n} trials as indicated in each table, with standard deviations obtained shown next to each parameter. Unless otherwise mentioned, parameter values were obtained through non-linear regression using the least squares fitting method to the appropriate model.

3.4 Results

3.4.1 Self-quenching as fluorescence marker for cTnC opening
The distance between residues 13 and 51 in cTnC are known to move 10Å further apart (19.21Å to 29.06Å) upon binding of Ca^{2+} by N-cTnC [30]. This structural transition is the crucial N-cTnC opening which initiates sarcomere contraction. Monitoring this structural change would thus enable a direct observation of N-cTnC activation and any change in this transition induced by Ca^{2+}-sensitizers. The labeling scheme employed in this study relies upon labeling of both residues 13 and 51 in cTnC with TAMRA. In order to establish the existence of significant distance dependent self-quenching within our doubly labeled cTnC mutant, we measured the emission spectra of both doubly and singly TAMRA labeled cTnC in the absence and presence of Ca^{2+}. For singly labeled cTnC no significant intensity change resulted from the addition of Ca^{2+} while a large intensity change (~30%) resulted for doubly labeled cTnC (see Figure 1). Because no increase in intensity is observed for the singly labeled cTnC in the presence of Ca^{2+} we can rule out environmentally dependent changes underlying the doubly labeled cTnC’s intensity increase upon Ca^{2+} binding. Thus the double labeled cTnC must undergo intensity changes arising from a change in distance between cTnC residues 13 and 51 whereon the TAMRA molecules have been labeled. This Ca^{2+}-induced opening of N-cTnC increases the distance between the two residues and results in a reduced self-quenching which subsequently increases the fluorescence intensity. Having linked the observable fluorescence intensity change with the opening of the N-domain of cTnC allows for the direct measurement of impacts made by Ca^{2+}-sensitizers on this key structural transition.
Figure 1. (Left) Singly labeled cTnC showing no intensity change upon addition of Ca$^{2+}$. Purple dashed trace shows fluorescence intensity in the Mg$^{2+}$-state while the green solid trace shows the intensity in the presence of saturating Ca$^{2+}$. (Right) Doubly labeled cTnC showing a large Ca$^{2+}$-induced fluorescent intensity change. Dashed purple trace shows the Mg$^{2+}$-state intensity while the solid green trace shows the Ca$^{2+}$-state intensity.

3.4.2 Ca$^{2+}$-Titrations

The sensitization efficacy of each Ca$^{2+}$-sensitizer was assessed at the cTnI-cTnC, cTn, whole TF and whole TF with cTnT(T204E) levels of reconstitution using fluorescence based in vitro Ca$^{2+}$-titrations. Through controlled addition of a 14 mM Ca$^{2+}$ solution to continuously stirred and temperature controlled protein samples and subsequent fitting of the normalized intensity versus pCa curve by the Hill equation we were able to extract the pCa$_{50}$ and Hill coefficient for each Ca$^{2+}$-sensitizer under each level of reconstitution. We hypothesized that the level of reconstitution would effect the efficacy of each Ca$^{2+}$-sensitizer according to its mechanism of action, confirming or contesting the suggested functionality of each and establishing an efficient method for future Ca$^{2+}$-sensitizer development and testing.
Figure 2. Representative Ca$^{2+}$-titration data from TF samples containing cTnC(13C-51C) double labeled with TAMRA. Samples are either in the presence of 100 μM Ca$^{2+}$-sensitizer or vehicle (control). At high pCa (low Ca$^{2+}$) the fluorescence intensity is at a minimum indicating maximum FRET efficiency, as the pCa decreases (Ca$^{2+}$ increases) more and more cTnC molecules are bound to Ca$^{2+}$ resulting in increased separation between the two TAMRA molecules, which in turn reduces the FRET efficiency and increases the fluorescence intensity.

3.4.3 Ca$^{2+}$-sensitizer impact on pCa$_{50}$ and cooperativity at the IC level of reconstitution

All Ca$^{2+}$-sensitizers produce an increased Ca$^{2+}$-sensitivity at this level, however the sensitivity increase by levosimendan is not significant by T-test (Table 1). The Hill coefficient was increased for EMD 57033 and bepridil, while levosimendan and pimobendan appeared to decrease the Hill slope with levosimendan producing the only statistically significant change in Hill slope.

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Table 1. Numerical results from fitting of the Hill equation (Eq. 1) to Ca\textsuperscript{2+} titration curves for cTnI-cTnC samples treated with Ca\textsuperscript{2+}-sensitizer or control. Parameter standard deviation is given to the right of each parameter value. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>IC</th>
<th>pCa\textsubscript{50}</th>
<th>n\textsubscript{H}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 11)</td>
<td>5.11 ± 0.25</td>
<td>1.31 ± 0.26</td>
</tr>
<tr>
<td>EMD 57033</td>
<td>5.28 ± 0.13\textsuperscript{**}</td>
<td>1.42 ± 0.22</td>
</tr>
<tr>
<td>Levosimendan</td>
<td>5.18 ± 0.33</td>
<td>1.26 ± 0.38\textsuperscript{*}</td>
</tr>
<tr>
<td>Bepridil</td>
<td>5.38 ± 0.13\textsuperscript{**}</td>
<td>1.40 ± 0.23</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5.31 ± 0.17\textsuperscript{**}</td>
<td>1.21 ± 0.39</td>
</tr>
</tbody>
</table>

3.4.4 Ca\textsuperscript{2+}-sensitizer impact on pCa\textsubscript{50} and cooperativity at the ICT level of reconstitution

Samples reconstituted with cTnT present showed a significant increase in Ca\textsuperscript{2+}-sensitivity compared to IC only samples (control from Table 1 compared to control from Table 2). This cTnT based sensitization may result from a cTnT mediated scaffolding effect which brings cTnI and cTnC into proper alignment for physiological functioning likely through the IT arm. The Ca\textsuperscript{2+}-sensitizing effect of EMD 57033 and levosimendan is blunted into insignificance at this level of reconstitution while bepridil and pimobendan maintain a significant increase in pCa\textsubscript{50} (Table 2). This implies that the mechanism of action for EMD 57033 and levosimendan is either partially saturated or blocked by cTnT while bepridil and pimobendan must enhance Ca\textsuperscript{2+}-sensitivity by a distinctly different mechanism from that of cTnT.
Table 2. Numerical results from fitting of the Hill equation (Eq. 1) to Ca\textsuperscript{2+} titration curves for cTnI-cTnC-cTnT samples treated with Ca\textsuperscript{2+}-sensitizer or control. Parameter standard deviation is given to the right of each parameter value. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>ICT</th>
<th>pCa\textsubscript{50} ± std</th>
<th>n\textsubscript{H} ± std</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.27 ± 0.25</td>
<td>1.27 ± 0.28</td>
</tr>
<tr>
<td>EMD 57033</td>
<td>5.32 ± 0.23</td>
<td>1.37 ± 0.28</td>
</tr>
<tr>
<td>Levosimendan</td>
<td>5.35 ± 0.19</td>
<td>1.40 ± 0.28*</td>
</tr>
<tr>
<td>Bepridil</td>
<td>5.48 ± 0.12**</td>
<td>1.40 ± 0.25*</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5.43 ± 0.10**</td>
<td>1.24 ± 0.20</td>
</tr>
</tbody>
</table>

3.4.5 Ca\textsuperscript{2+}-sensitizer impact on pCa\textsubscript{50} and cooperativity at the TF level of reconstitution

Control samples reconstituted into whole thin filaments showed an additional increase in Ca\textsuperscript{2+}-sensitivity over ICT samples as well as a significant increase in Hill slope (control from Table 2 compared to control from Table 3). These effects likely result from the additional cooperativity found in thin filaments arising from cTnT's positioning on actin and its ability to alter the position of Tm upon cTn binding to Ca\textsuperscript{2+} which in turn conducts structural changes to neighboring cTn complexes. EMD 57033, bepridil, and pimobendan all appeared to elevate the Ca\textsuperscript{2+} sensitivity of the TF however only bepridil's sensitization was statistically significant at this level of reconstitution.
Table 3. Numerical results from fitting of the Hill equation (Eq. 1) to Ca\textsuperscript{2+} titration curves for thin filament samples treated with Ca\textsuperscript{2+}-sensitizer or control. Parameter standard deviation is given to the right of each parameter value. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>TF</th>
<th>pCa\textsubscript{50}</th>
<th>n\textsubscript{H}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.49 ± 0.15</td>
<td>1.59 ± 0.33</td>
</tr>
<tr>
<td>EMD 57033</td>
<td>5.52 ± 0.14</td>
<td>1.65 ± 0.58</td>
</tr>
<tr>
<td>Levosimendan</td>
<td>5.44 ± 0.12</td>
<td>1.27 ± 0.35</td>
</tr>
<tr>
<td>Bepridil</td>
<td>5.57 ± 0.14**</td>
<td>1.51 ± 0.26</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5.55 ± 0.20</td>
<td>1.27 ± 0.22</td>
</tr>
</tbody>
</table>

3.4.6 Ca\textsuperscript{2+}-sensitizer impact on pCa\textsubscript{50} and cooperativity for cTnT(T204E) containing TF samples

Incorporation of cTnT(T204E) into TF samples reduced both the Ca\textsuperscript{2+}-sensitivity and Hill slope in accordance with previous studies (control from Table 3 compared to control from Table 4) [28, 29]. Under these conditions all Ca\textsuperscript{2+}-sensitizers appeared to bring up the Ca\textsuperscript{2+}-sensitivity of these TF samples with levosimendan, bepridil, and pimobendan doing so to a statistically significant extent (Table 4). This leads to the notion that the underlying cause behind cTnT(T204E)'s reduction in pCa\textsubscript{50} and Hill slope can be remedied by the use of Ca\textsuperscript{2+}-sensitizers, particularly levosimendan which brought up the pCa\textsubscript{50} and Hill slope most significantly.
Table 4. Numerical results from fitting of the Hill equation (Eq. 1) to Ca\(^{2+}\) titration curves for cTnT(T204E) containing thin filament samples treated with Ca\(^{2+}\)-sensitizer or control. Parameter standard deviation is given to the right of each parameter value. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>TF T204E (n = 5)</th>
<th>pCa(_{50})</th>
<th>n(_H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.42 ± 0.10</td>
<td>1.36 ± 0.16</td>
</tr>
<tr>
<td>EMD 57033</td>
<td>5.45 ± 0.15</td>
<td>1.42 ± 0.28</td>
</tr>
<tr>
<td>Levosimendan</td>
<td>5.53 ± 0.10**</td>
<td>1.51 ± 0.12**</td>
</tr>
<tr>
<td>Bepridil</td>
<td>5.52 ± 0.13**</td>
<td>1.44 ± 0.14</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>5.51 ± 0.16**</td>
<td>1.28 ± 0.35</td>
</tr>
</tbody>
</table>

3.4.7 Kinetic role of Ca\(^{2+}\)-sensitizers and reconstitution level on cTn and TF deactivation

The effect on relaxation is a critical consideration for drugs which impact the Ca\(^{2+}\)-sensitivity of the sarcomere since an increased Ca\(^{2+}\)-sensitivity is often associated with slowed deactivation which may impair diastolic function in the heart. By performing rapid removal of Ca\(^{2+}\) from cTnC by mixing protein sample with BAPTA in a stopped flow setup and monitoring the change in fluorescence intensity with respect to time, the rate of closing of N-cTnC can be computed by fitting this fluorescence decay with a simple mono-exponential function and extracting the rate constant. This allows for elucidation of the effects of each Ca\(^{2+}\)-sensitizer on cardiac relaxation at the IC, cTn or TF level. The impact on deactivation rate by the various Ca\(^{2+}\)-sensitizers showed less dependence on reconstitution level than did the Ca\(^{2+}\)-titration parameters (Table 5). EMD 57033 did not display a significant effect on deactivation rate. This is not surprising since its impact on Ca\(^{2+}\)-sensitivity was the most modest of all the drugs tested. Thus it could be surmised that EMD 57033 does not exert a strong influence on the deactivation rate at the level of cTn or
TF likely because its mechanism of action lies beyond the thin filament. For levosimendan an overall tendency to slow the rate of deactivation, particularly pronounced in the TF cTnT(T204E) samples indicates a stabilization of the cTn conformation in which cTnI is bound to Ca$^{2+}$-saturated cTnC. Bepridil, which in the titration experiments proved to be the most potent direct Ca$^{2+}$-sensitizer lead to a consistent, albeit not always significant increase in the rate of deactivation at all levels of reconstitution. This is counter to the usual reasoning which says an increase in Ca$^{2+}$-sensitivity tends to result through a more stable IC interaction with subsequently slower deactivation. This could be explained by a destabilization of cTnI-cTnC's interaction compared to control, despite shifting the equilibrium of cTnC toward the open state, thus making initial cTnI-cTnC interaction more likely while increasing their rate of dissociation upon rapid removal of Ca$^{2+}$. Pimobendan on the other hand which also sensitizes the thin filament to Ca$^{2+}$ results in a decrease in the rate of deactivation, the hallmark of enhanced cTnI-cTnC-Ca$^{2+}$stability.
**Figure 3.** Stopped flow BAPTA induced Ca\(^{2+}\) dissociation curves in TF samples. Rapid mixing of 10 mM BAPTA with Ca\(^{2+}\)-saturated TF samples either with or without Ca\(^{2+}\)-sensitizer induces fast removal of Ca\(^{2+}\) from N-cTnC and subsequent dissociation of cTnI from N-cTnC and N-cTnC hydrophobic patch closing. The exponential decrease in fluorescence intensity with respect to time indicates the conformational change from open to closed hydrophobic patch in N-cTnC. Representative traces above show increased rate of closing for N-cTnC in the presence of Bepridil and EMD 57033 with a slight reduction in this rate for Pimobendan and Levosimendan.
Table 5. Kinetic parameters for Ca\(^{2+}\)-dissociation induced structural changes in cTnC for all levels of reconstitution. Parameters are given plus or minus one standard deviation. *Indicates a significant difference from wild type at the 90% level while **indicates significance at the 95% level.

<table>
<thead>
<tr>
<th>Ca(^{2+})-Sensitizer</th>
<th>Kinetic rate (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC (n=5)</td>
</tr>
<tr>
<td>Control</td>
<td>29.4 ± 11.0</td>
</tr>
<tr>
<td>EMD 57033</td>
<td>27.5 ± 12.7</td>
</tr>
<tr>
<td>Levosimendan</td>
<td>29.8 ± 7.3</td>
</tr>
<tr>
<td>Bepridil</td>
<td>37.4 ± 10.3**</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>25.8 ± 5.1</td>
</tr>
</tbody>
</table>

3.5 Discussion

New Ca\(^{2+}\)-sensitizing agents that target the thin filament directly offer promise in the treatment of heart failure. Development of such drugs benefits from a clear understanding of their mechanism of action. The more complete the molecular level picture of drug action the more easily these and future agents can be tailored to fit a specific need. Traditional methods of characterizing Ca\(^{2+}\)-sensitizers rely on techniques such as NMR, MS, X-ray crystallography, and cardiac myocyte or fiber studies [10-27]. In this study we have demonstrated a new fluorescence based approach to characterize four important Ca\(^{2+}\)-sensitizers in vitro. One benefit of this technique is that many drugs can be tested with only a marginal increase in time, thus allowing for faster development and iteration of drug design. Additionally, the model being used represents the simplest units involved in the Ca\(^{2+}\)-regulation of force under physiological conditions, and thus reduces the number of confounding variables compared to tissue studies. Through use of standard in vitro fluorescence equipment, i.e. a spectrofluorometer and stopped
flow device, we were able to quantify the effect of each Ca\textsuperscript{2+}-sensitizer on cTnC's Ca\textsuperscript{2+}-sensitivity and relaxation kinetics at various levels of reconstitution and in the presence of a known Ca\textsuperscript{2+}-sensitivity and cooperativity blunting cTnT phosho-mimic. Interestingly, by examining the parameters of pCa\textsubscript{50}, n\textsubscript{H}, and k\textsubscript{relaxation}, at the IC, cTn and TF levels of reconstitution and in the presence of cTnT(T204E) we obtained a picture of how these parameters depend on the presence of cTnT, actin, and tropomyosin. In the following sections we discuss how our data confirm or contest the prevailing understanding of each Ca\textsuperscript{2+}-sensitizer’s mechanism of action, we also discuss the role of protein-protein interactions in cTn and the TF on Ca\textsuperscript{2+}-sensitivity and relaxation kinetics.

3.5.1 Dependence of cTnC's Ca\textsuperscript{2+}-induced opening on reconstitution level

Several important observations can be made by examining the non-drug treated samples at the various levels of reconstitution and in the presence of cTnT(T204E) (controls from tables 1-5). First is the importance of cTnT on Ca\textsuperscript{2+}-sensitivity. Going from cTnI-cTnC to cTnI-cTnC-cTnT (cTn) we see a significant increase in Ca\textsuperscript{2+}-sensitivity. The presence of cTnT allows for formation of the IT arm, an important structural interaction between cTnI and cTnT which may play a scaffolding role bringing cTnI and cTnC into proper alignment facilitating binding of cTnI to cTnC's hydrophobic patch in the presence of Ca\textsuperscript{2+} [43]. The presence of cTnT may also enhance the Ca\textsuperscript{2+}-sensitivity by positioning cTnI's cardiac specific N-terminal extension into the appropriate conformation with respect to cTnC, this region of cTnI has been implicated as playing a key role in the enhancement of cTnC's Ca\textsuperscript{2+}-sensitivity [44]. Additionally there is a possibility that the N-domain of cTnT directly stabilizes cTnC's Ca\textsuperscript{2+}-bound conformation [45].
Going from cTn to the full TF means inclusion of actin, and tropomyosin. These two filamentous proteins are thought to be essential for the cooperativity of the thin filament by acting as physical contacts between cTn units and conducting conformational changes longitudinally to neighboring cTn's, thus enhancing their Ca$^{2+}$-sensitivity [46]. Support for this is seen in the increased Hill coefficient for TF control as compared to cTn control (1.59 compared to 1.27). The additional increase in Ca$^{2+}$-sensitivity seen when going from cTn to full TF likely results from the enhanced cooperativity and thus does not imply a true increase in cTnC Ca$^{2+}$-affinity, but represents an apparent increase in Ca$^{2+}$-binding due to enhanced cooperativity. Incorporation of cTnT(T204E) into TF reduces the pCa$_{50}$ (5.42 compared to 5.49) and the Hill coefficient (1.36 compared to 1.59), in agreement with previous studies [28, 29], however this has not previously been reported through direct measurement of N-cTnC opening, as in this study. This offers further support for the notion that cTnT(T204E) interferes with conduction of structural changes initiated at cTnC from reaching neighboring cTn units and enhancing their activation, although an additional influence on cTnC's Ca$^{2+}$-sensitivity through alteration of cTnT's proper scaffolding of cTnI in relation to cTnC may also be involved.

3.5.2 Insights into Ca$^{2+}$-sensitizer mechanisms of action

3.5.2.1 EMD 57033

Through an NMR study it was found that EMD 57033 appears to bind C-cTnC, although the importance of this binding site is unclear [7, 19]. It is thought that the primary mechanism of action for EMD 57033 to directly affect the sarcomere is through enhancement of the force per crossbridge [47]. Our data is consistent with this notion of a “down stream” mechanism of action for EMD 57033 since both kinetic and Ca$^{2+}$-sensitivity data showed little change compared to
control at most levels of reconstitution. This implies there is no direct effect on the cTnC-cTnI switch event. The most widely accepted mechanism of action for EMD 57033 is at the actin-myosin interface. Since our model does not incorporate crossbridges or the dynamic process of crossbridge feedback there would be no way for EMD 57033 to impact the thin filament via this process. Only Ca\textsuperscript{2+}-sesnsitivity at the cTnI-cTnC level of reconstitution showed a significant increase upon addition of EMD 57033 (table 1). The cTnI-cTnC level of reconstitution showed lower Ca\textsuperscript{2+}-sensitivity than either the cTn or full TF levels of reconstitution indicating that the additional protein-protein interactions conferred additional stability to N-cTnC’s open conformation. Perhaps at the cTnI-cTnC level EMD 57033 was able to bind nonspecifically to some of the exposed hydrophobic surface area of the cTnI-cTnC complex not yet involved in a protein-protein interaction and impart some additional stability to the open conformation of cTnC.

3.5.2.2 Levosimendan

Levosimendan has been shown to bind both to C-cTnC and N-cTnC, although the binding of C-cTnC is not considered physiologically important, and levosimendan’s action as a Ca\textsuperscript{2+}-sensitizer is attributed to its Ca\textsuperscript{2+}-dependant N-cTnC binding [16, 48, 49]. The stabilization of cTnI binding to cTnC’s hydrophobic patch has been claimed as a likely mechanism of action for this drug [50]. A trend of slight sensitization was seen in the titration data (tables 1-4), however this sensitization was only statistically significant in TF with cTnT(T204E) present, our Ca\textsuperscript{2+}-titrations also showed an increase in n\textsubscript{H} with this phospho-mimic. The rate of Ca\textsuperscript{2+}-dissociation induced N-cTnC closing also showed a trend towards slower deactivation, however this was only statistically significant in the presence of cTnT(T204E). Taken together our findings show levosimendan does likely enhance cTnI’s affinity for cTnC’s hydrophobic cleft, but that this
effect is much more noticeable when the normal Ca\(^{2+}\)-sensitivity/cooperativity of the TF is disrupted. Thus it appears levosimendan directly counteracts the effect of cTnT(T204E) in the thin filament. This is an ideal behavior for a Ca\(^{2+}\)-sensitizer, working to enhance Ca\(^{2+}\)-sensitivity and cooperativity but only under conditions where the normal Ca\(^{2+}\)-sensitivity is impaired. From our data, it is unlikely that levosimendan would have any negative impact on diastole, a common issue amongst Ca\(^{2+}\)-sensitizers. It should be noted that levosimendan’s function in the presence of other physiologically relevant cTn or TF mutations/posttranslational modifications would offer more specific understanding of how this drug would work in different disease models.

3.5.2.3 Bepridil

Bepridil is known to bind directly to the hydrophobic patch of N-cTnC and stabilize its open conformation [13]. Unlike levosimendan this binding to cTnC is not Ca\(^{2+}\)-dependant, additionally it has been proposed that bepridil sterically inhibits the binding of cTnI to cTnC’s hydrophobic patch [51]. Our data show a marked Ca\(^{2+}\)-sensitization at all levels of reconstitution (tables 1-4) implying increased stability of cTnC with Ca\(^{2+}\) bound at site II and with its hydrophobic pocket open and bound to cTnI. In most cases bepridil was clearly the most potent Ca\(^{2+}\)-sensitizer, likely owing to its ability to bind cTnC in the presence or absence of Ca\(^{2+}\), thus having a stronger effect on Ca\(^{2+}\)-sensitivity at lower levels of free Ca\(^{2+}\). Based on the results from the titration experiments with bepridil one might anticipate a slowed rate of closing for N-cTnC upon Ca\(^{2+}\)-chelation by BAPTA, however our data show an increase in N-cTnC closing rate. Remembering that bepridil appears to sterically interfere with cTnI's binding to cTnC's hydrophobic pocket helps to clear up this apparent contradiction. Since the triggering event for N-cTnC closing in the stopped flow experiment is removal of Ca\(^{2+}\) from site II of cTnC by BAPTA, it becomes apparent that the rate of cTnI ejection from cTnC's hydrophobic patch will
actually be faster in the presence of bepridil owing to the steric destabilization. And since the total closing time for N-cTnC can be considered as the sum of the Ca\(^{2+}\)-removal time, the cTnI ejection time, and the hydrophobic pocket closing time, it makes sense for bepridil to increase deactivation rate under these conditions since Ca\(^{2+}\)-chelation time and N-cTnC closing time should be minimally affected. Thus, based on our in vitro data bepridil would likely be a powerful enhancer of systolic force and ironically an enhancer of relaxation rate. It is important to note that simply because the rate of Ca\(^{2+}\)-dissociation induced relaxation is enhanced does not imply that diastole in vivo will be preserved. In fact, seeing how potently bepridil enhances Ca\(^{2+}\)-sensitivity in a non-Ca\(^{2+}\)-specific manner may result in incomplete relaxation at diastolic Ca\(^{2+}\) levels in the sarcomere despite the faster kinetics of N-cTnC closing.

### 3.5.2.4 Pimobendan

Pimobendan appears to enhance the affinity of cTnC for Ca\(^{2+}\) and cTnI thus sensitizing troponin to the Ca\(^{2+}\) signal. Since pimobendan increases cTnC's affinity for Ca\(^{2+}\) even at the lowest levels of Ca\(^{2+}\) it has been thought this drug may slow relaxation kinetics and disrupt diastole. In human cardiac muscle strips the rate of relaxation indeed appeared to be reduced [52]. Our results appear to confirm that not only does pimobendan have a direct and powerful sensitizing effect on the contractile proteins from the level of cTnI-cTnC up to full TF but that a reduction in relaxation of cTn/TF likely underlies the reduced relaxation rate seen in human tissue samples. Unlike bepridil which appears to reduce cTnI's affinity for cTnC's hydrophobic patch, pimobendan is not thought to sterically inhibit this interaction and the reduced N-cTnC closing rates from this study support this notion. Pimobendan appears to act most similarly to levosimendan but with greater potency both at sensitizing cTnC to Ca\(^{2+}\) and in reducing the rate of relaxation, likely by increasing the affinity of cTnI for cTnC's hydrophobic patch. This
increased potency likely owning to pimobendan's non-Ca\textsuperscript{2+}-specific interaction with cTnC making it more effective at the lower concentrations of Ca\textsuperscript{2+}. Taken together these findings show that pimobendan would be a good Ca\textsuperscript{2+}-sensitizer to use only when an improvement of systolic function much outweighs the risk of a reduction in diastolic function.

### 3.6 Conclusion

The central hypothesis of this study appears to have been validated, namely that the level of reconstitution of the troponin complex affects the efficacy of a given Ca\textsuperscript{2+}-sensitizer in a manner dependant on its mechanism of action. The fluorescence based approach used in this study independently confirms the mechanisms of action for four well known Ca\textsuperscript{2+}-sensitizers while adding new insights on the efficacy of these sensitizers in the presence of Ca\textsuperscript{2+}-blunting phospho-mimic mutation, cTnT(T204E). The effect on relaxation of each Ca\textsuperscript{2+}-sensitizer was also determined, something which has previously been difficult to determine and is important in assessing potential diastolic interference. Thus, this study offers several new insights on thin filament function and Ca\textsuperscript{2+}-sensitizer action which were obtained using a new and efficient fluorescence based method, amenable to high throughput for drug screening and design.

### 3.7 Acknowledgments

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3.8 References


Chapter 4: Dynamic Equilibrium of cTnC's Hydrophobic Cleft and Its Modulation by Ca$^{2+}$ Sensitizers and a Ca$^{2+}$ Sensitivity blunting phosphomimic, cTnT(T204E)

4.1 Abstract

Several studies have suggested that conformational dynamics are important in cardiac troponin C's (cTnC's) regulation of thin filament activation, however little direct evidence has been offered to support these claims. In this study, a dye homodimerization approach is developed and implemented which allows for determination of the dynamic equilibrium between open and closed conformations in cTnC's hydrophobic cleft, referred to as the open and closed states respectively. Modulation of this equilibrium by Ca$^{2+}$, cardiac troponin I (cTnI), cardiac troponin T (cTnT), Ca$^{2+}$-sensitizers, and a Ca$^{2+}$ desensitizing phosphomimic of cTnT (cTnT(T204E)) is characterized. Isolated cTnC contained a small open state population in the absence of Ca$^{2+}$, which increased significantly upon addition of saturating levels of Ca$^{2+}$. This suggests the Ca$^{2+}$ induced activation of thin filament ultimately arises from an increase in the probability of hydrophobic cleft opening. Inclusion of cTnI increased the open state population of cTnC while inclusion of cTnT had the opposite effect. Samples containing Ca$^{2+}$-desensitizing cTnT(T204E) showed a slight but insignificant decrease in open state probability compared to samples with cTnT(wt), while Ca$^{2+}$-sensitizer treated samples generally increased open state probability. This suggests that increasing or decreasing the probability of hydrophobic cleft opening results in altered Ca$^{2+}$ sensitivity.
4.2 Introduction

The cardiac troponin complex (cTn) transduces the sarcomeric Ca\(^{2+}\) transient into the cyclic contraction and relaxation of the myocardium responsible for moving blood throughout the body. A hetero-trimeric protein assembly, cTn consists of troponin I which inhibits force generation, troponin C which is able to bind Ca\(^{2+}\) and remove cTnI’s inhibition of force, and troponin T which tethers cTn to tropomyosin and actin. When the sarcomeric Ca\(^{2+}\) concentration is low cTn in conjunction with tropomyosin inhibits the strong actin-myosin binding required for force generation and as Ca\(^{2+}\) concentration increases this inhibition is released. The troponin subunit responsible for this Ca\(^{2+}\) dependence is cTnC, which possesses a single regulatory Ca\(^{2+}\) binding site within its N-terminal half. When Ca\(^{2+}\) binds to this site a shift in cTnC’s helices B and C away from helices N, A, and D partially exposes hydrophobic residues in this region, known as the hydrophobic cleft, to which cTnI’s regulatory region (cTnI residues 150-165) can bind. As the regulatory region and cTnC bind, the inhibitory region of cTnI (cTnI residues 130-149) is pulled away from actin’s surface thereby relinquishing its inhibition of strong actin-myosin binding. This, in conjunction with tropomyosin movement away from myosin binding sites on actin, enables strong actin-myosin binding which generates force [1]. Thus, the events leading to cardiac contraction represent a tightly coupled cascade of structural transitions beginning with Ca\(^{2+}\) binding to cTnC and proceeding through to strong actin-myosin binding. This illustrates the central role cTnC has in cardiac contraction and explains why mutations or pharmaceuticals which alter cTnC function have a large impact on cardiac contractility [2-5]. But despite its importance and the many studies focused on it cTnC has yet to be fully characterized.
In recent years the essential role of dynamics in protein function has become increasingly clear. The current view is that most allosteric proteins exist in a dynamic conformational equilibrium between various physiologically relevant macrostates [6, 7]. Thus, to fully understand how a protein functions it is necessary to characterize the structure and function of accessible macrostates, the equilibrium between these states, and how ligand binding alters this equilibrium or changes which macrostates are accessible. An increasing body of evidence suggests cTnC exists in conformational equilibrium and that Ca\(^{2+}\) regulates this equilibrium [8-10]. Robinson et al. used FRET structural and kinetic data to show that in the absence of Ca\(^{2+}\) the cTnI-cTnC complex exists in dynamic equilibrium between “closed” and “primed-closed” conformations (although he did determine the constant for this equilibrium process). In this case “closed” indicates that the hydrophobic cleft is in a compact conformation disallowing binding by cTnI’s regulatory region and “primed-closed” indicates a conformation conceived of by Robinson wherein cTnC’s hydrophobic cleft is slightly ajar and to which cTnI’s regulatory region can bind via an induced fit mechanism. Robinson suggests Ca\(^{2+}\) binding causes cTnC to entirely adopt the primed-closed conformation and inclusion of cTnI establishes a dynamic equilibrium in which 65% of the time cTnC is in the open state (hydrophobic cleft fully open) and the other 35% of the time it is in the primed-closed state. This is in contrast to a study by Cordina et al. which suggests the hydrophobic cleft is able to access the fully open conformation in the absence of cTnI and perhaps even in the absence of Ca\(^{2+}\). Cordina showed isolated cTnC’s hydrophobic cleft is fully open up to 15% of the time in the absence of Ca\(^{2+}\) and approximately 27% of the time when Ca\(^{2+}\) is bound. Thus, according to Cordina Ca\(^{2+}\) binding to cTnC increases the amount of cTnC in the open conformation at any given time and what Robinson calls the primed-closed state Cordina suggests is a dynamic equilibrium between open and closed conformations.
Cordina’s model implies cTnI binds to cTnC via selective binding of cTnI's regulatory region to the open state of cTnC's hydrophobic cleft, instead of by an induced fit mechanism as Robinson proposes. Thus from the current literature on cTnC’s dynamic equilibria it is unclear 1) by which mechanism cTnI’s regulatory region binds with cTnC’s hydrophobic cleft, and 2) if Ca\(^{2+}\) binding to cTnC induces an ajar "primed-closed" structure or if what appears as a single macrostate is in fact a dynamic equilibrium between open and closed conformations. In addition to these fundamental questions regarding the role and nature of dynamic equilibria in cTnC, there is also much to be gained by understanding how these equilibria are modulated by various agents, such as cTnI, cTnT, Ca\(^{2+}\) sensitizers, or Ca\(^{2+}\) desensitizing mutations/post-translational modifications.

In the current study, we aimed to resolve some of these unanswered questions regarding dynamic equilibria in cTnC using an innovative TAMRA dimerization based approach. When in close proximity TAMRA molecules readily form non-fluorescent homodimers, which display a hypsochromic shift of 34 nm, giving the dimer a characteristic absorbance peak at 520 nm while monomeric TAMRA has a peak absorbance at 554 nm. So, by measuring the absorbance at 520 nm and 554 nm the amount of TAMRA monomer and dimer in a sample can be found based on the spectra of the pure monomer and pure dimer (see “Absorbance data processing” for more details) [11, 12]. TAMRA labeling residues 13C and 51C in cTnC, which reside on opposite sides of the hydrophobic cleft, links the relative abundance of dimeric TAMRA with the conformation of the hydrophobic cleft. This allows information regarding cTnC’s structure to be extracted from the spectroscopically determined dimeric TAMRA abundance. Quantitatively relating the dimer abundance with the structure of the hydrophobic cleft was achieved using a model derived in this study which gives TAMRA dimerization probability as a function of 13C-
51C separation distance. This model showed that when cTnC residues 13C and 51C are close enough to allow direct contact between the two TAMRA molecules (approximately 28.7 Å via molview) the probability of TAMRA dimerization is near unity, and for 13C-51C separations greater than 28.7 Å dimerization is impossible. Thus monomeric TAMRA equates to the open state of cTnC (13C-51C separations greater than 28.7 Å) while dimeric TAMRA corresponds to the closed/primed-closed states (13C-51C distances less than 28.7 Å) [13]. Therefore, dynamic equilibria between open and closed/primed-closed conformations would produce a corresponding equilibria between monomeric and dimeric TAMRA, while the absence of dynamic equilibrium would show entirely dimeric TAMRA in the absence of Ca$^{2+}$ and entirely monomeric TAMRA in the presence of Ca$^{2+}$.

Absorbance spectra measurements were carried out in the presence and absence of Ca$^{2+}$ for cTnC(13C/51C)$_{TAMRA2}$, cTnI(wt)-cTnC(13C/51C)$_{TAMRA2}$, cTnI(wt)-cTnC(13C/51C)$_{TAMRA2}$-cTnT(wt), and cTnI-cTnC(13C/51C)$_{TAMRA2}$-cTnT(T204E), where cTnT(T204E) is a known Ca$^{2+}$ desensitizing phosphorylation mimic. Additionally, each of these treatments was conducted in the presence of either bepridil, levosimendan, or pimobendan, three well known Ca$^{2+}$ sensitizers. The results show isolated cTnC contains an equilibrium between monomeric and dimeric TAMRA in both the presence and absence of Ca$^{2+}$, implying a dynamic equilibrium between open and closed conformations. This equilibrium was modulated by Ca$^{2+}$ binding which served to increase the population of TAMRA monomer/open cTnC from approximately 14.5% to 33.9%. This finding contradicts the notion of uniform adoption of a primed-closed state by cTnC upon Ca$^{2+}$ binding, and instead supports the hypothesis that what appears as a primed-closed conformation in Ca$^{2+}$-cTnC is actually a dynamic equilibrium between fully open and closed
conformations. The relatively large population of TAMRA monomer/open conformations in Ca\(^{2+}\)-cTnC also suggests conformational selection as the most likely mechanism by which cTnI binds to the hydrophobic cleft. Samples containing cTnI showed significantly reduced abundance of dimeric TAMRA in the absence of Ca\(^{2+}\), an effect mostly attributed to steric clash between the regulatory region of cTnI with TAMRA. Samples containing cTnI in the presence of saturating Ca\(^{2+}\) showed a significant increase in TAMRA monomer/open cTnC consistent with the notion that cTnI binding stabilizes the open conformation of cTnC. Inclusion of cTnT reduced the amount of cTnI-dimer clash in the absence of Ca\(^{2+}\), most likely by decreasing the mobility of cTnI via the stabilizing effect of the IT arm. The Ca\(^{2+}\) desensitizing phosphorylation mimic, cTnT(T204E), appears to further reduce the cTnI-dimer clash, suggesting reduced collisional rate between cTnI’s regulatory region and cTnC may explain how cTnT(T204E), which has minimal direct contact with cTnC, is able to reduce Ca\(^{2+}\) sensitivity of the troponin complex. Addition of Ca\(^{2+}\) sensitizing agents increased the TAMRA monomer/open population in cTnC in both the presence and absence of Ca\(^{2+}\), consistent with the view that these drugs stabilize the open conformation of cTnC. All sensitizers showed an enhanced impact on cTnC’s dynamic equilibrium in the presence of Ca\(^{2+}\) or cTnI, indicating that increased opening of the hydrophobic cleft induced by Ca\(^{2+}\) or cTnI facilitates increased sensitizer binding, which in turn further increases the probability of hydrophobic cleft opening. Taken together these results show that dynamic equilibria are central to cTnC’s function and illustrate how cTnI(wt), cTnT(wt) and cTnT(T204E), as well as Ca\(^{2+}\) sensitizers can modulate this equilibrium to tune the Ca\(^{2+}\) sensitivity of the troponin complex.
4.3 Materials and Methods

Mutant generation, protein expression, and labeling

A recombinant double cysteine mutant of cTnC (cTnC(T13C/N51C)), as well as wild type cTnI (cTnI(wt)), wild type cTnT (cTnT(wt)), and a cTnT with phosphorylation mimicking glutamic acid at residue 204 (cTnT(T204E)) were generated from wild type rat protein clones using approaches similar to those previously reported [14-17]. Briefly, using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA), rat cDNA clones of wild-type cTnC, cTnI, and cTnT sub-cloned into the plasmid pSBETa were used as template DNA to generate cTnC(T13C/N51C), cTnI(wt), cTnT(wt), and cTnT(T204E). Note that cTnC(T13C/N51C)’s endogenous cysteines, Cys-35 and Cys-84, have each been substituted with serine. The regulatory function of the double cysteine cTnC mutant, used before in our laboratory, was verified by testing its ability to participate in the Ca\(^{2+}\) dependent regulation of acto-S1 ATPase activity (data not shown). Recombinant cTnC, cTnI and cTnT clones were overexpressed in *E. coli* strain BL21(DE3) cells and purified as previously described [18-21].

Residues 13 and 51 in cTnC were chosen as labeling sites because they are not part of any alpha helices or Ca\(^{2+}\) coordination sites, thus labeling to these residues should have minimal impact on cTnC’s function, while simultaneously affording the labeled molecules a high degree of mobility. Labeling of cTnC(T13C/N51C) with tetramethylrhodamine-5-maleimide (TAMRA, Setareh Biotech) was accomplished by eliminating the presence of reducing agent, in this case DTT, to facilitate exposure of the sulfhydryl groups in the cysteine residues. Subsequent incubation with 4-5 molar excess TAMRA overnight produced a mixture of unlabeled, singly labeled and doubly labeled cTnC(T13C/N51C). Excess DTT was then added to terminate the
labeling reaction. The labeling process was repeated once more to increase the abundance of double labeled cTnC(T13C/N51C). Chromatographic separation of unlabeled, single labeled, and double labeled cTnC(T13C/N51C) was carried out on a DEAE column. Resolution of the singly and doubly labeled protein was only partial and the final sample utilized for this study contained a small amount of singly labeled cTnC. In order to account for the effect of singly labeled cTnC(T13C/N51C) on our TAMRA monomer/dimer measurements knowledge of the labeling ratio was required (see Absorbance data processing for more details). The labeling ratio was determined by finding the total TAMRA concentration and dividing it by the cTnC(T13C/N51C) concentration. The TAMRA concentration was determined spectroscopically by dividing the deconvoluted TAMRA absorbances (see Absorbance data processing) at 554 nm and 520 nm by their respective extinction coefficients (\( \varepsilon_{554} = 66,100 \text{ cm}^{-1}\text{M}^{-1} \) and \( \varepsilon_{520} = 64,200 \text{ cm}^{-1}\text{M}^{-1} \)) and adding these values together to obtain the total concentration of TAMRA present in the sample [11]. The concentration of cTnC(T13C/N51C) was determined by measuring the absorbance at 280 nm, adjusting this absorbance by a correction factor based on the total TAMRA concentration, and then dividing this adjusted absorbance by cTnC(T13C/N51C)'s extinction coefficient \( \varepsilon_{280} = 4,470 \text{ cm}^{-1}\text{M}^{-1} \) (via biomol.net's protein extinction coefficient tools). Correction factors for TAMRA absorbance at 280 nm were determined by serially diluting protein free TAMRA and recording its absorbance at 280 nm as a function of peak absorbance (either at 554 nm or 520 nm).

Reconstitutions

Doubly labeled cTnC(T13C/N51C) was reconstituted with 20% excess cTnI to yield the binary complex, or with 20% excess cTnI and cTnT to yield the ternary complex. Inclusion of 20%
excess cTnI and cTnT was done to ensure all cTnC(T13C/N51C) was complexed [17, 21, 22]. Reconstitution was achieved by mixing proteins in the appropriate ratio and gradually reducing the concentration of urea from 6-0 M and KCl from 0.5-0.15 M via dialysis. This was followed by a final dialysis into titration buffer, which consists of 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM nitrilotriacetic acid (NTA), 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 150 mM KCl, 5 mM MgCl2, and 1 mM dithiothreitol (DTT) at pH 7.10. Multiple experiments were performed on reconstituted samples within 5 days, and no protein degradation was observed by electrophoretic analysis over this time period.

Ca\(^{2+}\)-sensitizer preparation

The three Ca\(^{2+}\)-sensitizers used in this study levosimendan (Sigma-Aldrich), bepridil (Sigma-Aldrich) and pimobendan (Merck) were dissolved in enough dimethylformamide (DMF) to make 6 mM drug stock solutions. Stock solutions were kept at 4°C to prevent degradation and were remade every 2 weeks.

Absorbance spectra measurements

Absorbance spectra measurements were conducted at 22 °C on samples containing 1 µM cTnC(13C/51C)\textsubscript{TAMRA2}, cTnI(wt)-cTnC(13C/51C)\textsubscript{TAMRA2}, cTnI(wt)-cTnC(13C/51C)\textsubscript{TAMRA2}-cTnT(wt), or cTnI-cTnC(13C/51C)\textsubscript{TAMRA2}-cTnT(T204E) in titration buffer containing 1.67% DMF by volume. Ca\(^{2+}\) free measurements were performed in Ca\(^{2+}\) free titration buffer, while Ca\(^{2+}\) saturated samples contained 10 mM Ca\(^{2+}\). Drug samples contained 100 µM of their respective Ca\(^{2+}\)-sensitizer while control samples did not. Absorbance readings were obtained for the wavelength range 450-700 nm. All data was saved and exported to Excel for analysis.
Extraction of the fractional abundance (relative to moles TAMRA) of monomer and dimer was performed as described in Absorbance spectra processing.

Absorbance data processing

The absorbance spectra of a mixed population of monomeric and dimeric TAMRA is a weighted average of the absorbance spectra of the pure monomer and pure dimer, with weights equal to the percent abundance of that species in solution. Thus, if one knows the spectra of the pure monomer and pure dimer the fraction of monomer and dimer in a mixed population can be obtained. Spectra for the pure monomer and dimer were obtained from the study by Christie et al.[11], who created a high purity dimeric TAMRA species which dissociates into pure monomer in reducing conditions. The absorbance spectrum of the pure dimer showed an extinction coefficient at 520 nm of 64,200 M-1 cm-1 (relative to moles of TAMRA) with a 554nm/520nm ratio of 0.38, meaning that 38% of the absorbance at the dimer peak wavelength contributes to the absorbance at the monomer peak wavelength. The absorbance spectrum of pure monomer showed an extinction coefficient at 554 nm of 66,100 M-1 cm-1 relative to moles TAMRA, with a 520nm/554nm of 0.42. The absorbance spectra collected in this study were deconvoluted using a method similar to that of West and Pearce [12]. First the peak intensity at 554 nm is assumed to arise entirely from monomeric TAMRA, $A_{554M1} = A_{554}$. This means that the contribution of monomer to the absorbance at 520 nm is $0.42A_{554M1}$. This allows us to make an initial estimate of the absorbance at 520 nm arising from dimer, $A_{520D1} = A_{520} - 0.42A_{554M1}$. This in turn allows us to update our estimate of the monomer contribution to absorbance at 554 nm, $A_{554M2} = A_{554} - 0.38A_{520D1}$. Which allows us to update our estimate of the absorbance at 520 nm arising from dimer, $A_{520D2} = A_{520} - 0.42A_{554M2}$. This process is repeated several more times until the values...
converge, thus giving us the absorbance at 554 nm arising from monomer and the absorbance at 520 nm arising from dimer. Dividing the deconvoluted monomer and dimer peak absorbances by their respective extinction coefficients ($\varepsilon_{554} = 66,100 \text{ cm}^{-1}\text{M}^{-1}$ and $\varepsilon_{520} = 64,200 \text{ cm}^{-1}\text{M}^{-1}$) yields the concentrations of monomeric and dimeric TAMRA in the sample. Finally, dividing the concentration of dimeric TAMRA by the total TAMRA concentration gives the fractional abundance of dimeric TAMRA. The presence of singly labeled cTnC(T13C/N51C) in these samples serves to reduce the apparent fractional abundance of dimeric TAMRA. This is because the singly labeled species is unable to participate in dimerization but gets included when determining the total concentration of TAMRA. This causes the fractional abundance of dimeric TAMRA species to appear less than it actually is. In order to adjust from the apparent to the true fractional abundance of dimer we need to multiply by the adjustment factor shown in equation 1 below, which removes single labeled cTnC's contribution from the total TAMRA concentration.

$$\text{Labeling Ratio Adjustment Factor} = \frac{(\text{Labeling ratio}/2)}{(\text{Labeling ratio} - 1)}$$

Statistical analysis

All data was analyzed via two-way ANOVA using GraphPad Prism 7.02, comparisons were made using TUKEY’s honest significant difference (p < 0.05).

4.4 Results and Discussion

4.4.1 The cTnC(T13C/N51C)TAMRA-TAMRA labeling scheme, a binary reporter

The tendency of TAMRA to form non-covalent homodimers is well documented ([11, 23, 24]. TAMRA dimerization is governed by the reaction shown below.
\[ D \xrightarrow{k_1 \quad k_1} M + M \]  

2) The ratio of the forward and backward rate constants (k_1/k_{-1}) is known as the dissociation constant (K_d) and when the TAMRA concentration in solution is near or above this value, 1.37 \times 10^{-4} \text{ M} for TAMRA [25], the concentration of dimer becomes significant. So long as the bulk concentration of TAMRA is well below the dissociation constant, as in the present study, the expected concentration of dimer is approximately zero. However when two or more TAMRA molecules are labeled to the same protein, and the labeling sites are sufficiently close such that interaction between TAMRA’s xanthene rings can occur, intramolecular dimerization becomes possible [11, 23, 24]. For two TAMRA molecules attached via linkers of length R (14.36 Å in this case via molView) to residues within a single protein the probability of dimerization can be modeled as a function of the separation distance, d, between the labeled residues. To derive this model each TAMRA is considered to freely diffuse in a shell with outer radius R (the maximum linker length) and inner radius r (the minimum linker length, approximately 9.36 Å in this case via molView) centered on the residue to which it is labeled. Thus, within this shell the concentration of TAMRA is \( (1/\text{Avogadro’s number})/V_{\text{shell}} \). When the inter-residue separation distance d is sufficiently small such that overlap between the two shells occurs the volume of this overlap region is given by equation 3.

\[ V_{\text{overlap}} = V_{RR} - 2V_{Rr} + V_{rr} \]  

3) Where \( V_{RR} \) is the intersectional volume of two spheres of radius R with centers separated by distance d. \( V_{Rr} \) is the same thing but with one of the spheres having the minimal linker length r, and finally \( V_{rr} \) is the case for two spheres with radius r. Each of these values can be calculated as a function of the separation distance d according to equation 4.
\[ V_{rr} = \frac{\pi (R + r - d)^2 (d^2 + 2dr - 3r^2 + 2dR + 6rR - 3R^2)}{12d} \]  

The equations for \( V_{RR} \) and \( V_{\pi} \) are simply cases of equation 4 where \( R \) and \( r \) both equal either the maximal or minimal linker length. The total TAMRA concentration within this overlap region is given by equation 5.

\[ [\text{TAMRA}]_{\text{total}} = \frac{2/\text{Avogadro's Number}}{V_{\text{shell}}} \]  

And the total rate of dimer formation within this overlap region is given below.

\[ k_{-1} [M]^2 V_{\text{overlap}} \]  

Because our system consists of only two TAMRA molecules, if one of those molecules is in the monomer state the other must be as well. Thus, in our quantized system there are only two possible rates of dimerization, these are when both molecules of TAMRA are in the monomer state and thus \([M] = [\text{TAMRA}]_{\text{total}}\) or when both are in the dimer state and \([M] = 0\). So, for a large number of such systems the average rate of dimer formation is given by equation 7.

\[ \text{Rate}_{\text{Dimer Formation}} = F(M) k_{-1} [\text{TAMRA}]_{\text{total}}^2 V_{\text{overlap}} \]  

Where

\[ F(M) = \frac{\text{Number of systems in the monomer state}}{\text{Total number of systems}} \]  

The average rate of dissociation for the two TAMRA system can be found in a similar manner, however one key point is that when the system is in the dimer state both TAMRA molecules must be present somewhere in the overlap region. This means that when the system is in the dimer state the dimer concentration in the overlap region is that given in equation 9.
Thus, within the quantized system the total rate of dimer dissociation is either 0 or

\[
\frac{k_D [\text{TAMRA}]_{\text{Total}} \left( \frac{V_{\text{shell}}}{V_{\text{overlap}}} \right)}{2} = \frac{k_D [\text{TAMRA}]_{\text{Total}} V_{\text{shell}}}{2} \tag{9}
\]

As before the average rate of dissociation for many such systems is

\[
\text{Rate}_{\text{Dimer Dissociation}} = F(D) \frac{k_D}{2} [\text{TAMRA}]_{\text{Total}} V_{\text{shell}} \tag{10}
\]

Where

\[
F(D) = \frac{\text{Number of systems in the dimer state}}{\text{Total number of systems}} \tag{11}
\]

Having expressions for the average rates of dimerization and dissociation and knowing that at steady state these rates must equal, we set equations 7 and 11 equal to one another. Knowing that \(F(M)\) and \(F(D)\) must add to one, we can replace \(F(M)\) with \(1-F(D)\). Solving for \(F(D)\) yields the probability of TAMRA being in the dimer state as a function of separation distance between the two labeled residues

\[
F(D) = \frac{1}{1 + \frac{K_d}{2 [\text{TAMRA}]_{\text{Total}} V_{\text{shell}} / V_{\text{overlap}}} \tag{12}
\]

Although equation 13 was derived for the purposes of this study, the result is generally applicable to any case in which a protein or other substrate is double labeled with a dye capable of homodimerization and can be applied so long as the dissociation constant and linker structure
is known. Plugging in the parameters for the specific case of cTnC(T13C/N51C)TAMRA2 and plotting the probability of TAMRA dimerization as a function of 13C-51C separation distance gives figure 1.

![Graph showing dimerization probability as a function of separation distance.](image)

**Figure 1.** The probability of dimerization as a function of 13C-51C separation distance according to the model developed in this study.

As can be seen in figure 1, the probability of dimerization as a function of 13C-51C separation distance predicts binary behavior, with nearly 100% dimerization at distances less than 28.7 Å, and 0% dimerization for distances greater than this. This binary quality of the TAMRA labeling scheme informs interpretation of the following results. Because cTnC residues 13 and 51 lie on opposite sides of the hydrophobic cleft and the distance between these residues is approximately 21.7 Å in the absence of Ca$^{2+}$ (closed state), 23.9 Å in the presence of Ca$^{2+}$ (proposed primed-
closed state), and 28.9 Å in the presence of both Ca$^{2+}$ and cTnI (open state) we can equate dimeric TAMRA with the closed/primed-closed states (13C-51C distances less than 28.7 Å) while monomeric TAMRA corresponds with the open state of the hydrophobic cleft (13C-51C separations greater than 28.7 Å) [9, 14]. If cTnC exists in conformational equilibrium between open and closed/prime-closed conformations this will appear as an equilibrium between monomeric and dimeric TAMRA species, with the relative abundance of monomeric and dimeric TAMRA corresponding to the relative abundance of the open and closed/primed-closed conformations respectively.

4.4.2 Absorbance spectra measurements

Absorbance spectra such as those shown in figure 2, were measured for cTnC(T13C/N51C)$_{TAMRA2}$, cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$, cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(wt), and cTnI(wt)-cTnC(T13C/N51C)-cTnT(T204E) in the presence or absence of saturating Ca$^{2+}$. Each of these treatments was then repeated in the presence of 100 µM levsimendan, pimobendan, or bepridil. All absorbance spectra were deconvoluted to give the fractional abundance of TAMRA in the monomeric and dimeric state and this data was analyzed via two-way ANOVA and Tukey’s honest significant difference (see tables 1 and 2). These results are discussed below.
Figure 2. Representative absorbance spectra collected from Mg$^{2+}$-cTnC(T13C/N51C)$_{\text{TAMRA2}}$ (dimeric TAMRA dominates), Ca$^{2+}$-cTnC(T13C/N51C)$_{\text{TAMRA2}}$ (dimeric and monomeric TAMRA nearly equal), and Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{\text{TAMRA2}}$ (monomeric TAMRA dominates) illustrating how Ca$^{2+}$ and cTnI influence the abundance of monomeric and dimeric TAMRA species which in turn alter the relative absorbance at 554 nm and 520 nm.
Table 1. Percentage of TAMRA population in the dimer state in the absence of Ca$^{2+}$. Values given as mean ± Std. Dev. Drug treated samples with an * superscript differ significantly from the vehicle control for that level of reconstitution and vehicle controls without a common letter superscript differ significantly ($P < 0.05$) as analyzed by two-way ANOVA and the TUKEY test. I, C T, and T(T204E) stand for cardiac troponins I, C, T and T(T204E) respectively, the abbreviations indicate which of the troponins are present at a given reconstitution level. $^tR \times S =$ Reconstitution Level × Sensitizer interaction effect.

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<th></th>
<th>Mg$^{2+}$-cTnC(T13C/N51C)$_{TAMRA2}$</th>
<th>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$</th>
<th>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(wt)</th>
<th>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(T204E)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle ($n = 22$)</td>
<td>Levosimendan ($n = 24$)</td>
<td>Bepridil ($n = 24$)</td>
<td>Pimobendan ($n = 8$)</td>
</tr>
<tr>
<td></td>
<td>85.5 ± 2.0$^a$</td>
<td>85.1 ± 2.3</td>
<td>84.1 ± 1.6</td>
<td>57.8 ± 5.0$^*$</td>
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<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$</td>
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<td>Bepridil ($n = 20$)</td>
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<td></td>
<td>42.4 ± 3.1$^b$</td>
<td>37.1 ± 5.7$^*$</td>
<td>40.7 ± 2.7</td>
<td>26.0 ± 5.3$^*$</td>
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<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(wt)</td>
<td>Vehicle ($n = 30$)</td>
<td>Levosimendan ($n = 22$)</td>
<td>Bepridil ($n = 19$)</td>
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<tr>
<td></td>
<td>54.7 ± 3.5$^c$</td>
<td>50.8 ± 4.3$^*$</td>
<td>52.9 ± 3.1</td>
<td>33.3 ± 2.3$^*$</td>
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<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(T204E)</td>
<td>Vehicle ($n = 28$)</td>
<td>Levosimendan ($n = 18$)</td>
<td>Bepridil ($n = 20$)</td>
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<tr>
<td></td>
<td>56.5 ± 2.9$^c$</td>
<td>51.0 ± 3.0$^*$</td>
<td>53.7 ± 2.1</td>
<td>34.7 ± 4.1$^*$</td>
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$^tP$-value

<table>
<thead>
<tr>
<th>Reconstitution Level</th>
<th>Sensitizer</th>
<th>R×S$^t$</th>
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<tbody>
<tr>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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</table>
Table 2. Percentage of TAMRA population in the dimer state in the presence of Ca\textsuperscript{2+}. Values given as mean ± Std. Dev. Drug treated samples with an * superscript differ significantly from the vehicle control for that level of reconstitution and vehicle controls without a common letter superscript differ significantly (P < 0.05) as analyzed by two-way ANOVA and the TUKEY test. I, C, T, and T(T204E) stand for cardiac troponins I, C, T and T(T204E) respectively, the abbreviations indicate which of the troponins are present at a given reconstitution level. \( R \times S = \) Reconstitution Level × Sensitizer interaction effect.

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+}-cTnC(T13C/N51C)\textsubscript{TAMRA2}</th>
<th>Vehicle ((n = 22))</th>
<th>Levosimendan ((n = 23))</th>
<th>Bepridil ((n = 24))</th>
<th>Pimobendan ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>66.1 ± 2.4\textsuperscript{a}</td>
<td>63.6 ± 3.6</td>
<td>64.1 ± 4.6</td>
<td>41.0 ± 3.1\textsuperscript{*}</td>
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<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+}-cTnI(wt)-cTnC(T13C/N51C)\textsubscript{TAMRA2}</th>
<th>Vehicle ((n = 25))</th>
<th>Levosimendan ((n = 24))</th>
<th>Bepridil ((n = 21))</th>
<th>Pimobendan ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.3 ± 3.9\textsuperscript{b}</td>
<td>25.6 ± 5.2\textsuperscript{*}</td>
<td>27.8 ± 4.8</td>
<td>15.4 ± 2.1\textsuperscript{*}</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+}-cTnI(wt)-cTnC(T13C/N51C)\textsubscript{TAMRA2}-cTnT(wt)</th>
<th>Vehicle ((n = 30))</th>
<th>Levosimendan ((n = 22))</th>
<th>Bepridil ((n = 19))</th>
<th>Pimobendan ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.3 ± 5.8\textsuperscript{b}</td>
<td>30.2 ± 6.0\textsuperscript{*}</td>
<td>29.8 ± 7.9\textsuperscript{*}</td>
<td>15.8 ± 4.2\textsuperscript{*}</td>
<td></td>
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</tbody>
</table>

<table>
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<tr>
<th>Ca\textsuperscript{2+}-cTnI(wt)-cTnC(T13C/N51C)\textsubscript{TAMRA2}-cTnT(T204E)</th>
<th>Vehicle ((n = 28))</th>
<th>Levosimendan ((n = 18))</th>
<th>Bepridil ((n = 20))</th>
<th>Pimobendan ((n = 9))</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.2 ± 5.0\textsuperscript{b}</td>
<td>30.6 ± 4.2</td>
<td>31.0 ± 5.9</td>
<td>19.7 ± 2.7\textsuperscript{*}</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstitution Level</td>
</tr>
<tr>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

4.4.3 Conformational equilibrium in cTnC

Our results show Mg\textsuperscript{2+}-cTnC(T13C/N51C)\textsubscript{TAMRA2} samples contain 85.5% TAMRA dimer. To put it another way, at any given moment 85.5% of the Mg\textsuperscript{2+}-cTnC(T13C/N51C)\textsubscript{TAMRA2} population must be in a closed or primed-closed conformation in order for the two TAMRA molecules to be close enough to dimerize. The 14.5% of the Mg\textsuperscript{2+}-cTnC(T13C/N51C)\textsubscript{TAMRA2} population which contains TAMRA monomers must be in an open conformation. This suggests cTnC is never fully deactivated even in the absence of Ca\textsuperscript{2+}. Although the notion of incomplete deactivation of cTnC has been proposed in various studies [9, 21, 26], this is the first time that
the incomplete deactivation of cTnC has been shown directly. A previous NMR study suggested that if an open population exists in Mg\(^{2+}\)-cTnC that this population must be less than 15%, which is in agreement with our value of 14.5%. Upon addition of saturating Ca\(^{2+}\) to cTnC the dimer population is reduced to 66.1% (monomer abundance increased to 33.9%), thus Ca\(^{2+}\) induces a significant 19.4% decrease in the closed population of cTnC even in the absence of cTnI and cTnT. Our value of 33.9% opening in Ca\(^{2+}\)-cTnC(T13C/N51C)\_TAMRA\_2 is in reasonable agreement with a previous NMR study [8] which showed approximately 27% opening in Ca\(^{2+}\)-cTnC.

### 4.4.4 Effect of cTnI on cTnC's conformational equilibrium

The TAMRA dimer population in Mg\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\_TAMRA\_2 is significantly reduced compared to Mg\(^{2+}\)-cTnC(T13C/N51C)\_TAMRA\_2 (42.4% compared to 85.5%). This drastic reduction in dimer population likely results from two main causes. First, steric clash of cTnI with dimerized TAMRA molecules would disrupt dimer formation. Because the regulatory region of cTnI interacts directly with the hydrophobic cleft of cTnC it must pass through the region where the TAMRA dimer forms. Thus the relatively mobile cTnI can interfere with TAMRA dimer formation and reduce the dimer population without subsequent changes in cTnC's conformation. The second possible cause is a shift in the conformational equilibrium in cTnC induced by the presence of cTnI either through direct binding to cTnC's hydrophobic cleft or via alteration in the relative stabilities of cTnC's closed and open conformations. Unfortunately the approach used in this study is unable to directly determine the contributions of each effect. However, by comparing our results to FRET distance measurements taken in a previous study by Robinson et al. we can obtain some insight [9]. In our study, addition of saturating Ca\(^{2+}\) to cTnC induces a 19.4% increase in TAMRA monomer, in Robinson's study this same transition resulted in a 2.2
Å increase in distance between the labeled residues 12 and 51 in cTnC, thus we obtain a conversion factor of 8.82 %/Å with which we can convert between FRET distance change and expected TAMRA population change. In Robinson’s study addition of cTnI to Mg\(^{2+}\)-cTnC caused a relatively small 0.6 Å increase in FRET distance, using our conversion factor we would expect a 5.3% increase in the monomer population. Our data however shows an increase of 43.1% in the monomer population. Obviously addition of cTnI to Mg\(^{2+}\)-cTnC(T13C/N51C)\(_{\text{TAMRA2}}\) has a much larger impact on the TAMRA dimer/monomer equilibrium than would be expected based on FRET structural data. This discrepancy between our results and FRET structural results is explained by the fact that FRET distance values will be unaffected by steric clash with cTnI, while TAMRA dimerization will be inhibited by the presence of cTnI. We suggest that Mg\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\(_{\text{TAMRA2}}\) most likely exists in the closed state approximately 80.2% of the time, but that steric clash with cTnI reduces the TAMRA dimer population by an additional 37.8%. Finally, according to Robinson’s FRET structural data the addition of Ca\(^{2+}\) and cTnI causes between a 6.6 Å - 7.2 Å opening in cTnC’s hydrophobic cleft, using our conversion factor this corresponds to an expected dimer population in Ca\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\(_{\text{TAMRA2}}\) of 27.3% - 22.0% which is in good agreement with our measured dimer population of 31.3%. This implies that Ca\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\(_{\text{TAMRA2}}\) does not suffer from significant steric clash induced dimer disruption. This makes sense because in the presence of Ca\(^{2+}\) cTnI will spend much of its time bound to cTnC, greatly reducing probability of a cTnI collision with TAMRA dimer. In summary, we suggest Ca\(^{2+}\) induces an approximate 48.9% increase in the open conformation of cTnC when in the presence of cTnI going from approximately 80.2% dimer in the Mg\(^{2+}\) state to 31.3% dimer in the Ca\(^{2+}\) state.
4.4.5 Effect of cTnT and cTnT(T204E) on cTnC's conformational equilibrium

Incorporation of cTnT(wt) or cTnT(T204E) into the troponin complex in the Mg\(^{2+}\) state resulted in an increase in dimer population compared to Mg\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\_TAMRA\_2 samples. In the presence of Ca\(^{2+}\), cTnT(wt) and cTnT(T204E) also increased dimer population compared to Ca\(^{2+}\)-cTnI(wt)-cTnC(T13C/N51C)\_TAMRA\_2 samples but the shift was smaller. From FRET distance measurements between residues 13 and 51 in cTnC we do not see significant structural changes in the hydrophobic cleft induced by cTnT in the Mg\(^{2+}\) state, which implies cTnT is not altering cTnC structure or equilibrium, but is instead affecting cTnI's rate of collision with cTnC and thereby the TAMRA dimer [9, 14]. Thus, we conclude that cTnT(wt) and cTnT(T204E) increase the TAMRA dimer population in cTnC by reducing the ability of cTnI to collide with cTnC. The reason cTnT(wt) and cTnT(T204E) increase dimer population more in the Mg\(^{2+}\) state than in the Ca\(^{2+}\) state is because the Mg\(^{2+}\) state suffers from significant steric clash induced dimer disruption while the Ca\(^{2+}\) state does not, and since cTnT appears to reduce this steric clash it will naturally have a larger effect in the Mg\(^{2+}\) state than in the Ca\(^{2+}\) state. The reduced collisional rate of cTnI with cTnC in the presence of cTnT likely results from formation of the IT arm, a rigid structure which spatially restricts cTnI’s movements.

Reduction in the rate of collision between cTnI’s regulatory region and cTnC’s hydrophobic cleft might be thought to reduce the Ca\(^{2+}\) sensitivity of cTnC, however Ca\(^{2+}\) titrations from a previous study show an increase in Ca\(^{2+}\) sensitivity instead of a reduction upon addition of cTnT(wt) [2]. The most likely explanation is that cTnT scaffolds cTnI via the IT arm which reduces the rate of collision between cTnI and cTnC while simultaneously increasing the population of cTnI bound to cTnC, either by causing cTnI to collide with cTnC in a more optimum orientation for binding,
or by reducing the rate of cTnI dissociation from cTnC’s hydrophobic cleft once bound. Thus even though there are less collisions between cTnI and cTnC in the presence of cTnT the total probability of cTnI being bound to cTnC remains about the same or even increases.

In the absence of Ca$^{2+}$, samples containing cTnT(T204E), a known Ca$^{2+}$ desensitizing phosphorylation mimic, contain more TAMRA dimer than samples containing cTnT(wt) (although this trend is not significant at the 0.05 level). Thus, it appears that cTnT(T204E)’s ability to reduce the collisional rate between cTnI and cTnC is even greater than that of cTnT(wt). As was the case with cTnT(wt), a reduction in collisional rate between cTnI and cTnC by itself would be expected to reduce the Ca$^{2+}$ sensitivity of the troponin complex. In the case of cTnT(wt) this effect is likely mitigated by improvement in the collisional orientation of cTnI and cTnC, or by reduction in the rate of dissociation of cTnI from cTnC once bound, however for cTnT(T204E) it is unlikely that a further improvement in cTnI cTnC binding would occur via either of these mechanisms. Thus we propose that the Ca$^{2+}$ desensitization of the troponin complex induced by cTnT(T204E) occurs at least partially from a reduction in the rate of collision between cTnI and cTnC [3, 27].

4.4.6 Modulation of cTnC’s dynamic equilibria by Ca$^{2+}$ sensitizers

The difference between dimer abundance in vehicle and drug treated samples at the same reconstitution level provides a convenient way to quantify the impact each sensitizer has on cTnC’s dynamic equilibrium. This difference is represented mathematically below.

\[
\text{Difference in dimer population} = D\%_{\text{vehicle}} - (F_{\text{unbound}}D\%_{\text{vehicle}} + F_{\text{bound}}D\%_{\text{drug}})
\]
Where $F_{\text{bound}}$ and $F_{\text{unbound}}$ represent the fractions of drug bound and unbound cTnC respectively and $D\%_{\text{vehicle}}$ and $D\%_{\text{drug}}$ represent the equilibrium population of dimeric TAMRA in the absence of drug bound cTnC and in the presence of fully drug bound cTnC. This expression can be simplified to the following.

$$\text{Difference in dimer population} = F_{\text{bound}}(D\%_{\text{vehicle}} - D\%_{\text{drug}}) \quad (15)$$

The above expression makes it clear that the difference in dimer population between vehicle and drug treated samples depends both on the degree of drug binding as well as the extent to which the drug reduces the dimer population relative to drug unbound cTnC. It should be noted that a reduction in the dimer population in the vehicle sample will result in a reduction in the observed difference between drug treated and untreated samples, even if the behavior of the drug remains the same, a point we will return to shortly.

Tables 3 and 4 show the difference in dimer abundance between vehicle and drug treated samples at each level of reconstitution. As can be seen, all calcium sensitiser containing samples showed reduced dimer abundance at all levels of reconstitution in both the presence and absence of Ca$^{2+}$. This implies all sensitizers tested in this study work by shifting the dynamic equilibrium of cTnC towards the fully open state, which possesses higher Ca$^{2+}$ affinity than closed cTnC. Despite this common mechanism of action each sensitiser showed distinct behavior which will be discussed in depth below.
Table 3. Reduction in dimer population for each sensitizer treated sample compared to vehicle control at each level of reconstitution in the Mg$^{2+}$ state.

<table>
<thead>
<tr>
<th></th>
<th>Levosimendan</th>
<th>Bepridil</th>
<th>Pimobendan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$-cTnC(T13C/N51C)</td>
<td>0.43</td>
<td>1.43</td>
<td>27.69</td>
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<tr>
<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)</td>
<td>5.38</td>
<td>1.77</td>
<td>16.42</td>
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<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)TAMRA2-cTnT(wt)</td>
<td>3.90</td>
<td>1.80</td>
<td>21.34</td>
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<td>Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)TAMRA2-cTnT(T204E)</td>
<td>5.46</td>
<td>2.80</td>
<td>21.80</td>
</tr>
</tbody>
</table>

Table 4. Reduction in dimer population for each sensitizer treated sample compared to vehicle control at each level of reconstitution in the Ca$^{2+}$ state.

<table>
<thead>
<tr>
<th></th>
<th>Levosimendan</th>
<th>Bepridil</th>
<th>Pimobendan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$-cTnC(T13C/N51C)</td>
<td>2.47</td>
<td>2.03</td>
<td>25.09</td>
</tr>
<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)</td>
<td>5.71</td>
<td>3.51</td>
<td>15.8</td>
</tr>
<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)TAMRA2-cTnT(wt)</td>
<td>5.15</td>
<td>5.49</td>
<td>19.49</td>
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<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)TAMRA2-cTnT(T204E)</td>
<td>4.59</td>
<td>4.19</td>
<td>15.43</td>
</tr>
</tbody>
</table>

Levosimendan showed almost no impact on the dimer population of Mg$^{2+}$-cTnC, but showed significant dimer population reduction in the presence of cTnI and/or Ca$^{2+}$. These results imply levsimendan is unable to substantially bind with cTnC in the absence of Ca$^{2+}$ and cTnI at the concentrations tested, likely because the probability of hydrophobic cleft opening is too low. When the probability of hydrophobic cleft opening is increased, either by cTnC binding with Ca$^{2+}$ or cTnI, levsimendan is able to induce a much greater effect on the dynamic equilibrium in
cTnC. The greater than 12 fold increase in dimer population reduction upon inclusion of cTnI suggests cTnI and levosimendan binding is cooperative. There does not appear to be any major influence of cTnT on levosimendan action. Taken together levosimendan appears to amplify the extent of cTnC opening induced by Ca$^{2+}$ or cTnI binding.

Overall bepridil induced the least significant reduction in dimer abundance of the three Ca$^{2+}$ sensitizers tested. Although bepridil displays a slightly larger impact on dimer population in the presence of cTnI, this increase is significantly smaller than that seen for levosimendan. This is most likely because bepridil competes with cTnI for binding to cTnC’s hydrophobic cleft. As seen in levosimendan, bepridil has a significantly larger impact on cTnC’s dynamic equilibrium in the presence of Ca$^{2+}$, this is again attributed to Ca$^{2+}$ increasing the probability of hydrophobic cleft opening, to which bepridil is known to bind [5, 28, 29]. Essentially bepridil displays the same behavior as levosimendan, that is, enhanced impact on cTnC’s dynamic equilibrium in the presence of Ca$^{2+}$ and (to a lesser extent) cTnI.

Pimobendan induced dramatic reduction in cTnC’s dimer population at all levels of reconstitution in both the presence and absence of Ca$^{2+}$. However, a quick observation of the trends in table 3 and 4 shows the opposite of what was observed for levosimendan and bepridil, namely the impact of pimobendan on cTnC’s dynamic equilibrium appears to decrease in the presence of cTnI and Ca$^{2+}$. Recalling equation 15, in the case that drug binding induces nearly full opening of cTnC ($D_{\text{drug}}^{\%} = 0$) then the observed difference between vehicle and drug treated dimer populations will be inversely proportional to the vehicle dimer population.
Difference in dimer population = $F_{bound}D_{\%vehicle}$

This would explain why in the presence of Ca$^{2+}$ the observed impact of pimobendan on cTnC is reduced at every level of reconstitution, a trend which would otherwise elude rationalization. Tables 5 and 6 were generated by dividing the values reported for pimobendan in tables 3 and 4 by $D_{\%vehicle}$ for that reconstitution level. Thus, the values reported in tables 5 and 6 are the percent difference between the dimer populations of vehicle and pimobendan treated samples, and in the case where $D_{\%drug} = 0$ (which seems to be the case for pimobendan) this percent difference is equivalent to the fraction of pimobendan bound cTnC. Looking at tables 5 and 6 the familiar trends of increased sensitizer impact in the presence of Ca$^{2+}$ and cTnI are seen. Thus after accounting for changes in $D_{\%vehicle}$ pimobendan displays increased binding to cTnC in the presence of Ca$^{2+}$ and cTnI, the same general trends observed for levosimendan and bepridil.

**Table 5.** Percent change in dimer population for pimobendan compared to vehicle control at each level of reconstitution in the Mg$^{2+}$ state.

| Mg$^{2+}$-cTnC(T13C/N51C)$_{TAMRA2}$ | Pimobendan | 32.37 |
| Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$ | Pimobendan | 38.69 |
| Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(wt) | Pimobendan | 39.03 |
| Mg$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(T204E) | Pimobendan | 38.61 |
Table 6. Percent change in dimer population for pimobendan compared to vehicle control at each level of reconstitution in the Ca$^{2+}$ state.

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>Pimobendan</th>
<th>Ca$^{2+}$-cTnC(T13C/N51C)$_{TAMRA2}$</th>
<th>37.96</th>
</tr>
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<tbody>
<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$</td>
<td>Pimobendan</td>
<td>50.54</td>
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<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(wt)</td>
<td>Pimobendan</td>
<td>55.19</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$-cTnI(wt)-cTnC(T13C/N51C)$_{TAMRA2}$-cTnT(T204E)</td>
<td>Pimobendan</td>
<td>43.89</td>
<td></td>
</tr>
</tbody>
</table>
4.5 References


