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Interaction of cardiac troponin with cardiotonic drugs:

A structural perspective

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Abstract

Over the forty years since its discovery, many studies have focused on understanding the role of troponin as a myofilament based molecular switch in regulating the Ca^{2+} -dependent activation of striated muscle contraction. Recently, studies have explored the role of cardiac troponin as a target for cardiotonic agents. These drugs are clinically useful for treating heart failure, a condition in which the heart is no longer able to pump enough blood to other organs. These agents act via a mechanism that modulates the Ca^{2+} -sensitivity of troponin; such a mode of action is therapeutically desirable because intracellular Ca^{2+} concentration is not perturbed, preserving the regulation of other Ca^{2+} -based signaling pathways. This review describes molecular details of the interaction of cardiac troponin with a variety of cardiotonic drugs. We present recent structural work that has identified the docking sites of several cardiotonic drugs in the troponin C - troponin I interface and discuss their relevance in the design of troponin based drugs for the treatment of heart disease.

Keywords

cardiac muscle contraction; myofilament; troponin; cardiotonic drugs; calcium sensitizers

Introduction

The Ca^{2+} -dependent regulation of striated muscle contraction by troponin has been a fascinating area for biochemical and biophysical study since its discovery ~ 40 years ago by Professor S. Ebashi. Troponin is a heterotrimeric complex that is comprised of the Ca^{2+} -binding subunit troponin C (TnC), inhibitory subunit troponin I (TnI), and an elongated molecule troponin T (TnT) that binds both TnC and TnI and also to tropomyosin. At sub-micromolecular Ca^{2+} concentrations, the troponin-tropomyosin complex sterically hinders strong, force-producing cross bridges between actin and myosin. At micromolecular concentrations, Ca^{2+} binds to the regulatory domain of TnC, resulting in a cascade of structural changes in troponin and the movement of tropomyosin deeper into the groove of the actin strand, thus revealing actin binding sites for myosin attachment and releasing inhibition. Thus, the interaction of troponin and tropomyosin propagates the regulatory signal along the thin filament and as such acts as a Ca^{2+} -sensitive molecular switch of the thin filament (for reviews on the thin filament regulatory system, see [1-3]).

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Structural studies have contributed a great deal to the understanding of the molecular details of the role of troponin in regulating striated muscle contraction. The first three-dimensional structure of skeletal TnC (sTnC) was solved by X-ray crystallography in 1985 [4,5]. In this structure, the two N-domain (sNTnC) Ca^{2+} -binding sites were unoccupied, while the C-domain (sCTnC) was in two Ca^{2+} -bound state. By comparing the crystal structure of the apo N-domain with its homologous Ca^{2+} -bound C-domain, Herzberg et al. [6] proposed a model for the Ca^{2+} -induced conformational change in sNTnC that involved a 'closed' to 'open' transition accompanied with the exposure of the hydrophobic pocket. The first direct structural evidence describing the Ca^{2+} -induced 'closed' to 'open' conformational change in sNTnC came in 1995 when the NMR solution structures of sTnC in 4Ca^{2+} state [7] and of sNTnC in apo and 2Ca^{2+} states [8] were determined. Subsequently, the X-ray structures of sTnC- 2Ca^{2+} [9] and sTnC- 4Ca^{2+} [10] were reported. These structures provide detailed features of the Ca^{2+} -binding loops that were not as well defined in the NMR structures. The main difference between the X-ray and solution structures of sTnC is in the central linker that connects the N- and C-domains; while a long α -helix in the crystal form, this linker is unstructured and flexible in solution. The response of cardiac TnC (cTnC) to Ca^{2+} -binding was unknown until the determination of the NMR solution structures of cTnC- 3Ca^{2+} [11] and the regulatory domain of cTnC (cNTnC) in both the apo and Ca^{2+} states [12]. Strikingly, cNTnC remains essentially 'closed' in the Ca^{2+} state, unlike sNTnC, a consequence of the defunct site I. The significant reduction in the hydrophobic surface of cNTnC suggested that the mode of interaction between cTnC-cTnI maybe different than that between sTnC-sTnI. However, both the regulatory domains of sTnC and cTnC assume similar 'open' conformations when bound to switch region of sTnI and cTnI regions (sTnI₁₁₅₋₁₃₁ and cTnI₁₄₇₋₁₆₃), respectively [13,14]. This indicates that the pathways involved in initiating skeletal and cardiac muscle contraction are structurally very similar; however, the kinetics and thermodynamics of the pathways must differ for the two systems to account for the different physiological behavior of the two muscle types [15]. NMR studies of TnC with various TnI peptides have yielded detailed structural information on the structure of TnC when bound to TnI [16-19], on the structure of TnI inhibitory peptide [20,21], and on the overall topology of TnC-TnI arrangement [22-32]. The high-resolution structures of TnC-TnI available are the X-ray structure of sTnC- 2Ca^{2+} -sTnI₁₋₄₇ [33], the NMR structures of cNTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃ [13], sNTnC(rhodamine)- 2Ca^{2+} -sTnI₁₁₅₋₁₃₁ [14], and cCTnC- 2Ca^{2+} -cTnI₁₂₈₋₁₄₇ [34], the X-ray structure of the core domain cardiac troponin complex, cTnC- 3Ca^{2+} -cTnI₃₄₋₂₁₀-cTnT₁₈₂₋₂₈₈ [35], and the X-ray structures of skeletal troponin complex in both the apo and Ca^{2+} -state, sTnC-apo-sTnI₁₋₁₈₂-sTnT₁₅₆₋₂₆₂ and sTnC- 4Ca^{2+} -sTnI₁₋₁₈₂-sTnT₁₅₆₋₂₆₂ [36]. In the structure of sTnC- 2Ca^{2+} -sTnI₁₋₄₇, the 31-residue long sTnI α -helix (residues 3-33) stretches on the surface of the sTnC and stabilizes its compact conformation by multiple contacts with both TnC domains [33]. The corresponding region of cTnI (cTnI₃₄₋₇₁) was found to bind to the hydrophobic cleft of the C-domain of cTnC [19] and exhibit a similar conformation and orientation as observed in the structure of cTnC- 3Ca^{2+} -cTnI₃₄₋₂₁₀-cTnT₁₈₂₋₂₈₈ [35]. In the structure of cNTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃, the bound cTnI₁₄₇₋₁₆₃ peptide adopts an α -helical conformation spanning residues 4-12 in the 17-residue peptide. The C-terminus of the peptide is unstructured and the N-terminus of the peptide interacts with the center of the hydrophobic pocket. The most important finding is that the α -helical region interacts with the AB helical interface and stabilizes the opening conformation of cNTnC- Ca^{2+} . The corresponding sTnI₁₁₅₋₁₃₁ peptide adopts a similar structure and a similar mode of interaction with the N-domain of sTnC as observed in the structure of sNTnC(rhodamine)- 2Ca^{2+} -sTnI₁₁₅₋₁₃₁ [14]. The backbone atoms of the cNTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃ structure superimposes to 1.5 Å with the corresponding regions in the cardiac troponin structure. In the structure of cCTnC- 2Ca^{2+} -cTnI₁₂₈₋₁₄₇, residues L134-K139 of cTnI forms a helix and residues R140-R147 adopts an extended conformation. With the helical region interacting with the E and H helices of cCTnC- 2Ca^{2+} , the highly basic R140-R147 region containing R140, R144, R145, and R147 makes potential electrostatic contacts with the acidic residues present on the linker region (beginning of the E-helix) including E94, E95, and E96.

The conformation and orientation of cTnI₁₂₈₋₁₄₇ in this structure are similar to those reported from an electron spin labeling work which shows that a section of the inhibitory region (cTnI₁₂₉₋₁₃₇) displays a helical profile, with the C-terminal residues 139-145 displaying no discernible secondary structure characteristics [37]. While most of the inhibitory region of cTnI is not visualized in the cTnC·3Ca²⁺·cTnI₃₄₋₂₁₀·cTnT₁₈₂₋₂₈₈ structure, the conformation and orientation of the N-terminal and switch regions of cTnI are in good agreement with those observed from the binary complexes. The overall core domain structure (Figure 1A) is dominated by α -helical elements and can be divided into structurally distinct subdomains, denoted as the regulatory head (consisting of the N-domain of cTnC and the switch region of cTnI) and the IT arm (consisting of the C-domain of cTnC, cTnI₄₂₋₁₃₆ and cTnT₂₀₃₋₂₇₁). The subdomains are connected by flexible linkers including the one connecting the two domains of cTnC and the inhibitory region of cTnI. The arrangement of cTnC and cTnI in this structure is antiparallel, with the N-terminal domain of TnI interacting with the C-terminal domain of TnC and *vice versa*. This agrees with the earlier proposal emerged from integrating various biochemical and biophysical data (for a review, see [1]). Two other α -helices of cTnI are observed in this structure, cTnI₁₆₄₋₁₈₈ and cTnI₉₀₋₁₃₆. While cTnI₁₆₄₋₁₈₈ is free of contact with cTnC and cTnT, cTnI₉₀₋₁₃₆ forms a coiled-coil with a portion of the T2 fragment of cTnT, cTnT₂₂₆₋₂₇₁, as predicted previously [38]. Upstream from the coiled-coil, cTnT₂₀₄₋₂₂₀ forms another α -helix (Figure 1A). The N-terminal extension of cTnI is not present in the core domain structure. The overall orientation of the cTnI in this complex would direct the cardiac specific region to extend upwards toward the N-domain of cTnC. Direct interaction of this region of cTnI with cTnC has been demonstrated [28,32,39,40]. This interaction has been reported to influence conformational exchange in the regulatory domain by shifting the equilibrium to favor an open conformation that exposes the hydrophobic cleft. These contacts are disrupted by protein kinase A (PKA) phosphorylation of S22 and S23 and loss of these interactions shifts the conformational equilibrium in cTnC towards a more closed state. A recent study has shown that bisphosphorylation extends and stabilizes the C-terminal helix region of cTnI₁₋₃₂, weakening the interaction with cTnC·Ca²⁺ and bends over so its N-terminal acidic residues make electrostatic contacts with the inhibitory region of cTnI [41]. The recent published 3.0 Å X-ray structure of skeletal troponin complex (Figure 1B) in the Ca²⁺-activated state, sTnC·4Ca²⁺·sTnI₁₋₁₈₂·sTnT₁₅₆₋₂₆₂, and a 7.0 Å structure in the Ca²⁺-free state, sTnC·apo·sTnI₁₋₁₈₂·sTnT₁₅₆₋₂₆₂ [36], have provided mechanistic features that are not found in the cardiac troponin structure. The main difference between the cardiac troponin complex and the skeletal troponin complex resides in the fact that the central helix is preserved in the skeletal version in Ca²⁺ bound state which plays a key role in stabilizing the inhibitory region of sTnI (Figure 1B). Loss of Ca²⁺ causes the rigid central helix of sTnC to collapse and to release the inhibitory region of sTnI [42]. A recent study has shown that sTnI₁₃₉₋₁₈₂ in the sTnC·sTnI·sTnT2 ternary complex constitutes a mobile actin binding domain that tumbles independently of the core domain and that this tumbling is more restricted through the closer contact with the core domain at high Ca²⁺ concentrations [43]. This study also proposed a structure for the mobile domain that consists of two helices connected by a β -bulge [43]. Based on the cardiac core domain structure, Takeda et al [35] proposed a “drag and release” model for cTnC-cTnI interaction. In this model, the movement of the switch region of TnI towards the hydrophobic cleft of N-domain of TnC drags the adjoining inhibitory region from actin causing its release from actin, and the C-terminus of cTnI is carried along with this motion. As such, the C-terminal half of cTnI toggles between cTnC and actin-tropomyosin during the relaxation and contraction cycle and this movement is controlled by the C-domain of cTnC together with the N-domain of cTnI and the IT arm. A similar model based on the structure of skeletal troponin in both the apo and Ca²⁺-saturated states is also proposed [36]. Robinson *et al.* [44] further propose that as cTnI is released from actin-tropomyosin, residues within the inhibitory region switch from a β -turn/coil to an extended quasi- α -helical conformation and the switch region remains α -helical. This is based on FRET measurements of the Ca²⁺-dependent distance change between the inhibitory and the switch regions of cTnI in a cardiac

troponin complex reconstituted in the presence of tropomyosin, actin, and myosin subfragment-1 (S1). An alternative mechanism based on NMR relaxation data for sTnI in the Tn complex [45] proposes that any structure in the C-terminal regions of sTnI (sTnI₁₃₁₋₁₈₂) is nascent, and stabilized only upon binding to actin [46].

It is clear from the structural biology of the troponin complex described above that the interaction of cTnC and cTnI plays a critical role in transmitting the Ca^{2+} -signal to the other myofilament proteins in heart muscle contraction. As such, the cTnC-cTnI interface constitutes a potential target for cardiotonic agents that can modulate the Ca^{2+} -sensitivity of the myofilaments in diseased hearts [47,48]. This approach is considered more beneficial than some others because it avoids an overload of intramolecular Ca^{2+} that would perturb the regulation of other Ca^{2+} -based signaling pathways, leading to a series of undesirable side effects and making the heart less efficient. As a result, a group of Ca^{2+} -sensitizers have been developed. One good example is levosimendan, a novel Ca^{2+} -sensitizer discovered by using cTnC as a target protein [49]. This drug has proved to be a well-tolerated and effective treatment for patients with severe decompensated heart failure (for reviews, see [3,50,51]). High resolution structures of the troponin-drug complexes are limited; however, recent structural studies have identified the docking sites of a group of cardiotonic drugs (Figure 2) in the cTnC-cTnI interface. In this review, we focus on the available structural data describing drug binding to the troponin complex with the goal of delineating the mode of action of different cardiotonic drugs. This analysis will provide a comprehensive molecular insight into the concepts underlining the Ca^{2+} -sensitizing effects of these compounds and the potential of cTnC-cTnI interface as a target for future rational drug design for the treatment of heart disease. We first present the five compounds (bepridil, TFP, levosimendan, anapoe, and W7) that bind to the N-domain followed by EMD 57033 that interacts with the C-domain of cTnC.

Bepridil

Bepridil (Figure 2) is a Ca^{2+} channel blocker and a calmodulin (CaM) antagonist. Early evidence indicated that it is able to enter cardiomyocytes and bind to the thin filament [52]. Studies have demonstrated direct binding of bepridil to cTnC·3 Ca^{2+} with a K_D of $\sim 10\ \mu\text{M}$ and found that it enhances Ca^{2+} binding to the regulatory site of cTnC both free in solution and in skinned fiber bundles [53]. Using stopped flow fluorescence techniques, MacLachlan et al. showed that bepridil slows the Ca^{2+} release from the N-domain of cTnC [54]. An NMR study, using Met residues as markers, identified three bepridil binding sites in cTnC [55]. By monitoring amide chemical shift changes, another NMR study detected only one bepridil binding site ($K_D = \sim 140\ \mu\text{M}$) in the N-domain of cTnC when in complex with cTnI [56]. Thus, it is likely that the interaction of cTnI with cTnC blocks two of the three bepridil sites that may not be functionally relevant. All of these studies agree that binding of bepridil requires site II in cTnC to be occupied by Ca^{2+} . The X-ray structure of cTnC·3 Ca^{2+} ·3bepridil provided detailed structural data on the interaction of cTnC and bepridil [57]. In this structure, two bepridil molecules pull the N- and C-domains together to result in a compact structure for cTnC while a third bepridil appears to stabilize an 'open' cTnC conformation by binding to the center of the hydrophobic pocket, much like the switch region cTnI₁₄₇₋₁₆₃. Interpretation of the structural data in relation to the biochemical and functional data led to the conclusion that the two bepridils located in the hydrophobic cavity between the two cTnC domains would be replaced by cTnI in the troponin complex. The binding of bepridil impedes closure of the N-domain hydrophobic pocket, thereby slowing Ca^{2+} release from site II and enhancing the Ca^{2+} -sensitivity of the myofilament.

The fact that both the switch region of cTnI, cTnI₁₄₇₋₁₆₃, and bepridil can induce a structural 'opening' in cTnC· Ca^{2+} by interacting with the hydrophobic patch prompted us to explore whether cTnI₁₄₇₋₁₆₃ and bepridil compete for cTnC· Ca^{2+} [58]. Using 2D $\{^1\text{H}, ^{15}\text{N}\}$ HSQC

NMR spectroscopy, we examined the binding of bepridil to cTnC- Ca^{2+} in the absence and presence of cTnI₁₄₇₋₁₆₃ and of cTnI₁₄₇₋₁₆₃ to cTnC- Ca^{2+} in the absence and presence of bepridil. The results show that bepridil and cTnI₁₄₇₋₁₆₃ bind to cTnC- Ca^{2+} simultaneously but the affinity of cTnI₁₄₇₋₁₆₃ for cTnC- Ca^{2+} is reduced ~3.5 fold by bepridil and vice versa. This is consistent with a NMR study showing a weaker binding of bepridil to cTnC in the cTnC-cTnI complex than to isolated cTnC [56]. The observed negative cooperativity between bepridil and cTnI for cTnC suggests that the effect of bepridil on muscle fibers may result from a combination of many integrated effects within the myofibrillar lattice. The NMR structure of the cTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃-bepridil ternary complex reveals a binding site for cTnI₁₄₇₋₁₆₃ primarily located on the A/B interhelical interface and a binding site for bepridil in the hydrophobic core of cTnC- Ca^{2+} (Figure 3 and Figure 7A). In the structure, the N-terminus of the peptide clashes with part of the bepridil molecule due to both the steric hindrance and repelling force between the positively charged R147 of cTnI₁₄₇₋₁₆₃ and the partial positive charge of the pyrrolidine group of bepridil, which explained the observed negative cooperativity.

This study has important implications with respect to the design of Ca^{2+} -sensitizers. Figure 3 summarized the effects of Ca^{2+} , cTnI₁₄₇₋₁₆₃, and bepridil on cTnC. Ca^{2+} binding to cTnC induces little structural changes but sets the stage for cTnI₁₄₇₋₁₆₃ binding (A to B). A large 'closed' to 'open' conformational transition occurs in cTnC upon binding bepridil (B to C) or cTnI₁₄₇₋₁₆₃ (B to D). Despite the simultaneous presence of the two ligands that are capable of inducing the opening of cTnC, the domain is, in fact, more closed than if either one of the ligands was complexed to cTnC alone. This is the result of the steric clash between the two ligands. Ideally, a Ca^{2+} -sensitizer would increase the Ca^{2+} -binding affinity of cTnC and enhance the cTnC-cTnI interaction, corresponding to driving the equilibrium to the right (Figure 3, from A to B to C/D to E). Clearly, bepridil itself is not an ideal Ca^{2+} -sensitizer because it decreases the binding affinity of cTnI for cTnC- Ca^{2+} , but its chemical structure could be modified to eliminate the clash with cTnI and stabilize the cTnC-cTnI interaction. The high resolution NMR structure of the cTnC- Ca^{2+} -bepridil-cTnI₁₄₇₋₁₆₃ complex together with the detailed binding mechanism not only contribute to the understanding of the mode of action of bepridil in cardiac troponin but also provide useful information for the design of cardiotonic drugs in general.

Trifluoperazine (TFP)

Like bepridil, the antipsychotic phenothiazine derivative TFP (Figure 2) is a CaM antagonist. TFP was shown to bind TnC in a Ca^{2+} specific manner [59] and its affinity ranges from 10 - 30 μM [60,61]. TFP has been shown to increase the Ca^{2+} affinity of TnC and the Ca^{2+} sensitivity of muscle preparation [61,62]. Using NMR spectroscopy and chemical shift mapping, 3-4 TFP binding sites were identified in cTnC-3 Ca^{2+} [55]. This study suggested that there are two drug binding sites in the N-domain hydrophobic pocket, with one site (demarcated by M45, M60, and M80) conferring Ca^{2+} -sensitizing effects, and the other site (containing M47, M81, and M85) also being capable of coordinating ligand, but being capable of inhibiting the association of cTnC with cTnI or cTnI peptides. This finding is verified in the X-ray structures of cTnC- Ca^{2+} -2TFP in two different crystal forms (pdb code: 1WRK and 1WRL), which are deposited in the protein data bank by Takeda et al. but not yet published. The structure is a dimer with four TFP molecules shared between two cTnC- Ca^{2+} domains. Two TFP molecules fit snugly in the hydrophobic pocket of each cTnC- Ca^{2+} domain with the $-\text{CF}_3$ group of each TFP pointed inwards, towards the hydrophobic cleft (Figure 7B). When compared to the NMR structure of cTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃-bepridil, it appears that one of the TFP molecules would be completely replaced by the cTnI₁₄₇₋₁₆₃ peptide. This is in agreement with the data of Kleerekoper et al. [55].

Levosimendan and analogs

Levosimendan (Figure 2), a pyridazinone derivative, is the most widely studied Ca^{2+} -sensitizer to date. This drug has been proved to be a well-tolerated and effective treatment for patients with severe decompensated heart failure [50] and is currently marketed under the trade name Simdax (Abbott). Levosimendan treatment enhances cardiac efficiency without an increase in oxygen demand associated with enhanced contractility induced by other agents, such as dobutamine, currently in clinical use (see review by Endoh [47]). Levosimendan increases myofilament Ca^{2+} sensitivity by a molecular mechanism that appears to involve direct binding to the N-domain of Ca^{2+} -saturated cTnC [49,63], although this was challenged by a study showing that levosimendan did not bind to cTnC or a cTnC/cTnI complex [64]. A later report has shown that the inability to observe levosimendan binding to cTnC was in part due to sample degradation and the use of a recombinant cTnC having C35 and C84 mutated to serines [63]. It appears that C84 is necessary for the interaction of levosimendan and cTnC. Further more this report shows that levosimendan is capable of binding to both domains of cTnC. The exact location of the docking site of this drug on cTnC remains unclear due to the lack of a high resolution structure of the cTnC-levosimendan complex.

Sorsa *et al.* used NMR chemical shift mapping to demonstrate levosimendan binding to the N-domain of cTnC in the presence of the cTnI peptides, cTnI₃₂₋₇₉ and cTnI₁₂₈₋₁₈₀, corresponding to the N-terminal and the inhibitory-switch regions of cTnI [65]. In the presence of these peptides, no significant levosimendan-induced chemical shift changes were detected in the C-domain of cTnC, indicating that the two levosimendan binding sites to the C-domain is blocked by the cTnI peptides. Levosimendan induced amide chemical shift changes were detected throughout the N-domain of cTnC in the presence of the cTnI peptides. A number of N-domain resonances were either broadened beyond detection or exhibited multiple chemical shifts. A large number of both polar and non-polar residues that exhibit chemical shift changes upon levosimendan binding precluded identification of the levosimendan-binding site on cTnC. Sorsa *et al.* attributed the numerous N-domain chemical shift changes to levosimendan altering either the dynamic equilibrium or the rate of exchange between 'open' and 'closed' N-domain conformations by partially stabilizing defunct Ca^{2+} -site I in cTnC- Ca^{2+} (Figure 4). The authors estimate that the dissociation constant of levosimendan binding to cTnC- 3Ca^{2+} -cTnI₃₂₋₇₉-cTnI₁₂₈₋₁₈₀ is $\geq 200 \mu\text{M}$.

In order to understand the exact location and effect of the pharmacophores of levosimendan on cTnC, a detailed structure-to-activity relationship (SAR) analysis of a group of levosimendan analogs has shown that both the hydrogen-bond donor and acceptor groups (=N-NH-, -CO) on the pyridazinone ring or on the mesoxalonitrile hydrozone moieties of the molecule are important for stabilizing the Ca^{2+} -saturated conformation of cTnC [66]. Using 2D $\{^1\text{H}, ^{15}\text{N}\}$ HSQC NMR spectroscopy, we investigated the binding of levosimendan and four of its analogs (Figure 5) to cTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃ and cTnC- Ca^{2+} -cTnI₁₄₄₋₁₆₃ [67]. Among the derivatives, only those containing the chiral methyl group induced chemical shift changes, suggesting that this group may constitute a key pharmacophore responsible for the binding of pyridazinone derivatives to cTnC- Ca^{2+} . Interestingly, levosimendan only binds cTnC- Ca^{2+} -cTnI₁₄₄₋₁₆₃ but not cTnC- Ca^{2+} -cTnI₁₄₇₋₁₆₃, indicating that in addition to the chiral methyl group, the three residue (RRV) N-terminal extension in cTnI₁₄₄₋₁₆₃ may be essential for stabilizing levosimendan binding to cTnC- Ca^{2+} . Our results suggest that the pyridazinone ring of levosimendan interacts with the center of the hydrophobic pocket toward the AB helical interface while the mesoxalonitrile hydrozone moieties interact with the N-terminus of the cTnI₁₄₄₋₁₆₃ peptide. This network of interactions would create a binding site for levosimendan and the net result is the stabilization of the hydrophobic pocket and the open conformation of cTnC- Ca^{2+} , which could be the rationale for the Ca^{2+} -sensitizing property of levosimendan.

Anapoe

Anapoe is a detergent agent (Figure 2) used to optimize crystal quality of the $sTnC \cdot 4Ca^{2+} \cdot sTnI_{1-182} \cdot sTnT_{156-262}$ complex [36]. In the X-ray structure of $sTnC \cdot 4Ca^{2+} \cdot sTnI_{1-182} \cdot sTnT_{156-262} \cdot anapoe$ (Figure 6), the molecule interacts specifically with the hydrophobic side chains of eight amino residues of $sTnC$ that comprise the pocket and the geometry of the cavity (Figure 7C). Together with three hydrophobic residues (M121, L122, and L125) of the switch region of $sTnI$, the hydrophobic head of anapoe is located in the hydrophobic Met-rich pocket, whereas the polar residues (D119 and R123) of the switch region of $cTnI$ and the polar tail of the detergent molecule extend out of the hydrophobic cavity. This network of interactions contribute to the stabilization of the N-domain hydrophobic pocket, corresponding to the facilitation of the binding of Ca^{2+} to the regulatory sites and the observed enhancement of the contractile force of muscle fibers [36]. The binding site of anapoe on $sTnC$ is very similar to that of bepridil on $cTnC$ in the $cTnC \cdot Ca^{2+} \cdot cTnI_{147-163} \cdot bepridil$ complex (Figure 7A, 7C). In both cases, the small molecule fits into the hydrophobic pocket together with the switch region of TnI . Note that the affinity of anapoe for $sTnC \cdot 4Ca^{2+} \cdot sTnI_{1-182} \cdot sTnT_{156-262}$ ($K_D = \sim 0.64$ mM) is much weaker than that of bepridil for $cTnC \cdot Ca^{2+} \cdot cTnI_{147-163}$ ($K_D = \sim 80$ μ M). This is probably the result of a more extensive hydrophobic contact made by bepridil (two aromatic rings) to $cTnC$ than anapoe (one aromatic ring) to $sTnC$ (Figure 7A, 7C). The position of anapoe in the $sTnC$ - $sTnI$ switch and that of bepridil in the $cTnC$ - $cTnI$ switch should prompt efforts to find a cardiotonic drug that could modulate the Ca^{2+} -sensitive interaction of the regulatory domain of $cTnC$ with the switch region of $cTnI$.

W7

Like bepridil and TFP, another CaM antagonist, W7 [N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide] (Figure 2) has been frequently used to explore a wide range of physiological processes that are mediated by Ca^{2+} - CaM . There is considerable evidence that CaM and its antagonists modulate the Ca^{2+} -signaling pathway in cardiac myocytes. The fact that another Ca^{2+} -binding protein $cTnC$, exists in abundance in cardiac myocytes has prompted studies on the role of W7 in the interplay of troponin- and myosin-based pathways of Ca^{2+} -activation in skeletal and cardiac muscle, which showed that in both skeletal and cardiac muscle fibers, W7 inhibits the maximum ATPase activity and tension development [68]. It is unclear whether this effect is achieved by the direct interaction of W7 and $cTnC$. We have used 2D $\{^1H, ^{15}N\}$ and 2D $\{^1H, ^{13}C\}$ HSQC NMR spectroscopy to monitor W7 binding to $cTnC$. We examined the binding of W7 to the separated N- and C-domains of $cTnC$ [69]. In the titration of $cTnC \cdot 2Ca^{2+}$, the spectral peaks originating from a subset of residues changed nonuniformly, and could not be well-described as single-site binding. A global fit of the $cTnC \cdot 2Ca^{2+}$ titration data to a two-site, sequential binding model yielded a dissociation constant (K_D) of 0.85-0.91 mM for the singly bound state, with the second dissociation constant fit to 3.40-3.65 mM. The titration data for $cTnC \cdot Ca^{2+}$ was globally fit to single-site binding model with a K_D of 0.15-0.30 mM. The data are consistent with W7 binding to each domain's major hydrophobic pocket. Titration of $cTnC \cdot 3Ca^{2+}$ with W7 shows that residues throughout the sequence, including the N- and C-domains and the central linker, are affected [70]. Analysis of the binding stoichiometry and the trajectories of chemical shift changes indicate that W7 binding occurs at multiple sites. To address the issue of whether multiple binding is relevant within the troponin complex, W7 was titrated to a $cTnC$ - $cTnI$ complex; $cTnC \cdot 3Ca^{2+} \cdot cTnI_{34-71} \cdot cTnI_{128-163}$. In the presence of the N-terminal (residues $\sim 34-71$), the inhibitory (residues $\sim 128-147$), and the switch (residues $\sim 147-163$) regions of $cTnI$, W7 induces chemical shift changes only in the N-domain and not in the C-domain or the central linker of $cTnC$. The results indicate that in the presence of $cTnI$, W7 no longer binds to multiple sites of $cTnC$, but instead binds specifically to the N-domain and the binding ($K_D = \sim 0.5$ mM)

can occur together with the switch region of cTnI. Hence, W7 may play a role in directly modulating the Ca^{2+} -sensitivity of the regulatory domain of cTnC and the interaction of the switch region of cTnI and cTnC. We propose that the position of W7 in the cardiac troponin complex is analogous to that of anapoe in the skeletal troponin complex (Figure 6). This is based on the observation that the binding of the switch region of cTnI to the N-domain of cTnC is the same in the X-ray structure of cTnC- 3Ca^{2+} -cTnI₃₁₋₂₁₀-cTnT₁₈₃₋₂₈₈ complex [35] and that of sTnC- 4Ca^{2+} -sTnI₁₋₁₈₂-sTnT₁₅₆₋₂₆₂ complex [36]. This is supported by mapping the residues (G34, G42, G49, T53, E66, G68, G70, D73, and V79) that undergo W7-induced chemical shift changes of cTnC- 3Ca^{2+} -cTnI₃₄₋₇₁-cTnI₁₂₈₋₁₆₃ on the X-ray structure of sTnC- 4Ca^{2+} -sTnI₁₋₁₈₂-sTnT₁₅₆₋₂₆₂ complex (Figure 6). These residues are conserved from human cTnC to chicken sTnC based on primary sequence alignment and they cluster around the binding site of anapoe (Figure 6). This is also supported by the comparable affinities of W7 binding to cTnC- 3Ca^{2+} -cTnI₃₄₋₇₁-cTnI₁₂₈₋₁₆₃ ($K_D = \sim 0.5$ mM) and anapoe binding to sTnC- 4Ca^{2+} -sTnI₁₋₁₈₂-sTnT₁₅₆₋₂₆₂ (K_D of ~ 0.64 mM). Thus, the biologically relevant binding site of W7 is in the N-domain of cTnC and it may play a role in directly modulating the Ca^{2+} -sensitivity of the regulatory domain of cTnC and the interaction of the switch region of cTnI and cTnC in cardiac myocytes.

EMD 57033

EMD 57033 (Figure 2) is a thiadiazinone derivative. It has been found to increase the Ca^{2+} -sensitivity of both myofibrillar ATPase and force development by skinned muscle fibers [71, 72]. *In vivo* studies have also demonstrated that EMD 57033 enhances cardiac contractile function without affecting Ca^{2+} -homeostasis [73-77]. The myofibrillar Ca^{2+} -sensitizing effect of EMD 57033 is stereo-specific. EMD 57033 is the (+)-enantiomer of a racemate. The (-)-enantiomer, EMD 57439, exhibits no Ca^{2+} -sensitizing activity but acts as a pure phosphodiesterase III inhibitor [71,72]. The drawback in the development of EMD 57033 as a Ca^{2+} -sensitizer is because of observations that whereas treatment with EMD 57033 results in consistent positive inotropic effects, its effect on relaxation is impaired in some heart muscle preparations [72,78]. However, Tsutsui *et al* showed that EMD 57033 increased contractility of isolated myocytes, studied at 37°C (close to human body temperature), from hearts of control dogs and dogs with induced heart failure with no effect on diastolic cell length or mechanical dynamics [77]. Studies in conscious dogs with and without induced heart failure showed a dramatic effect of EMD 57033 on contractility in both control and failed hearts with improvement of mechanical efficiency and without compromise of diastolic function [74]. Furthermore, studies with open chest pig hearts stressed by a stunning protocol, also demonstrated an improvement of contractility with improved mechanical efficiency [79]. A related study has shown that EMD 57033 is potentially effective in acutely restoring systolic function in a mouse model harboring a proteolytic truncation of cTnI, cTnI₁₋₁₉₃, associated with myocardial stunning [80]. Taken together, these studies indicate that the potential undesirable effects of EMD 57033, such as diastolic abnormalities and impaired relaxation, may not be a problem after all.

Recently, several key studies have provided new insights in the understanding of the molecular mechanism underlining the Ca^{2+} -sensitizing effects of EMD 57033. Evidence that EMD 57033 binds to the C-domain of cTnC has come from both direct binding measurements [81] and NMR chemical shift changes [64,82]. This indicates that EMD 57033 has a different mechanism of action than bepridil, TFP, W7, anapoe, and levosimendan. With EMD 57033, there appears to be an amplification of the hydrophobic surface in the C-domain of cTnC that modifies the interaction of cTnC with cTnI rather than an increase in Ca^{2+} -binding to the regulatory domain of cTnC. Measurement of the NMR spectra in the presence of cTnI peptide indicates a competition between cTnI₃₄₋₇₁ and EMD 57033, whereas the inhibitory region cTnI₁₂₈₋₁₄₇ did not share a common binding site with EMD 57033 [83]. Thus a straightforward

mechanism for the Ca^{2+} -sensitizing effect of EMD 57033 is that this drug disrupts and therefore weakens the interaction of cTnI₃₄₋₇₁ with cTnC in the myofilaments, and consequently, enhances the binding of the C-terminal region of cTnI to cTnC. Since this interaction is Ca^{2+} -dependent, the apparent Ca^{2+} -sensitivity of the contractile system is enhanced. This hypothesis is supported by a study showing that EMD 57033 can restore the Ca^{2+} -sensitivity of myofilaments containing pseudophosphorylated cTnI, cTnI-S41E-S43E and a familiar hypertrophic cardiomyopathy (FHC) mutant α -tropomyosin (E1-0G); both of the modifications surround the C-domain of cTnC [84]. In the high resolution structure of cTnC·2 Ca^{2+} ·EMD 57033 (Figure 7D), the drug molecule is orientated such that the chiral group of EMD 57033 fits deep in the hydrophobic pocket and makes several key contacts with the protein [83]. This stereo-specific interaction explains why the (-)-enantiomer of EMD 57033 is inactive. Since the methyl group attached to the chiral carbon of EMD 57033 makes extensive contacts with methyl groups of I112, L117, and I148, located on the β -sheet connecting the two Ca^{2+} binding sites, these contacts would be weakened or lost if the stereo-specificity of the chiral carbon is changed. Support of this hypothesis comes from the data showing that the (-)-enantiomer, EMD 57439, induces no chemical shift changes of cTnC·2 Ca^{2+} , suggesting a lack of interaction between the two (Wang and Sykes, unpublished data).

Structural comparison

Based on the structural analysis of cTnC· Ca^{2+} ·cTnI₁₄₇₋₁₆₃·bepridil, cTnC· Ca^{2+} ·2TFP, sTnC·4 Ca^{2+} ·sTnI₁₋₁₈₂·sTnT₁₅₆₋₂₆₂·anapoe, and cTnC·2 Ca^{2+} ·EMD57033, and the proposed mechanisms for the interaction of levosimendan and W7 with cTnC, it seems that cardiotonic drugs modulate the Ca^{2+} -response of troponin *via* two molecular mechanisms. One is binding directly to the regulatory domain (*e.g.* bepridil, TFP, levosimendan, W7, and anapoe) and together with the switch region of cTnI, exerting its effect by altering the dynamic equilibrium between 'closed' and 'open' cTnC conformations. The other is binding to the C-domain (*e.g.* EMD 57033) of cTnC and exerting its effect by modulating the Ca^{2+} -dependent cTnC-cTnI interaction and thereby the apparent Ca^{2+} -sensitivity of the contractile system. One way to understand the differences between the two mechanisms is to analyze and compare the binding pockets between different drugs (Figure 7). For the N-domain mechanism, the common binding site is in the hydrophobic pocket but each molecule adjusts its orientation on the hydrophobic surface to accommodate the switch region of cTnI. For the C-domain mechanism, the drug molecule fits deep in the hydrophobic cavity. This suggests that the selectivity of cTnC for cardiotonic drugs arise from the combination of a group of specific amino acids that can form a binding pocket and the geometry of the cavity. Kinetics and thermodynamics play an important role in fine-tuning the binding of these compounds in the cTnC-cTnI interface. Chemical modifications of these compounds to suit the binding cavity on the protein are potentially useful for the design of an ideal cardiotonic drug that can be used to enhance cTnC-cTnI interaction and thereby enhance cardiac contractility.

Perspective

Despite many medical advances, heart disease remains the leading cause of morbidity and mortality in western society. The search for novel approaches to attenuate this disease is presently receiving a long-needed boost with the development of cardiotonic agents (*e.g.* levosimendan) that can enhance cardiac contractility without requiring increased levels of intracellular Ca^{2+} . The data summarized here show that potential cardiotonic drugs target cardiac troponin and the mode of binding in cTnC-cTnI interface is similar but the pose in the hydrophobic pocket is different for different compounds. These structures not only illuminate the mechanism of the cardiotonic effects of these agents on cardiac troponin but also suggest new directions for future rational drug design in the therapy of heart disease. The structural data describing the interaction of cardiac troponin with cardiotonic drugs are essential for the

interpretation and understanding of the pathological, physiological, and pharmacological data. The information can of course be exploited to develop possible therapeutic intervention and diagnostics for the treatment of heart disease.

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Figure 1. Ribbon diagrams representing the X-ray structures of (A) the cTnC·3 Ca^{2+} ·cTnI₃₁₋₂₁₀·cTnT₁₈₃₋₂₈₈ complex (PDB 1J1E), and (B) the sTnC·4 Ca^{2+} ·sTnI₁₋₁₈₂·sTnT₁₅₆₋₂₆₂ complex (PDB 1YTZ). The protein subunits are color-coded as follows: green, TnC; cyan, TnI; and magenta, TnT. Red spheres indicate Ca^{2+} .

Figure 2.
The chemical structures of bepridil, TFP, levosimendan, W7, anapoe, and EMD 57033.

Figure 3.

An overview of the mechanism of and affinities for Ca^{2+} , bepridil, and the cTnI₁₄₇₋₁₆₃ peptide binding to cTnI. A, cTnI-apo; B, cTnI- Ca^{2+} ; C, cTnI- Ca^{2+} -bepridil; D, cTnI- Ca^{2+} -cTnI₁₄₇₋₁₆₃; E, cTnI- Ca^{2+} -cTnI₁₄₇₋₁₆₃-bepridil. The figure was adapted from Wang et al. [58].

Figure 4.

A proposed mechanism of the effects of levosimendan on the N-domain of cTnC. The figure was adapted from Li et al. [3].

Figure 5.
Levosimendan and four analogs

Figure 6.

Ribbon representation of the X-ray structure of the sTnC·4Ca²⁺·sTnI₁₋₁₈₂·sTnT₁₅₆₋₂₆₂·anapoe complex (PDB 1YTZ). The protein subunits are color-coded as follows: red, sTnC; blue, sTnI; and yellow, sTnT. Pink spheres indicate Ca²⁺. The residues corresponding to the cTnC residues G34, G42, G49, T53, E66, G68, G70, D73, and V79 are colored green. The figure was adapted from Li et al. [70].

Figure 7.

Structural comparison of several drug cNTnC·Ca²⁺ complexes, showing ribbon and surface representations of (A) the NMR structure of cNTnC·Ca²⁺·cTnI₁₄₇₋₁₆₃·bepridil (B) the X-ray structure of cNTnC·Ca²⁺·2TFP, (C) the X-ray structure of sNTnC·2Ca²⁺·sTnI₁₁₅₋₁₃₁·anapoe, and (D) the NMR structure of cCTnC·2Ca²⁺·EMD57033.