


RESEARCH ARTICLE

3-Chlorodiphenylamine activates cardiac troponin by a mechanism distinct from bepridil or TFP

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Despite extensive efforts spanning multiple decades, the development of highly effective Ca^{2+} sensitizers for the heart remains an elusive goal. Existing Ca^{2+} sensitizers have other targets in addition to cardiac troponin (cTn), which can lead to adverse side effects, such as hypotension or arrhythmias. Thus, there is a need to design Ca^{2+} -sensitizing drugs with higher affinity and selectivity for cTn. Previously, we determined that many compounds based on diphenylamine (DPA) were able to bind to a cTnC–cTnI chimera with moderate affinity ($K_d \sim 10\text{--}120 \mu\text{M}$). Of these compounds, 3-chlorodiphenylamine (3-Cl-DPA) bound most tightly (K_d of $10 \mu\text{M}$). Here, we investigate 3-Cl-DPA further and find that it increases the Ca^{2+} sensitivity of force development in skinned cardiac muscle. Using NMR, we show that, like the known Ca^{2+} sensitizers, trifluoperazine (TFP) and bepridil, 3-Cl-DPA is able to bind to the isolated N-terminal domain (N-domain) of cTnC (K_d of $6 \mu\text{M}$). However, while the bulky molecules of TFP and bepridil stabilize the open state of the N-domain of cTnC, the small and flexible 3-Cl-DPA molecule is able to bind without stabilizing this open state. Thus, unlike TFP, which drastically slows the rate of Ca^{2+} dissociation from the N-domain of isolated cTnC in a dose-dependent manner, 3-Cl-DPA has no effect on the rate of Ca^{2+} dissociation. On the other hand, the affinity of 3-Cl-DPA for a cTnC–cTnI chimera is at least an order of magnitude higher than that of TFP or bepridil, likely because 3-Cl-DPA is less disruptive of cTnI binding to cTnC. Therefore, 3-Cl-DPA has a bigger effect on the rate of Ca^{2+} dissociation from the entire cTn complex than TFP and bepridil. Our data suggest that 3-Cl-DPA activates the cTn complex via a unique mechanism and could be a suitable scaffold for the development of novel treatments for systolic heart failure.

Introduction

Heart failure occurs when the heart is unable to pump enough blood to meet the demands of the body (Erdmann, 1998; Kemp and Conte, 2012; Johnson, 2014). In the United States, over six million adults are living with heart failure, with the number of affected individuals projected to rise to over eight million by the year 2030 (Benjamin et al., 2018). Heart failure patients face poor long-term outcomes, with $\sim 50\%$ and 10% survival rates at 5 and 10 yr after diagnosis, respectively (Roger, 2013). In systolic heart failure, contraction of cardiac muscle is impaired, leading to a reduced ejection fraction (Chatterjee, 2012; Komamura, 2013). One way to enhance the strength of cardiac muscle contraction is to increase the responsiveness of the contractile apparatus to Ca^{2+} (Kass and Solaro, 2006; Davis et al., 2016). Thus, compounds that sensitize the contractile apparatus to Ca^{2+} are expected to be beneficial in the treatment of systolic heart failure.

Because of its key role in the regulation of muscle contraction, cardiac troponin C (cTnC) represents a logical target for the development of positive inotropic drugs (Kass and Solaro, 2006; Endoh, 2008; Li and Hwang, 2015). In fact, increasing Ca^{2+} sensitivity of the regulatory N-domain of cTnC by mutagenesis resulted in improved heart function, without adverse side effects, in a model of heart failure caused by myocardial infarction (Shettigar et al., 2016). However, despite extensive efforts, development of highly effective Ca^{2+} -sensitizing compounds has remained elusive. Currently, two Ca^{2+} -sensitizing compounds, levosimendan and pimobendan, are being used in clinical practice to treat heart failure in humans, albeit not in the United States (Lehmann et al., 2003; Kass and Solaro, 2006; Pollesello et al., 2016). One of these compounds, pimobendan, has been approved in the United States and worldwide to treat heart failure in dogs (Boyle and Leech, 2012). Both

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levosimendan and pimobendan are believed to elicit their positive inotropic effect through sensitization of the regulatory N-domain of cTnC to Ca^{2+} (Fujino et al., 1988; Pollesello et al., 1994). However, both of these compounds have molecular targets other than cTnC, potentially leading to undesirable side effects, such as hypotension (Hwang and Sykes, 2015; Pollesello et al., 2016).

Many compounds with Ca^{2+} -sensitizing properties have not entered clinical practice for use in heart failure patients for various reasons (Li et al., 2008). For instance, use of positive inotropes such as trifluoperazine (TFP) and bepridil in the treatment of heart failure is prevented by their off-target actions (Li et al., 2008). Although these compounds are not used clinically to treat heart failure, studies of interactions between these molecules and cTnC provide insight into features important for Ca^{2+} sensitization. A crystal structure suggests that the Ca^{2+} -sensitizing effect of bepridil arises from the ability of the molecule to bind into the hydrophobic cavity in the regulatory N-domain of cTnC, stabilizing the fully open state (Li et al., 2000). However, binding of bepridil into the hydrophobic pocket of cTnC competes with binding of cTnI (Wang et al., 2002), diminishing the overall cTn-activating effect. The ability of cTnC to bind the switch peptide of cTnI is a critical determinant of the overall Ca^{2+} sensitivity of the contractile apparatus (Siddiqui et al., 2016).

The ultimate goal of our research is to develop effective treatments for cardiovascular diseases. Recently, we determined that despite their small size, many compounds based on diphenylamine were able to bind moderately well ($K_d \sim 10\text{--}120 \mu\text{M}$) to a chimeric protein consisting of the regulatory N-domain of cTnC (cNTnC) and the switch region of cTnI (cNTnC-cSp chimera), with 3-chlorodiphenylamine (3-Cl-DPA) binding most tightly (K_d of $10 \mu\text{M}$; Cai et al., 2016). The goal of this work was to determine whether 3-Cl-DPA is able to sensitize cardiac muscle to Ca^{2+} and, if so, to explore the molecular mechanism of its action compared with that of bepridil and TFP, compounds known to sensitize the N-domain of isolated cTnC to Ca^{2+} (Li et al., 2008).

Materials and methods

Preparation of proteins for fluorescence studies

The recombinant human cTnC^{T53C} protein (with C35S and C84S substitutions) was generated, expressed, purified, and labeled with 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS) as previously described (Davis et al., 2007). The recombinant human cTnI and cTnT proteins were expressed and purified as previously described (Davis et al., 2007). Reconstitution of the cTn complex was performed as previously described (Davis et al., 2007).

Measurements of force versus pCa ($-\log [\text{Ca}^{2+}]$) in skinned ventricular trabeculae

The care and use of animals in this study were in accordance with a protocol approved by The Ohio State University Institutional Animal Care and Use Committee. The hearts of four female Wistar rats (3–5 months old) were rapidly isolated after induction of a deep level of anesthesia with isoflurane and placed in cold relaxing solution. A single cut was made

through the free wall of the right and left ventricles, and the hearts were soaked in 1% Triton X-100 for 30 min, with occasional manual swirling, to permeabilize the trabeculae on the endocardial surface. The trabeculae were studied as previously described (McConnell et al., 2015). Briefly, a trabecula was mounted in a temperature-controlled experimental chamber that was maintained at 15°C . The chamber was placed on the stage of an upright microscope (Labophot; Nikon). The trabecula was connected at one end to a servo-controlled torque motor (model 322C; Aurora Scientific) and at the other end to an isometric force transducer (model 403; Aurora Scientific). The motor and transducer were attached to three-way positioners, and the length of the trabecula was set by moving the motor or transducer to set the resting striation spacing (equated with sarcomere length). Striation spacing was determined using a digital camera that was mounted on the microscope and image analysis software. The distance spanned by ~ 20 striations was measured to calculate resting sarcomere length. Fiber width and depth were measured and fiber cross-sectional area was calculated, assuming an ellipsoidal cross section. The average resting sarcomere length of all 13 trabeculae was set to $2.12 \pm 0.03 \mu\text{m}$. The force versus pCa relationship was studied in ten trabeculae, first without, then with $100 \mu\text{M}$ 3-Cl-DPA (100 mM stock dissolved in DMSO). The force versus pCa relationship was also studied in three trabeculae, first without and then with $1 \mu\text{l/ml}$ DMSO. The trabecula was exposed to a series of pCa solutions with complete relaxation (in pCa 9.0 solution) between each activation. Resting force was measured (in pCa 9.0 solution) and was subtracted from the total peak force during each activation to calculate peak active force. Every third activation was performed with pCa 4.0 solution, and the force in that solution was used to normalize the force during each temporally neighboring submaximal activation. All of the solutions (preactivating, activating, and relaxing) that were used for a series of measurements (with or without 3-Cl-DPA and DMSO) were identical with respect to the concentration of 3-Cl-DPA and DMSO, so that the only variation was the pCa of the activating solution. All of the measurements were made at pH 7.0 and 15°C . The composition and preparation of the solutions was as previously described (Reiser et al., 2013). The force versus pCa data were fit with the logistic sigmoid function, mathematically equivalent to the Hill equation, as previously described (Black et al., 2000; Tikunova et al., 2002).

Determination of Ca^{2+} -binding sensitivities

All steady-state fluorescence measurements were performed using a Perkin-Elmer LS55 fluorescence spectrometer at 15°C . IAANS fluorescence was excited at 330 nm and monitored at 445 nm as microliter amounts of CaCl_2 were added to 2 ml of IAANS-labeled cTnC^{T53C} reconstituted into $0.15 \mu\text{M}$ cTn complex, in the absence or presence of increasing concentrations of 3-Cl-DPA or bepridil, in titration buffer (200 mM MOPS [to prevent pH changes upon addition of Ca^{2+}], 150 mM KCl, 2 mM EGTA, 3 mM MgCl_2 , and 0.025% Tween-80, pH 7.0). Tween-80 was excluded from the titration buffer for experiments performed in the presence of 3-Cl-DPA. Free $[\text{Ca}^{2+}]$ was calculated using a program (EGCA02) developed by Robertson and Potter (1984). Each

reported pCa_{50} represents an average of at least three titrations \pm SEM. The data were fit with a logistic sigmoid function, mathematically equivalent to the Hill equation, as previously described (Black et al., 2000; Tikunova et al., 2002).

Determination of Ca^{2+} dissociation rates

All kinetic measurements were performed using an Applied Photophysics model SX.18MV stopped-flow apparatus with a dead time of ~ 1.4 ms. IAANS fluorescence was excited at 330 nm with emission monitored through a 420–470 nm band-pass interference filter (Oriel). 10 mM EGTA in a stopped-flow buffer (10 mM MOPS and 150 mM KCl, pH 7.0) was used to remove 500 μ M Ca^{2+} from IAANS-labeled cTnC^{T53C} in isolation (1 μ M) or reconstituted into the cTn complex (0.3 μ M), in the absence or presence of compounds, in the stopped-flow buffer at 15°C. Increasing concentration of compounds were individually added to both stopped-flow reactants. The data were fit using a program by P. J. King (Applied Photophysics) that utilizes the nonlinear Levenberg–Marquardt algorithm. Each k_{off} represents an average of at least three separate experiments \pm SEM, each averaging at least five shots, which were fit with a single exponential equation.

NMR titration of cTnC and cNTnC–cSp chimera with 3-Cl-DPA, TFP, and bepridil

Sample preparation

Recombinant human cTnC, with the mutations C35S and C84S, was used for these experiments. The expression and purification of [^{15}N]-cTnC and [^{15}N]-cNTnC–cSp chimera (also containing C35S and C84S mutations) in *Escherichia coli* were as described previously (Cai et al., 2016). In this construct, the N-domain of cTnC with C35S, C84S substitutions (residues 1–90) is fused to cTnI_{136–163}. The cNTnC–cSp chimera also contains a C-terminal Gly-His₆ tag to aid purification. The full amino acid sequence of the cNTnC–cSp chimera was reported previously (Cai et al., 2016).

For each NMR sample, solid [^{15}N]-cTnC or cNTnC–cSp chimera was dissolved into 500 μ l NMR buffer containing 100 mM KCl, 10 mM imidazole in 90% H₂O/10% D₂O, and 0.5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid as chemical shift standard. Protein concentration was determined by integrating 1D 1H and 2D [1H , ^{15}N] heteronuclear single quantum coherence (HSQC) NMR spectral signals. For titrations into [^{15}N]-cTnC, the starting protein concentration was 0.48 mM. For titrations into [^{15}N]-cNTnC–cSp chimera, the starting protein concentration was 0.11 mM. To each sample, 5 μ l of 1 M $CaCl_2$ was added to ensure that the protein was Ca^{2+} saturated and the pH was adjusted with 1 M NaOH or 1 M HCl to 6.7. 1H , ^{15}N -NMR experiments are usually performed in slightly acidic conditions, because this slows the solvent hydrogen exchange rate of unprotected backbone amides so that they can be observed by NMR (Bai et al., 1993).

3-Cl-DPA was purchased from Toronto Research Chemicals. TFP and bepridil were purchased from Sigma. The compounds are insoluble in aqueous solutions and were thus dissolved in d_6 -DMSO to make stock solutions (97.2 mM 3-Cl-DPA, 67 mM TFP, and 67 mM bepridil), with concentrations determined by integrating 1D 1H NMR spectrum using 4,4-dimethyl-4-silapentane-1-sulfonic acid as an internal standard. The purity and structure of the compounds were verified by 1D 1H NMR

spectroscopy. The vials containing the compound solutions were wrapped in aluminum foil to protect the molecules from light-catalyzed degradation. To a 500- μ l NMR sample, aliquots of 0.2–10 μ l compound stock solution were added for each titration point. The sample was mixed thoroughly with each addition. Total volume increases were taken into account for data analyses. Both 1D 1H and 2D [1H , ^{15}N]-HSQC spectra were acquired at every titration point.

NMR spectroscopy

All NMR experiments were run on a Varian Inova 500-MHz spectrometer. All data were collected at 30°C. Both 1D 1H and 2D [1H , ^{15}N]-HSQC spectra were acquired at every titration point. The spectrometer is equipped with a triple resonance 1H , ^{13}C , ^{15}N probe and z -pulsed field gradients.

K_d values were calculated from NMR-based compound titrations. Amide NMR signals that showed large and linear perturbations during a titration were chosen for K_d calculation. For each individual amide signal, chemical shift changes were plotted as a function of ligand-to-protein ratio and fit to a 1:1 binding stoichiometry (protein + compound \leftrightarrow protein • compound) using xcrvfit, which fully accounts for protein concentration, ligand concentration, and dilution effects (www.bionmr.ualberta.ca/bds/software/xcrvfit). This yielded a K_d value for each amino acid residue analyzed that was then averaged to a single value \pm SEM.

Statistical analysis

Statistical significance was determined by a paired or unpaired two-sample t test (wherever applicable), using the statistical analysis software Minitab. The two means were considered to be significantly different when the P value was <0.05 . All data are reported as mean \pm SEM.

Results

Effect of 3-Cl-DPA on the Ca^{2+} sensitivity of force development in skinned ventricular trabeculae

Previously, we determined that 3-Cl-DPA was able to bind to the cNTnC–cSp chimera with an affinity of 10 μ M (Cai et al., 2016). For this study, we wanted to determine whether 3-Cl-DPA was a Ca^{2+} sensitizer of cardiac muscle. Fig. 1 shows that in the absence of 3-Cl-DPA, rat ventricular trabeculae develop force with a pCa_{50} ($-\log[Ca^{2+}]$ at which force is at 50% of maximal force) of 5.81 ± 0.03 . When force versus pCa experiments are repeated in the presence of 100 μ M 3-Cl-DPA, trabeculae develop force with a significantly higher pCa_{50} of 5.99 ± 0.03 . The presence of 3-Cl-DPA does not significantly affect the steepness of the force versus pCa curve (Hill coefficient [n_H] = 3.3 ± 0.2 in the absence of a compound versus n_H = 3.5 ± 0.3 in the presence of 100 μ M 3-Cl-DPA). DMSO alone did not cause a significant shift in the force versus pCa relationship (data not shown). The resting tension, in pCa 9.0, was measured after all of the force versus pCa data were collected in seven of the trabeculae that were used to study the effects of 3-Cl-DPA. This value was compared with the initial resting tension value immediately after the trabecula was mounted in the chamber and before exposure to 3-Cl-DPA. The initial and

final resting tensions, normalized with trabecula cross-sectional area (1.2 ± 0.4 kN/m² and 1.1 ± 0.4 kN/m², respectively), were not significantly different.

There was a small but significant decrease in maximal force (P_o) during the course of the control (no 3-Cl-DPA) force versus pCa measurements. The mean P_o in the first and the last activations with pCa 4.0 was 12.8 ± 1.1 and 11.2 ± 0.8 kN/m², respectively. There was also a small but significant decrease in P_o during the course of measurements with 3-Cl-DPA (10.8 ± 0.7 [first activation] and 10.0 ± 0.7 [last activation] kN/m²). We compared P_o in pCa 4.0 during the first activation with 3-Cl-DPA to the last activation in pCa 4.0 without 3-Cl-DPA to test whether the compound had any effect on P_o , and there was not a significant difference. Therefore, we conclude that 3-Cl-DPA had no significant effect on maximal force. Thus, our results indicate that 3-Cl-DPA results in ~ 1.5 -fold increase in the Ca²⁺ sensitivity of force development without altering the maximal or resting forces.

Effect of 3-Cl-DPA on the rate of Ca²⁺ dissociation from the regulatory N-domain of intact cTnC

Ca²⁺-sensitizing compounds typically increase the Ca²⁺-binding affinity of cTnC by slowing the rate of Ca²⁺ dissociation from the N-terminal regulatory domain of cTnC (for review, see Davis and Tikunova, 2008). To further elucidate the molecular mechanism by which 3-Cl-DPA sensitizes cardiac muscle to Ca²⁺, we compared the effect of 3-Cl-DPA on the rate of Ca²⁺ dissociation from the regulatory N-domain of intact IAANS-labeled cTnC^{T53C} to that of a known Ca²⁺-sensitizing compound, TFP. Fig. 2A (with representative stopped-flow traces shown in Fig. 2B) shows that while TFP dramatically slows the rate of Ca²⁺ dissociation from the N-domain of intact cTnC in a concentration-dependent manner, 3-Cl-DPA has no effect on the rate of Ca²⁺ dissociation at a concentration of up to 150 μ M. Note that in the presence of TFP, removal of Ca²⁺ from the N-domain of cTnC led to an increase

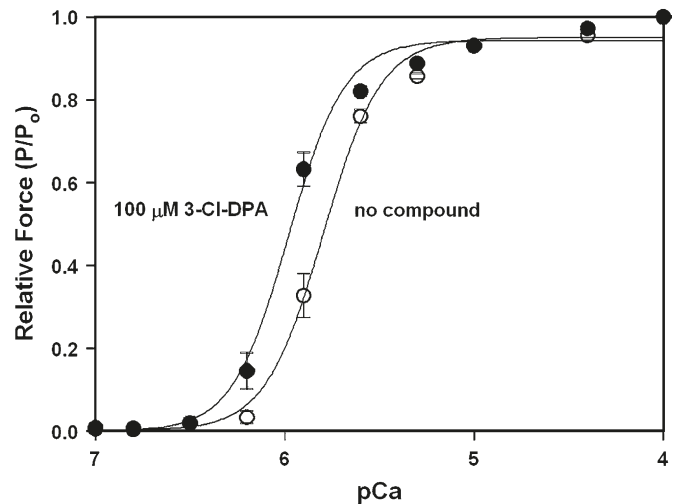


Figure 1. **Effect of 3-Cl-DPA on the Ca²⁺ sensitivity of force development in skinned ventricular trabeculae.** The curves show the Ca²⁺ dependence of force development in skinned ventricular trabeculae in the absence and presence of 100 μ M 3-Cl-DPA. Data represent the mean \pm SEM for 10 trabeculae. Data sets were individually normalized and fit with a logistic sigmoid.

rather than decrease in IAANS fluorescence observed in the absence of compound or presence of 3-Cl-DPA.

We are not able to unambiguously measure the Ca²⁺-binding affinity of the regulatory N-domain of IAANS-labeled cTnC^{T53C}, because, in addition to undergoing an increase in fluorescence upon Ca²⁺ binding to the N-terminal regulatory site of cTnC, the environmentally sensitive IAANS probe attached to Cys⁵³ undergoes a large increase in fluorescence upon Ca²⁺ binding to the C-terminal structural sites of isolated cTnC. Since the rate of Ca²⁺ dissociation from the N-domain of isolated cTnC is more than 1,000-fold faster than that from the C-terminal domain (C-domain), we are able to distinguish the rate of Ca²⁺ dissociation

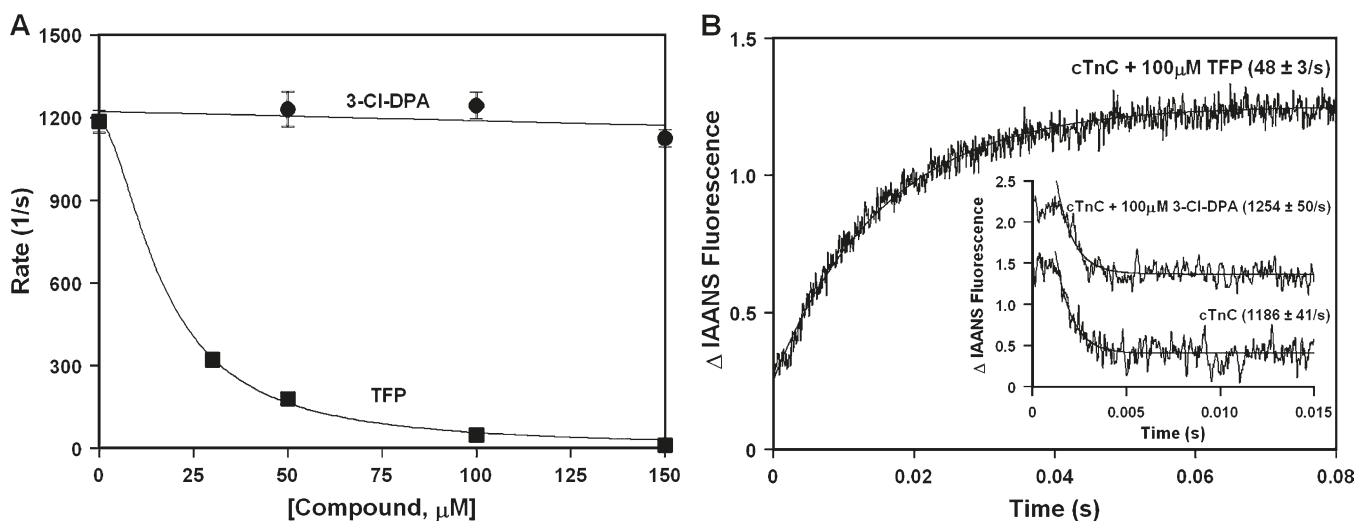


Figure 2. **Effect of 3-Cl-DPA on the rate of Ca²⁺ dissociation from the regulatory N-domain of intact cTnC.** (A) Plot of the apparent rates of Ca²⁺ dissociation from the N-domain of intact cTnC in the presence of increased concentrations of 3-Cl-DPA or TFP. Each data point represents an average of at least three measurements \pm SEM. (B) Representative stopped-flow traces as Ca²⁺ is removed from the N-domain of intact cTnC in the absence or presence of 100 μ M 3-Cl-DPA or TFP. The traces have been normalized and staggered for clarity. Rates of Ca²⁺ dissociation were measured following fluorescence of IAANS attached to Cys⁵³ of cTnC with C35S, T53C, and C84S substitutions.

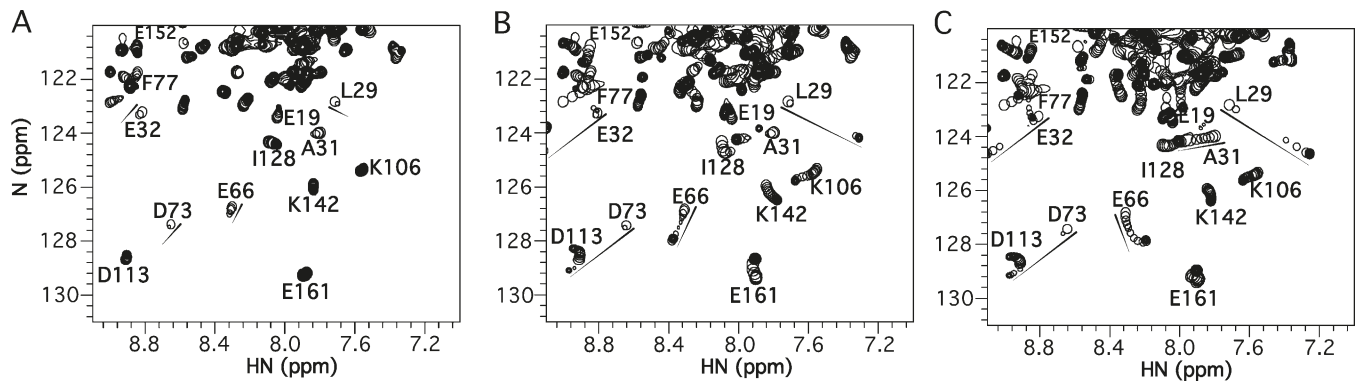


Figure 3. **Titrations of 3-Cl-DPA, TFP, and bepridil into Ca^{2+} -saturated intact cTnC.** (A–C) Titrations of 3-Cl-DPA (A), TFP (B), or bepridil (C) into ^{15}N -labeled cTnC, observed by 2D $[^1\text{H}, ^{15}\text{N}]$ -HSQC NMR spectra (amide HN ^1H chemical shift plotted along x axis, amide ^{15}N chemical shift plotted along y axis). For each titration, all spectra have been overlaid, with peaks plotted as a single contour level so that their progression can be tracked (shown by arrows). The final spectrum at the end of the titration is plotted conventionally with multiple contour levels. For 3-Cl-DPA, note many peaks disappear and do not reappear at the end of the titration.

from the N-domain of IAANS-labeled cTnC^{T53C} versus the rate from the C-domain using a stopped-flow apparatus, but we were not able to accurately distinguish the Ca^{2+} -binding sensitivity of the N-domain versus C-domain using steady-state fluorescence. However, we were able to determine that 100 μM 3-Cl-DPA did not significantly change the Ca^{2+} sensitivity of the regulatory N-domain of isolated cTnC using IAANS attached to Cys⁸⁴ of cTnC^{C35S}, whereas 100 μM bepridil led to ~ 6.5 -fold increase in the Ca^{2+} sensitivity of the N-domain of IAANS-labeled cTnC^{C35S} (data not shown). Thus, our results suggest that the molecular mechanism by which 3-Cl-DPA interacts with cTnC is different from that of TFP, bepridil, and several other Ca^{2+} -sensitizing compounds.

Titrations of 3-Cl-DPA, TFP, and bepridil into Ca^{2+} -saturated intact cTnC

Ca^{2+} -bound cTnC exists in a predominantly closed form that makes transient excursions to more open states (Sia et al., 1997; Eichmüller and Skrynnikov, 2005; Cordina et al., 2013). A previously published x-ray crystal structure demonstrated bepridil binds to the central hydrophobic cavity of the cTnC domain in its open conformation (Li et al., 2000). As bepridil is titrated into cTnC, NMR signals corresponding to the cTnC domain move as the conformational equilibrium shifts from the closed state to the open bepridil-bound form (Fig. 3 C). A previous study showed that bepridil binds cTnC with a K_d of 20 μM (Wang et al., 2002). Most peaks shift linearly, suggesting a 1:1 binding process. Some residue peaks move in a curved path during the titration, indicating that these residues are influenced by additional lower-affinity binding sites on cTnC (e.g., E66). Some peaks with large chemical shift changes (e.g., L29, E32, and D73) disappear during the titration and then reappear at saturation, consistent with an intermediate timescale exchange process, suggesting that bepridil binding to cTnC occurs on a timescale similar to the frequency difference between free and bound states for these residues (~ 200 Hz on a 500-MHz spectrometer). Some peaks in the C-terminal domain also shift, indicating fast timescale weak binding to this domain as well. The NMR spectra of the TFP titration into cTnC (Fig. 3 B) looks very similar to that of bepridil, suggesting a similar binding mode and accom-

panying closed-to-open conformational transition, except that TFP appears to bind with slightly slower kinetics (L29, E32, and D73 are in a slow-to-intermediate exchange timescale) and correspondingly tighter binding ($K_d < 20$ μM , though the exact K_d cannot be calculated because of multiple binding sites on the two cTnC domains).

For 3-Cl-DPA, the start of the titration begins similarly to bepridil and TFP, with peaks shifting in the same direction (see E32, D73, E66, A31, and L29), signifying a similar closed-to-open conformational change to accommodate small-molecule binding (Fig. 3 A). However, as the 3-Cl-DPA titration progresses, most cTnC peaks weaken in intensity and then disappear, except for those that are less influenced by the closed-to-open transition, mainly the first 20 amino acid residues in the sequence. Unlike the effects of bepridil and TFP, the disappearing signals do not reemerge at the end of the titration, indicating conformational flexibility within the fully saturated cTnC–3-Cl-DPA complex. Thus, it appears that 3-Cl-DPA binding does not stabilize a single dominant open conformation in cTnC like bepridil or TFP, instead giving rise to an ensemble of structural states.

Also seen in Fig. 3 A, NMR signals from the C-terminal domain of cTnC shift only slightly during the 3-Cl-DPA titration (e.g., K106, I128, K142, and E161), indicating very weak binding. Thus, 3-Cl-DPA binding appears to be much more specific for the N-terminal domain, which is also unlike bepridil or TFP. Although we cannot definitively determine the K_d of 3-Cl-DPA for intact cTnC, a K_d of 6 ± 1 μM was determined from the titration of 3-Cl-DPA into the isolated cTnC domain (data not shown).

Effect of 3-Cl-DPA on the Ca^{2+} -binding properties of the regulatory N-domain of cTnC reconstituted into the cTn complex

Surprisingly, our results indicate that despite binding to cTnC and sensitizing cardiac muscle to Ca^{2+} , 3-Cl-DPA has no effect on the rate of Ca^{2+} dissociation from the N-domain of intact cTnC. One possibility is that 3-Cl-DPA requires the presence of the other cTn subunits to exert its effect on Ca^{2+} binding. To test this hypothesis, we compared the effect of 3-Cl-DPA on the Ca^{2+} sensitivity of the N-domain of intact cTnC after reconstitution into

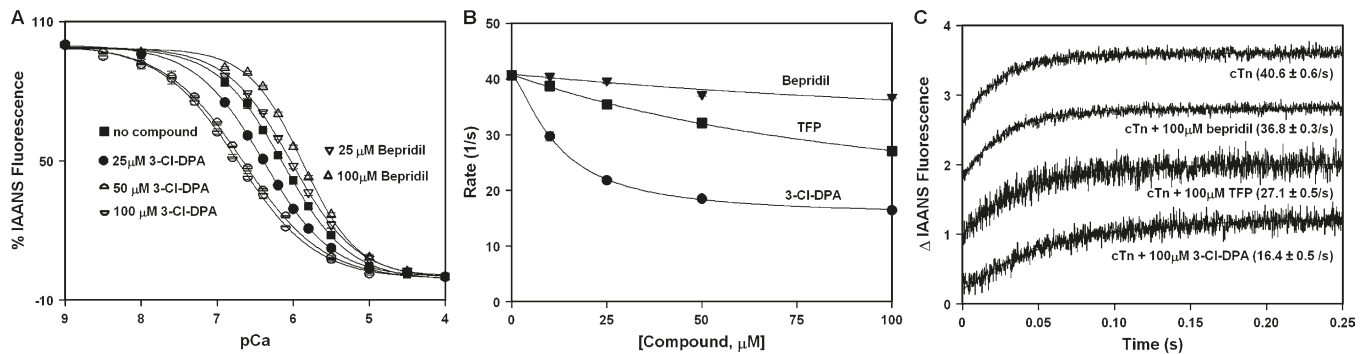


Figure 4. Effect of 3-Cl-DPA on the Ca²⁺ binding properties of the regulatory N-domain of cTnC reconstituted into the cTn complex. (A) Decreases in IAANS fluorescence occur when Ca²⁺ binds to labeled cTnC with C35S, T53C, and C84S substitutions, reconstituted into the cTn complex in the absence or presence of increasing concentrations of 3-Cl-DPA or bepridil. Data sets were individually normalized and fit with a logistic sigmoid. (B) Plot of the apparent rates of Ca²⁺ dissociation from the cTn complex in the presence of increased concentrations of 3-Cl-DPA, TFP, or bepridil. (C) Representative stopped-flow traces as Ca²⁺ is removed from the cTn complex in the absence or presence of 100 μM 3-Cl-DPA, TFP, or bepridil. The traces have been normalized and staggered for clarity. Rates of Ca²⁺ dissociation were measured following the fluorescence of IAANS attached to Cys⁵³ of cTnC with C35S, T53C, and C84S substitutions after reconstitution into the cTn complex.

the cTn complex (cTnC complexed with cTnI and cTnT) to that of bepridil. After reconstitution into the cTn complex, changes in fluorescence of IAANS-labeled cTnC^{T53C} primarily report N-domain Ca²⁺ binding and exchange (Davis et al., 2007). The Ca²⁺-induced decreases in IAANS fluorescence, which occur when Ca²⁺ binds to the N-domain of intact labeled cTnC^{T53C} reconstituted into the cTn complex in the absence or presence of small molecules, are shown in Fig. 4 A. In the absence of small molecules, the cTn complex exhibited a half-maximal Ca²⁺-dependent decrease in IAANS fluorescence with a pCa₅₀ of 6.13 ± 0.02. In the presence of increasing concentrations of 3-Cl-DPA, the Ca²⁺ sensitivity of the cTn complex significantly increases in a concentration-dependent manner (pCa₅₀ = 6.39 ± 0.01, pCa₅₀ = 6.65 ± 0.01, and pCa₅₀ = 6.73 ± 0.02 in the presence of 25, 50, and 100 μM 3-Cl-DPA, respectively). In contrast, bepridil actually significantly desensitizes the cTn complex to Ca²⁺, also in a concentration-dependent manner (pCa₅₀ = 6.02 ± 0.2 and pCa₅₀ = 5.88 ± 0.01 in the presence of 25 and 100 μM bepridil, respectively).

We also compared the effect of 3-Cl-DPA on the rate of Ca²⁺ dissociation from the N-domain of intact cTnC reconstituted into the cTn complex to that of bepridil and TFP. We have previously demonstrated that under identical experimental conditions, the rate of Ca²⁺ dissociation measured from IAANS-labeled cTnC^{T53C} reconstituted into the cTn complex was similar to that measured from the unlabeled wild-type cTnC reconstituted into the cTn complex (Davis et al., 2007). Thus, we are confident that IAANS-labeled cTnC^{T53C} accurately reports Ca²⁺ dissociation from the cTn complex. Stopped-flow experiments were conducted to determine the effect of increasing concentrations (ranging from 12.5 to 100 μM) of 3-Cl-DPA, bepridil, and TFP on the rate of Ca²⁺ dissociation from the cTn complex. Our results, shown in Fig. 4 B (with representative stopped-flow traces shown in Fig. 4 C), indicate that the ability of 3-Cl-DPA to slow the rate of Ca²⁺ dissociation from the cTn complex is much greater than that of either bepridil or TFP. Thus, 3-Cl-DPA requires the addition of cTnI and cTnT to sensitize cTnC to Ca²⁺, with the most likely essential binding partner being cTnI, since cTnT is not known to interact with

cTnC. Indeed, 3-Cl-DPA is able to slow Ca²⁺ dissociation from a cTnC-cTnI chimeric protein (data not shown).

Titration of 3-Cl-DPA, TFP, and bepridil into Ca²⁺-saturated cTnC-cSp chimera

The constant presence of cTnI residues 146–158 in the cTnC-cSp chimera keeps the Ca²⁺-saturated cTnC domain in an open conformation for >90% of the time, consistent with a high effective concentration of cTnI (Pineda-Sanabria et al., 2014; Siddiqui et al., 2016). As shown in Fig. 5 A, titration of 3-Cl-DPA into cTnC-cSp chimera yields large NMR chemical shift changes that are linear, indicative of a 1:1 complex, and all peaks corresponding to cTnC are present at 3-Cl-DPA saturation; that is, they do not disappear as they did in the cTnC-3-Cl-DPA complex (compare with Fig. 3 A). Thus, binding of cTnI prevents the conformational exchange processes that lead to NMR signal broadening in the cTnC-3-Cl-DPA complex. 3-Cl-DPA binds to cTnC-cSp chimera with a K_d of 10 ± 2 μM, demonstrating that 3-Cl-DPA binding is compatible with cTnI switch region binding, though it is slightly weakened in its presence (K_d of 6 μM for 3-Cl-DPA binding to the isolated cTnC domain).

In contrast to 3-Cl-DPA, when bepridil is titrated into cTnC-cSp chimera, NMR signals corresponding to cTnC weaken or disappear, indicating conformational exchange (Fig. 5 C). The binding affinity of bepridil to the cTnC-cSp chimera is very weak (K_d = 380 ± 60 μM; an order of magnitude weaker than it is to cTnC domain alone), indicating that the cTnI switch region strongly competes against bepridil for binding to cTnC. The binding of TFP (Fig. 5 B) is also similarly weakened (K_d = 110 ± 20 μM), indicating competition with cTnI for cTnC binding, too. Thus, bepridil and TFP both compete with the cTnI switch region for binding to cTnC, whereas 3-Cl-DPA binding is more compatible.

Discussion

Recently, we screened over 40 diphenylamine (DPA)-based compounds for binding to cTnC-cSp chimera in order to explore whether any of these compounds have potential for drug devel-

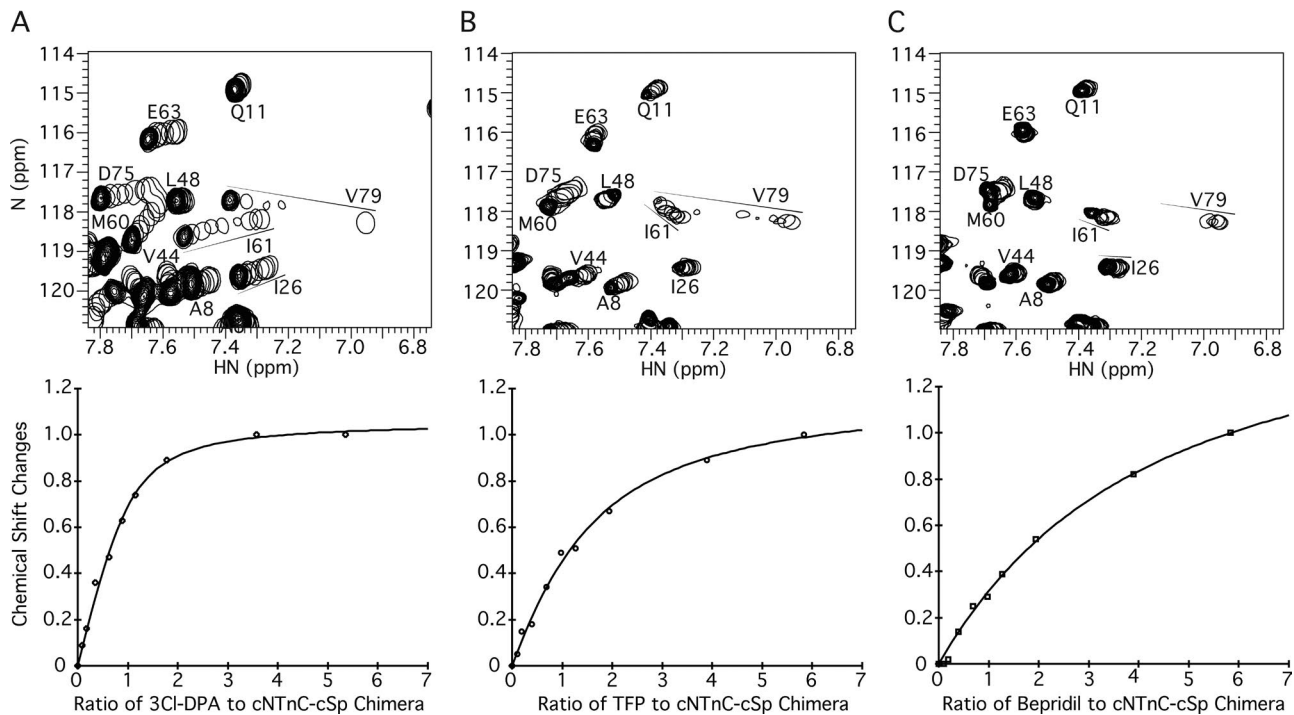


Figure 5. **Titrations of 3-Cl-DPA, TFP, and bepridil into Ca^{2+} -saturated cTnC-cSp chimera.** (A–C) Titrations of 3-Cl-DPA (A), TFP (B), or bepridil (C) into ^{15}N -labeled cTnC-cSp chimera, as observed by 2D $[^1\text{H}, ^{15}\text{N}]$ -HSQC NMR spectra (amide HN ^1H chemical shift plotted along x axis, amide ^{15}N chemical shift plotted along y axis). For each titration, all spectra have been overlaid, with peaks plotted as a single contour level so that their progression can be tracked (shown by arrows). The final spectrum at the end of the titration is plotted conventionally with multiple contour levels. Below each spectrum is a representative plot of chemical shift changes as a function of compound added, from which K_d is calculated. Note that the ratio of compound to cTnC-cSp chimera, rather than the absolute concentration of compound, is on the x axis. This is because plots of saturation versus compound concentration are typically performed when the protein concentration is much smaller than the K_d . However, because of the sensitivity limitation of multidimensional solution NMR, the protein concentration (0.11 mM) is greater than the K_d (10 μM in the case of 3-Cl-DPA). Thus, to avoid confusion, the protein/compound ratio is used instead.

opment (Cai et al., 2016). Of the DPA-based compounds, 3-Cl-DPA was able to bind to cTnC-cSp chimera most tightly (K_d of 10 μM), and thus, it was selected for further studies. Since molecules that bind to cTnC are not necessarily Ca^{2+} sensitizers, we evaluated the ability of 3-Cl-DPA to increase the Ca^{2+} sensitivity of force development in cardiac muscle. Our results show that 3-Cl-DPA is a Ca^{2+} sensitizer of cardiac muscle.

The Ca^{2+} sensitivity of cardiac muscle is determined by the Ca^{2+} -binding affinity of the regulatory N-domain of cTnC, which in turn is modulated by many factors (Li and Hwang, 2015). Physiologically, the most important of these factors is the effective concentration of cTnI switch region (Siddiqui et al., 2016). Binding of the cTnI switch region to the N-domain of cTnC locks it into an open conformation, in which the Ca^{2+} ion is fully ligated (either directly or indirectly) by Ca^{2+} -binding residues of the second EF hand (Ca^{2+} -binding helix-loop-helix motif) (Takeda et al., 2003). In contrast, when the Ca^{2+} -bound N-domain of cTnC is not bound to the cTnI switch region, it remains predominantly in the closed conformation (Sia et al., 1997), in which the EF hand loop residues Asp⁶⁷ and Ser⁶⁹ do not directly chelate the Ca^{2+} (Zhang et al., 2013) as they do in the open state (Takeda et al., 2003). Consequently, the N-domain of cTnC (dominated by the closed conformation) has a Ca^{2+} -binding affinity approximately one order of magnitude lower than that of the intact cTn complex (Davis and Tikunova, 2008). Like the cTnI switch region, small molecules can act as Ca^{2+} sensitizers by stabilizing the open

form of the N-domain of cTnC (for review, see Li et al., 2008; Li and Hwang, 2015).

Our results show that unlike bepridil or TFP, 3-Cl-DPA is unable to sensitize the N-domain of isolated cTnC to Ca^{2+} . Our NMR data suggest that saturation with bepridil or TFP stabilizes the open conformation of the N-domain of cTnC, consistent with a crystal structure of the complex between bepridil and Ca^{2+} -bound cTnC (Li et al., 2000). Our stopped-flow experiments demonstrate that stabilization of the open state by TFP results in dramatic slowing of the rate of Ca^{2+} dissociation from the N-domain of cTnC. On the other hand, our NMR studies suggest that 3-Cl-DPA binding does not stabilize the open state in the same manner as TFP or bepridil. In agreement with NMR studies, 3-Cl-DPA is not able to slow the rate of Ca^{2+} dissociation from the N-domain of cTnC. Thus, unlike bulky molecules such as TFP and bepridil, the small and flexible molecule 3-Cl-DPA is able to bind into the hydrophobic cavity of cTnC without formation of the fully open state. The inability of 3-Cl-DPA to stabilize the open state of cTnC likely results in its ineffectiveness in Ca^{2+} -sensitizing the N-domain of isolated cTnC and diminishes its Ca^{2+} -sensitizing potency in cardiac muscle.

We originally designed diphenylamine-based compounds based on structures of bepridil bound to the N-domain of cTnC (Li et al., 2000; Wang et al., 2002). We suspected that most of the free energy of bepridil binding derived from its benzylphenylamine group, whereas its bulky isopropyl group displaces the

switch region of cTnI from its optimal binding site. We found that benzylphenylamine bound to the cNTnC–cSp chimera with a K_d of 150 μ M, and the smaller diphenylamine molecule bound even better, with a K_d of 120 μ M (Cai et al., 2016). Out of all the diphenylamine derivatives we tested, 3-Cl-DPA bound most tightly, but we chose to determine the structure of the similar compound, 3-methyl-DPA bound to the cNTnC–cSp chimera, because the methyl group provides additional useful information for structural NMR studies (Cai et al., 2016). Our structures showed that 3-methyl-DPA was less disruptive of cTnI switch region binding than bepridil, as it was designed to be. Ile¹⁴⁸ and Met¹⁵³ of cTnI are displaced slightly, but all other major contacts between cNTnC and cTnI are preserved. 3-Cl-DPA likely binds in a manner similar to 3-methyl-DPA, because the chemical shift changes upon binding are very similar. In contrast, bepridil binding causes a marked displacement of the entire switch region as well as an additional opening of the cNTnC structure (Wang et al., 2002).

Our results suggest that 3-Cl-DPA can stabilize the open state of the N-domain of cTnC only in the presence of cTnI. In fact, 3-Cl-DPA acts as a Ca^{2+} sensitizer of the cTn complex, while bepridil actually desensitizes the cTn complex to Ca^{2+} . In that regard, bepridil acts similarly to the V44Q mutation in cTnC, which sensitizes isolated cTnC and cardiac muscle to Ca^{2+} (Tikunova and Davis, 2004; Norman et al., 2007) but has a Ca^{2+} -desensitizing effect on the cTn complex (Tikunova et al., 2010). Our results are consistent with that of an earlier study showing that bepridil had almost no effect on the rate of Ca^{2+} dissociation from the cTn complex (Varughese et al., 2011). The interference of bepridil with the binding of the switch region of cTnI to the hydrophobic cavity likely weakens the extent of Ca^{2+} sensitization that bepridil is able to achieve in cardiac muscle, compared with its large Ca^{2+} sensitizing effect on isolated cTnC (Solaro et al., 1986; Kischel et al., 1999; Piroddi et al., 2007).

The ability of cTnC to bind cTnI plays a major role in influencing its Ca^{2+} -binding properties (Siddiqui et al., 2016). Because cTnC transitions through multiple states during each heartbeat (Davis and Tikunova, 2008; Davis et al., 2016), the effect of compounds on each state of cTnC needs to be considered. We theorize that the most potent Ca^{2+} sensitization could be achieved by compounds that are able to stabilize the open state of cNTnC while enhancing or at least not interfering with the binding of cTnI, thus increasing Ca^{2+} sensitivity of cNTnC before and after binding of cTnI. In that regard, an “ideal” Ca^{2+} -sensitizing compound would act as the L48Q mutation in cTnC, which sensitizes isolated cTnC and the cTn complex to Ca^{2+} (Tikunova and Davis, 2004; Tikunova et al., 2010) while slightly enhancing the affinity of cTnC for cTnI (Wang et al., 2012). There are numerous ways in which the Ca^{2+} sensitivity of the heart can be altered. Although there is evidence that some cardiomyopathy-linked modifications that alter the Ca^{2+} sensitivity may be a part of the disease process (Gomes and Potter, 2004; Kimura, 2010), we have recently demonstrated that this is not always the case. For instance, the L48Q mutation in cTnC increases in vivo cardiac contractility without causing adverse side effects in a mouse model of heart failure (Shettigar et al., 2016). Thus, we believe that it is possible to enhance cardiac contractility through sensitizing cTnC to Ca^{2+} without causing disease.

Moreover, it should be noted that Ca^{2+} -sensitizing drugs are not intended for use in normal well-functioning hearts, in which the delicate balance between contraction and relaxation is properly regulated. Rather, the drugs are intended for diseased hearts with primarily systolic failure, as typically seen in familial dilated cardiomyopathy or ischemic heart disease. In such cases, it is anticipated that Ca^{2+} sensitizers will shift the contractile balance, improving the efficiency of cardiac contraction and attenuating (and possibly even reversing) the pathological remodeling process that leads to progressively dilated and thinned ventricles.

In conclusion, we determined that 3-Cl-DPA is a Ca^{2+} sensitizer of cardiac muscle, with a mechanism of action very different from that of known Ca^{2+} sensitizers, such as TFP and bepridil. The small size of the 3-Cl-DPA molecule makes it an excellent starting scaffold for the development of more potent Ca^{2+} -sensitizing compounds. Understanding the molecular mechanisms by which small molecules modulate Ca^{2+} and cTnI binding and exchange with cTnC is the first step in the development of drugs with high potency and specificity that could one day be used to treat systolic heart failure.

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