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Engineered Troponin C Constructs Correct Disease-related Cardiac Myofilament Calcium Sensitivity*

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Background: Improved myofilament Ca^{2+} sensitivity alleviates defects in thin filament bearing disease-causing mutations.
Results: By engineering the cardiac muscle Ca^{2+} sensor troponin C, aberrant myofilament Ca^{2+} sensitivity can be corrected *in vitro*.
Conclusion: Engineered TnC provides a novel and versatile avenue to reset disease-related myofilament Ca^{2+} sensitivity.
Significance: Engineered TnC could be a new therapeutic strategy for cardiac muscle diseases.

Aberrant myofilament Ca^{2+} sensitivity is commonly observed with multiple cardiac diseases, especially familial cardiomyopathies. Although the etiology of the cardiomyopathies remains unclear, improving cardiac muscle Ca^{2+} sensitivity through either pharmacological or genetic approaches shows promise of alleviating the disease-related symptoms. Due to its central role as the Ca^{2+} sensor for cardiac muscle contraction, troponin C (TnC) stands out as an obvious and versatile target to reset disease-associated myofilament Ca^{2+} sensitivity back to normal. To test the hypothesis that aberrant myofilament Ca^{2+} sensitivity and its related function can be corrected through rationally engineered TnC constructs, three thin filament protein modifications representing different proteins (troponin I or troponin T), modifications (missense mutation, deletion, or truncation), and disease subtypes (familial or acquired) were studied. A fluorescent TnC was utilized to measure Ca^{2+} binding to TnC in the physiologically relevant biochemical model system of reconstituted thin filaments. Consistent with the pathophysiology, the restrictive cardiomyopathy mutation, troponin I R192H, and ischemia-induced truncation of troponin I (residues 1–192) increased the Ca^{2+} sensitivity of TnC on the thin filament, whereas the dilated cardiomyopathy mutation, troponin T ΔK210, decreased the Ca^{2+} sensitivity of TnC on the thin filament. Rationally engineered TnC constructs corrected the abnormal Ca^{2+} sensitivities of the thin filament, reconstituted actomyosin ATPase activity, and force generation in skinned trabeculae. Thus, the present study provides a novel and versatile therapeutic strategy to restore diseased cardiac muscle Ca^{2+} sensitivity.

Prokaryotes, eukaryotes, and even viruses utilize Ca^{2+} and Ca^{2+}-binding proteins to perform specific duties (1–3). The most common motif used by proteins to bind Ca^{2+} is the EF-hand (4). EF-hand proteins help perform cellular functions by maintaining the structural integrity of multimeric protein complexes, altering protein interactions like switches, or simply buffering Ca^{2+} (5, 6). Ultimately, these Ca^{2+}-dependent processes are controlled by the Ca^{2+} signal and the Ca^{2+} binding properties of the protein (7, 8). A prime example of such behavior is the increased amplitude and decreased duration of cardiac muscle contraction after a surge of adrenaline. In this case, both the Ca^{2+} transient profile and the Ca^{2+}-dependent response of the protein are complimentarily modulated to affect contraction and relaxation (9, 10).

In the heart, troponin C (TnC)[2] is the Ca^{2+}-dependent, switch-like protein that helps regulate force development as an integral part of the contractile machinery (5). Different isoforms of the troponin complex (Tn) within an organism and between different species help to tune the response of TnC to Ca^{2+} to meet developmental and environmental demands of the heart (11–15). In this regard, TnC does not behave like a simple switch because the Ca^{2+} binding properties of the regulatory EF-hand of TnC are modulated by interactions with its protein binding partner, troponin I (TnI) (16, 17). The response of TnC to Ca^{2+} can be further adjusted by additional myofilament proteins (troponin T, actin, tropomyosin, and myosin) and by an assortment of posttranslational modifications to many of these proteins (5, 17–19). Thus, it would appear that TnC acts as a central hub converging information from the myofilament proteins to tune its response to the Ca^{2+} signal (5). Unfortunately, in many inherited and acquired cardiac diseases, the proper tuning of TnC to Ca^{2+} is disturbed (18, 20, 21).

Wide assortments of mutations, deletions, truncations, and aberrant posttranslational modifications of numerous myofilament proteins have been associated with various cardiac diseases (21–23). Alterations in the Ca^{2+} sensitivity of TnC and force development have been commonly observed to be one of many problems that arise in these complex dis-

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[2] The abbreviations used are: Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; DCM, dilated cardiomyopathy; RCM, restrictive cardiomyopathy; IAANS, 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium salt; Tm, tropomyosin.

orders (20, 21, 23–26). Strikingly, for any particular class of inherited cardiomyopathies, the apparent Ca^{2+} sensitivity of TnC and force development are typically altered in a qualitatively similar manner (20, 22, 25). Furthermore, pharmacological and genetic interventions that rectify the apparent Ca^{2+} sensitivity of cardiac muscle in transgenic animal models harboring cardiomyopathic genes show promise of alleviating the disease symptoms (27–29). For instance, modulating TnI, TnT, or tropomyosin (each of which can indirectly tune the Ca^{2+} sensitivity of TnC) counteracted the abnormal cardiac muscle Ca^{2+} sensitivities and ameliorated the disease symptoms (28–30). Ultimately, correcting the aberrant Ca^{2+} sensitivity may be part of an integrative approach to improving cardiac function in these complex cardiomyopathies (26).

Due to its central role as the Ca^{2+} sensor for cardiac muscle contraction, TnC stands out as an obvious and more versatile target to genetically modulate cardiac muscle Ca^{2+} sensitivity. Unfortunately, there are no pharmacological compounds that just target TnC (31). However, by directly engineering TnC, our laboratory has developed several TnC constructs, which behave as Ca^{2+} sensitizers or desensitizers in biochemical model systems and in muscle (16, 32, 33). By utilizing different design principles (32, 33), the intrinsic Ca^{2+} binding properties of TnC can be finely or grossly tuned. We have rationally engineered TnC to test whether the Ca^{2+} dependence of biochemical and physiological systems harboring disease-associated protein modifications could be reset. We chose to test this idea using three disease-related protein modifications (inherited and acquired) in TnI and TnT that exhibit physiologically abnormal increased or decreased Ca^{2+} sensitivities. We demonstrate that by specifically adjusting the Ca^{2+} binding properties of TnC, both the aberrant biochemical and the aberrant physiological Ca^{2+} sensitivity of the cardiac myofilaments can be corrected.

EXPERIMENTAL PROCEDURES

Materials—Phenyl-Sepharose CL-4B, sodium molybdate dihydrate, and EGTA were purchased from Sigma. IAANS and phalloidin were purchased from Invitrogen. Affi-Gel 15 affinity medium was purchased from Bio-Rad. Malachite green oxalate was purchased from Fisher Scientific.

Mutagenesis—TnC, TnI, and TnT mutants were constructed from their respective pET3a expression plasmids by primer-based site-directed mutagenesis and confirmed by DNA sequence analysis.

Protein Expression and Purification—The plasmid encoding human cardiac TnC was transformed into *Escherichia coli* BL21(DE3)pLysS cells (Novagen, San Diego, CA), whereas those for TnI and TnT were transformed into Rosetta™(DE3)pLysS cells (Novagen). TnC, TnI, and TnT were purified as described previously (15, 16). Rabbit skeletal actin and bovine ventricular cardiac tropomyosin (Tm) were purified from acetone powders as described previously (34, 35). Rabbit ventricular S1 was isolated from purified myosin after α-chymotrypsin digestion (36).

Fluorescent Labeling—TnC^{T53C} [3] and its constructs were labeled with the environmentally sensitive thiol reactive fluorescent probe IAANS as described previously (17).

Reconstitution of Tn Complex—The Tn complexes were prepared and reconstituted as described previously (17).

Reconstitution of Regulated Thin Filaments—Thin filaments were prepared in a reconstitution buffer containing 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.0, as described previously (17).

Steady-state Fluorescence—All steady-state fluorescence measurements were performed using a PerkinElmer Life Sciences LS 55 fluorescence spectrometer at 15 °C. IAANS fluorescence was excited at 330 nm and monitored at 450 nm as microliter amounts of $CaCl_2$ were added to 2 ml of each Tn complex or thin filament in 200 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.0, as described previously (17). The Ca^{2+} sensitivity was reported as a dissociation constant K_d, representing a mean of at least three titrations. The data were fit with the Hill equation.

Stopped-flow Fluorescent Measurements—Ca^{2+} dissociation rates were characterized using an Applied Photophysics model SX.20 stopped-flow instrument with a dead time of 1.4 ms at 15 °C. IAANS fluorescence was excited at 330 nm. The IAANS emission was monitored through a 510-nm broad band-pass interference filter for the thin filament. The filters were purchased from Oriel (Stratford, CT). Data traces (an average of 3–5 individual traces) were fit with a single exponential equation to calculate the kinetic rates. The working buffer used for the kinetic measurements was 10 mM MOPS, 150 mM KCl, 1 mM DTT, 3 mM $MgCl_2$ at pH 7.0. 10 mM EGTA was utilized to remove 200 μM Ca^{2+} from the thin filaments.

Actomyosin S1 ATPase Assay—Reconstituted thin filaments were formed in a buffer containing 5 mM $MgCl_2$, 30 mM MOPS, pH 7.0. The thin filaments were formed using 5 μM actin, 2 μM Tm, and 1.5 μM Tn. 0.2 μM myosin S1 was used in the assay. A final EGTA concentration of 0.5 mM and various amounts of Ca^{2+} were added to the reaction mixture to form the different *p*Ca values. The reactions were initiated by adding 3 mM ATP, and 15-μl aliquot reaction mixtures were terminated by the addition of 0.2 M ice-cold Δ'-pyrroline-5-carboxylic acid at different time intervals (typically every 4 min). ATPase activity was determined by analyzing the amount of phosphate released in a time course of up to 20 min. The malachite green assay was utilized to quantify the phosphate released during the reaction as described previously (37).

Skinned Muscle Chamber and Apparatus—Trabeculae were "T-clipped" and attached to hooks connected to a servo-controlled DC torque motor (Cambridge Technologies) and an isometric force transducer (model 403A, Cambridge Technolo-

[3] The mutant designations used are: TnC^{T53C}, Cys-less human cardiac TnC with T53C mutation; TnC^{T53C}_{IAANS}, TnC^{T53C} labeled with IAANS; Tn^{T53C}_{IAANS}, Tn^{T53C} labeled with IAANS; TnI R192H, TnI mutation with Arg-192 replaced with His; TnT ΔK210, TnT mutation with Lys-210 deleted; TnI-(1–192), truncated TnI with residues 193–210 removed; TnC M45Q, TnC mutation with Met-45 replaced with Gln; TnC S69D, TnC mutation with Ser-69 replaced with Asp; TnC S69D/D73N, TnC mutation with Ser-69 replaced with Asp and Asp-73 replaced with Asn; TnC M45Q/S69D, TnC mutation with Met-45 replaced with Gln and Ser-69 replaced with Asp.

gies) located in stainless steel troughs (38). A reticule on the eyepiece of the dissecting microscope was used to measure the width and depth of the trabecula. Cross-sectional area was calculated from the depth and width measurements by assuming an elliptical circumference. The motor and force transducer were set on a three-way positioner that can be moved to adjust the resting sarcomere length to ~2.2 μm as determined by the first-order diffraction pattern from a HeNe laser directed through the trabeculae. The analog output of the force transducer was digitized using a DaqBoard/2000 and the DaqView software. The temperature of the solution in the troughs was maintained at 15 °C by a thermocouple-controlled Peltier device.

Preparation of Rat Cardiac Trabeculae—All protocols were approved by the Institutional Animal Care and Use Committee. Rat cardiac trabeculae were harvested and prepared from male LBN-F1 rats (175–200 g) as described previously (39). Briefly, rats were anesthetized via intraperitoneal injection of pentobarbital sodium (Nembutal, 50 mg/kg), and the thoracic cavity was opened. Heparin (0.1 ml of 10,000 units/ml stock) was injected intracardially, and right ventricular trabeculae were harvested and placed overnight at 4 °C in a relaxing solution containing 1% Triton X-100. The trabeculae were used within 48 h of harvest. The mean maximal *F*/cross-sectional area of 23 trabeculae used in this study was 48 ± 3 millinewtons/mm^2.

Human Troponin Exchange in Rat Cardiac Trabeculae—After maximal force was measured in the pre-exchanged trabeculae, the trabeculae were shortened by 20% of the resting length and soaked in a Rigor Buffer (10 mM MOPS, 150 mM KCl, 20 mM 2,3-butanedione monoxime, 0.01% NaN_3, 0.5 mM DTT, and 3 mM $MgCl_2$) for 30 min. The temperature was then elevated to 25 °C, and the trabeculae were soaked in an exchange buffer consisting of the Rigor Buffer with 7–15 μM human Tn and 500 μM Ca^{2+} for 2.5 h. In the case of the mock Tn exchange, no Tn was added to the exchange buffer. The exchange buffer was briefly mixed in the chamber every 15 min. After exchange, the trabeculae were stretched back to their original length and transferred to a *p*Ca 9.0 solution with 20 mM 2,3-butanedione monoxime, and the passive tension was measured at 15 °C. The trabeculae were subsequently washed three times in *p*Ca 9.0 solution for 5 min each to remove residual 2,3-butanedione monoxime. Afterward, maximal tension at *p*Ca 4.0 was measured twice to determine the percentage of maximal force recovery. The trabeculae were then randomly contracted in solutions of varying [Ca^{2+}] with a maximal contraction performed in the middle and end to determine rundown. Muscles that exhibited greater than 20% rundown in the maximal force over the course of the force-*p*Ca experiments were excluded.

Quantification of Tn Exchange—The percentage of exchanged Tn was quantified for trabecula that underwent force measurements. After removing the T-clips, the trabeculae were extracted in sample buffer (50 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 0.1% bromphenol blue) by heating to 80 °C for 6 min with periodic vortexing. Each sample was subsequently clarified by centrifugation. The extracted proteins from an entire trabeculae were separated by SDS-PAGE on a 16 × 18 cm (Hoefer) 12% (29:1) acrylamide gel cooled to 8 °C. The portion of the gel containing TnI was then transferred to a polyvinylidene difluoride membrane, probed with an anti-TnI antibody (C5, Fitzgerald), and detected by Enhanced Chemiluminescence Plus (GE Healthcare). The films were scanned and quantified using an ImageQuant TL and software (GE Healthcare) (40). The variable size of each trabecula resulted in a varied amount of total TnI loading; therefore the amount of exchanged human TnI was expressed as a percentage of the total TnI (exchanged human and remaining rat endogenous TnI).

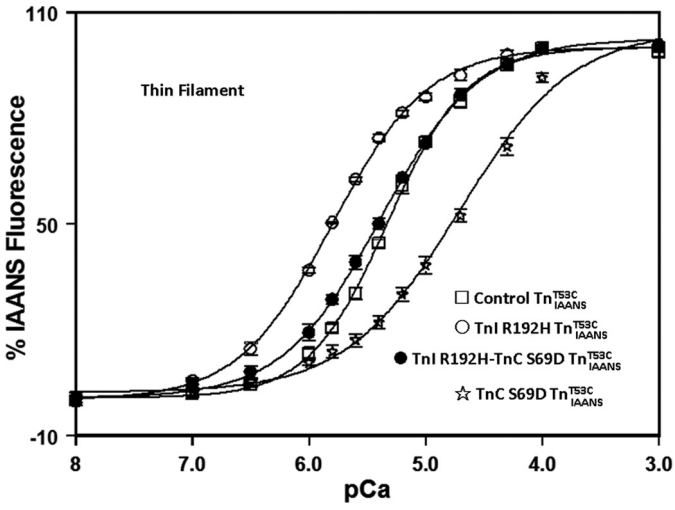

FIGURE 1. **An engineered TnC corrects RCM TnI R192H thin filament Ca^{2+} sensitivity.** The figure shows the Ca^{2+}-dependent changes in IAANS fluorescence for control Tn^{T53C}_{IAANS} (□), TnI R192H Tn^{T53C}_{IAANS} (○), TnI R192H-TnC S69D Tn^{T53C}_{IAANS} (●), and TnC S69D Tn^{T53C}_{IAANS} (☆) reconstituted thin filaments as a function of *p*Ca.

Statistical Analysis—Statistical significance was determined by analysis of variance followed by a post hoc *t* test using the statistical analysis software Minitab (State College, PA). Two means were considered to be significantly different when the *p* value was < 0.05. All data are shown as a mean value ± S.E.

RESULTS

Thin Filament Ca^{2+} Binding Studies—Our laboratory has developed a fluorescent troponin C, TnC^{T53C}_{IAANS}, which minimally affects cTnC function and reports the structural changes that occur in the regulatory domain of cTnC upon Ca^{2+} binding and dissociation on the thin filament (17, 32). This fluorescent TnC enabled the Ca^{2+} binding studies reported here.

Restrictive cardiomyopathy (RCM) is characterized by impaired ventricular filling due to an extremely stiff heart (24). Consistent with the diastolic dysfunction, RCM-associated contractile proteins typically sensitize actomyosin ATPase activity and force generation to Ca^{2+} (18, 24). As shown in Fig. 1, thin filament-bound control Tn^{T53C}_{IAANS} exhibited a Ca^{2+} sensitivity of 4.8 ± 0.2 μM (Fig. 1 and Table 1). Consistent with previous studies, thin filament Ca^{2+} sensitivity increased ~3-fold when the RCM associated mutation TnI R192H was incorporated into the Tn complex (Fig. 1 and Table 1) (18).

Ca^{2+} binding to an EF-hand is partially controlled by the number and position of acidic residues within the Ca^{2+}-binding loop (41). Previously, we have shown that the Ca^{2+} affinity of calmodulin (an EF-hand protein) could be decreased by modulating the position of acidic residues within the Ca^{2+}-

TABLE 1

Summary of thin filament Ca^{2+} sensitivity

Values marked with * are significantly different from the control values ($p < 0.05$). NA denotes a measurement that is not applicable.

Tn	pCa_{50}[a]	Ca^{2+} sensitivity	Hill coefficient	Relative change in Ca^{2+} sensitivity[b]	Ca^{2+} k_{off} (/s)	Relative change in Ca^{2+} k_{off}[b]
		μM				
Control	5.33 ± 0.02	4.8 ± 0.2	1.28 ± 0.06	NA	104.7 ± 0.5	NA
TnI R192H	5.80 ± 0.003*	1.59 ± 0.01*	1.07 ± 0.03*	↑ 3.0	73 ± 2*	↓ 1.4
TnI-(1–192)	6.15 ± 0.03*	0.71 ± 0.05*	1.14 ± 0.09	↑ 6.7	55.6 ± 0.6*	↓ 1.9
TnT ΔK210	4.81 ± 0.03*	15 ± 1*	1.19 ± 0.06	↓ 3.2	253 ± 7*	↑ 2.4
TnI R192H-TnC S69D	5.39 ± 0.01	4.08 ± 0.08	1.04 ± 0.03*	↑ 1.2	167 ± 4*	↑ 1.6
TnC S69D	4.71 ± 0.04*	20 ± 2*	0.89 ± 0.03*	↓ 4.1	224 ± 3*	↑ 2.1
TnI-(1–192)-TnCS69D/D73N	5.46 ± 0.01	3.46 ± 0.07	0.93 ± 0.04*	↑ 1.4	240 ± 7*	↑ 2.3
TnC S69D/D73N	4.78 ± 0.07*	17 ± 2*	1.12 ± 0.05	↓ 3.6	252 ± 12*	↑ 2.4
TnT ΔK210-TnC M45Q	6.01 ± 0.02*	0.98 ± 0.05*	0.77 ± 0.04*	↑ 4.8	169 ± 7*	↑ 1.6
TnTΔK210-TnCM45Q/S69D	5.38 ± 0.09	4.3 ± 0.8	0.89 ± 0.05*	↑ 1.1	84 ± 2*	↓ 1.2
TnC M45Q/S69D	5.80 ± 0.05*	1.6 ± 0.2*	1.26 ± 0.07	↑ 3.0	102 ± 2	↓ 1.0
TnC M45Q	6.23 ± 0.03*	0.59 ± 0.04*	1.45 ± 0.05	↑ 8.1	40 ± 5*	↓ 2.6

[a] The Ca^{2+} concentration at half-maximal fluorescent change.

[b] ↑ indicates increase in Ca^{2+} sensitivity or k_{off}, ↓ indicates decrease in Ca^{2+} sensitivity or k_{off}.

binding loop of its N-terminal domain (41). Utilizing a similar strategy, constructs that desensitize TnC to Ca^{2+} were also generated. One of the engineered TnC constructs, TnC S69D, desensitized thin filament Ca^{2+} binding ~4-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 1 and Table 1). Excitingly, when combined with TnI R192H, TnI R192H-TnC S69D Tn_{IAANS}^{T53C} exhibited a thin filament Ca^{2+} sensitivity and cooperativity that was indistinguishable from the control Tn_{IAANS}^{T53C} (Fig. 1 and Table 1). Thus, the increased thin filament Ca^{2+} sensitivity of an RCM mutation can be corrected through an engineered Ca^{2+}-desensitizing TnC.

Dilated cardiomyopathy (DCM) is another subtype of familial cardiomyopathy that is characterized by ventricular dilation and diminished systolic function of the left or both ventricles (25). Contrary to RCM, DCM is typically associated with decreased Ca^{2+} sensitivity of actomyosin ATPase activity and force generation (22, 25). Fig. 2 shows that the DCM TnT ΔK210 modification decreased thin filament Ca^{2+} sensitivity ~3-fold when compared with the control Tn_{IAANS}^{T53C} (Table 1). A Ca^{2+}-sensitizing TnC will be required to correct the DCM thin filament behavior. By mutating the hydrophobic pocket of the regulatory domain of TnC, Ca^{2+}-sensitizing TnC constructs can be engineered (32, 33). For instance, the TnC M45Q mutation increased thin filament Ca^{2+} sensitivity ~8-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 2*A* and Table 1). Upon combining the Ca^{2+}-sensitizing TnC M45Q mutation with the DCM TnT ΔK210 modification, the thin filament Ca^{2+} sensitivity was ~5-fold overcorrected (Fig. 2*A* and Table 1). Interestingly, the resultant change in Ca^{2+} sensitivity was roughly an additive effect of the two mutations. These data suggest that the Ca^{2+}-sensitizing TnC will need to be precisely tuned to correct the aberrant thin filament Ca^{2+} sensitivity.

To temper the strong Ca^{2+} binding of M45Q, the Ca^{2+}-desensitizing mutation S69D was introduced to fine-tune its Ca^{2+} sensitivity. TnC M45Q/S69D only sensitized thin filament Ca^{2+} sensitivity ~3-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 2*B* and Table 1). When TnC M45Q/S69D was combined with the DCM TnT ΔK210 modification, TnT ΔK210-TnC M45Q/S69D Tn_{IAANS}^{T53C} exhibited a thin filament Ca^{2+} sensitivity of 4.3 ± 0.8 μM that was nearly identical to control Tn_{IAANS}^{T53C}, yet with a slightly reduced cooperativity (Fig. 2*B* and Table 1). Thus, it is also possible to fine-tune the Ca^{2+} sensitivity of TnC and correct abnormally desensitized thin filament Ca^{2+} binding associated with a dilated cardiomyopathy.

Besides inherited cardiac muscle diseases, cardiac muscle Ca^{2+} sensitivity can also be adversely affected during acquired conditions such as ischemia reperfusion-induced injury (21). Proteolysis of myofilament proteins such as TnI has been proposed to play a key role in human myocardial ischemia/reperfusion injury (21, 42, 43). Consistent with its reported effect of sensitizing both actomyosin ATPase activity and force generation to Ca^{2+} (21), truncated TnI-(1–192) increased thin filament Ca^{2+} sensitivity ~7-fold when compared with Tn_{IAANS}^{T53C} (Fig. 3 and Table 1). To improve this extremely sensitized Ca^{2+} binding, we developed another Ca^{2+}-desensitizing TnC, S69D/D73N. TnC S69D/D73N decreased thin filament Ca^{2+} sensitivity ~4-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 3 and Table 1). When combined with the truncated TnI-(1–192), TnI-(1–192)-TnC S69D/D73N Tn_{IAANS}^{T53C} exhibited a thin filament Ca^{2+} sensitivity of 3.46 ± 0.07 μM, which was statistically indistinguishable from that of the control Tn_{IAANS}^{T53C}, albeit with a slightly reduced cooperativity (Fig. 3 and Table 1). Thus, the hypersensitized thin filament Ca^{2+} binding associated with an acquired cardiac disease can also be corrected by an engineered TnC.

Thin Filament Ca^{2+} Dissociation Rates—In addition to altering the steady-state Ca^{2+} binding properties of TnC, disease-related protein modifications have been shown to alter the rate of Ca^{2+} dissociation from TnC (44–46). Fig. 4 shows that the rate of Ca^{2+} dissociation from the thin filament reconstituted with control Tn_{IAANS}^{T53C} occurred at 104.7 ± 0.5/s (Table 1). Both disease-related Ca^{2+}-sensitizing modifications (TnI R192H and TnI-(1–192)) slowed the rate of Ca^{2+} dissociation 1.4–1.9-fold, whereas the disease-related Ca^{2+}-desensitizing modification (TnT ΔK210) accelerated the rate of Ca^{2+} dissociation ~2.4-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 4, *A–C*, and Table 1). Both correcting TnC constructs (TnC S69D and TnC S69D/D73N) engineered against the disease-related Ca^{2+}-sensitizing modifications accelerated the rate of Ca^{2+} dissociation 2.1–2.4-fold when compared with the control Tn_{IAANS}^{T53C} (Fig. 4, *A* and *C*, and Table 1). Interestingly, the correcting TnC construct M45Q/S69D designed against the disease-related Ca^{2+}-desensitizing modification had a negligible effect on the rate of Ca^{2+} dissociation (Fig. 4*B* and Table 1).

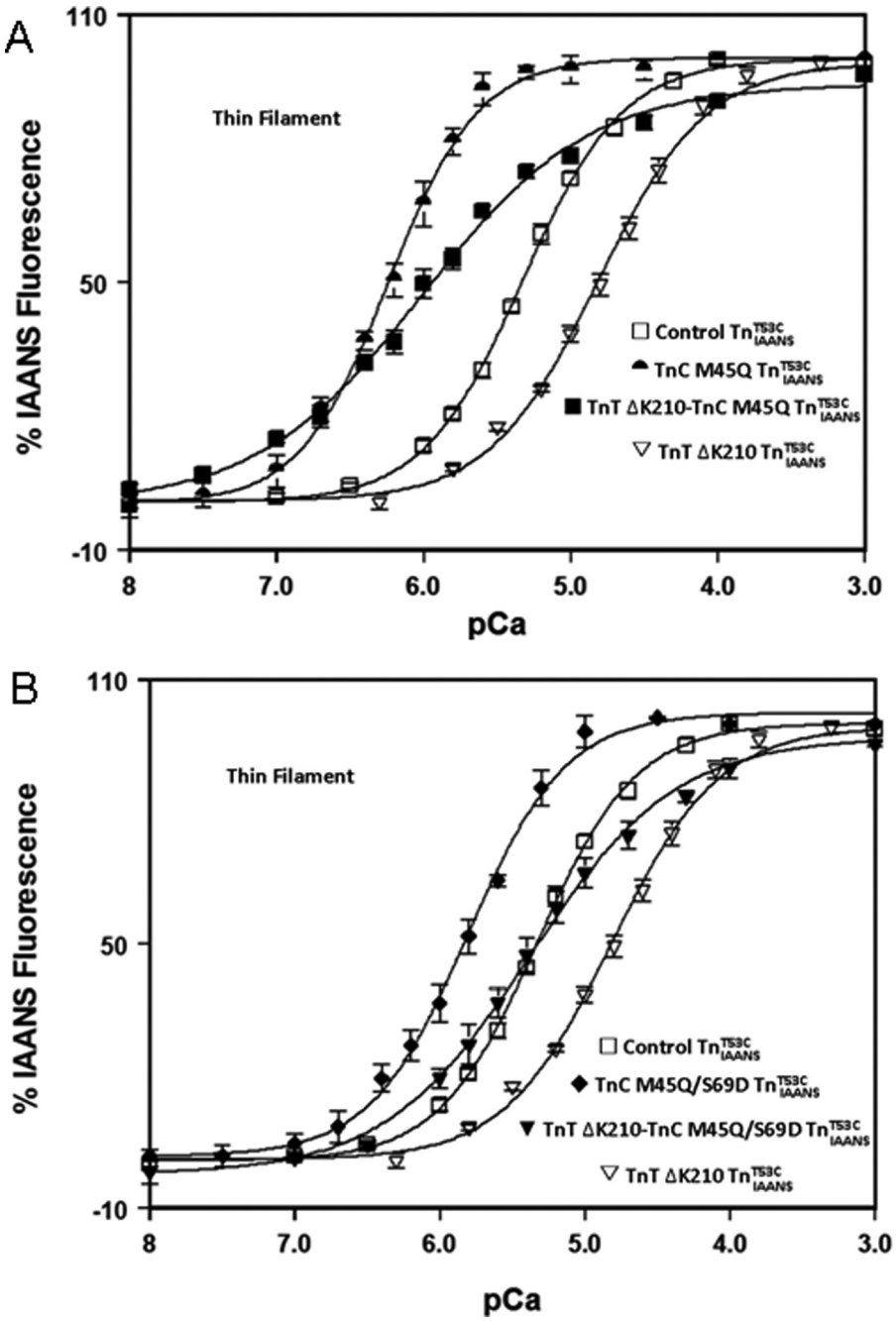

FIGURE 2. **An engineered TnC corrects DCM TnT ΔK210 thin filament Ca^{2+} sensitivity.** *A*, the Ca^{2+}-dependent changes in IAANS fluorescence for control Tn^{T53C}_{IAANS} (*open squares*), TnC M45Q Tn^{T53C}_{IAANS} (*semicircles*), TnT ΔK210-TnC M45Q Tn^{T53C}_{IAANS} (*filled squares*), and TnT ΔK210 Tn^{T53C}_{IAANS} (*inverted triangles*) reconstituted thin filaments as a function of *p*Ca. *B*, the Ca^{2+}-dependent changes in IAANS fluorescence for control Tn^{T53C}_{IAANS} (*open squares*), TnC M45Q/S69D Tn^{T53C}_{IAANS} (*filled diamonds*), TnT ΔK210-TnC M45Q/S69D Tn^{T53C}_{IAANS} (*filled inverted triangles*), and TnT ΔK210 Tn^{T53C}_{IAANS} (*open inverted triangles*) reconstituted thin filaments as a function of *p*Ca.

When combined, all of the correcting TnC constructs were able to reverse the effects of the disease-related protein modification on the rate of Ca^{2+} dissociation from the thin filament (Fig. 4, *A–C*, and Table 1).

Actomyosin S1 ATPase Assay—The thin filament Ca^{2+} binding studies demonstrated that it was feasible to engineer TnC constructs with appropriately tuned Ca^{2+} sensitivities to correct both abnormally decreased and abnormally increased Ca^{2+} binding associated with different cardiac dysfunctions. To further verify the significance of the corrected thin filament Ca^{2+} binding, the functional assay of thin filament actomyosin S1 ATPase was performed. For control Tn^{T53C}_{IAANS}, the Ca^{2+} sensitivity of the actomyosin ATPase activity occurred at 1.6 ± 0.1 μM (Fig. 5 and Table 2). Consistent with the thin filament Ca^{2+} binding studies, the RCM TnI R192H mutation sensitized the Ca^{2+}-dependent ATPase activity ~3-fold. TnC S69D desensitized the ATPase activity to Ca^{2+} ~4-fold when compared with the control Tn^{T53C}_{IAANS} (Fig. 5 and Table 2). When combined, TnI R192H-TnC S69D Tn^{T53C}_{IAANS} exhibited an actomyosin ATPase Ca^{2+} sensitivity of 1.5 ± 0.3 μM, which was indistinguishable

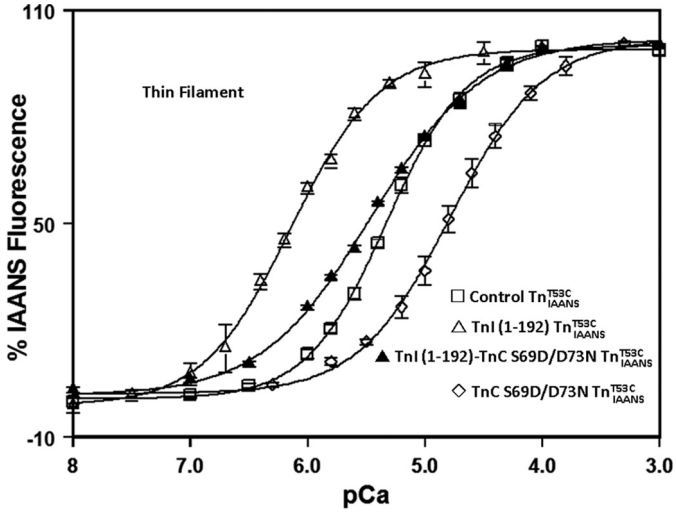

FIGURE 3. **An engineered TnC corrects ischemic TnI-(1–192) thin filament Ca^{2+} sensitivity.** The figure shows the Ca^{2+}-dependent increase in IAANS fluorescence for control Tn_{IAANS}^{T53C} (□), TnI-(1–192) Tn_{IAANS}^{T53C} (△), TnI-(1–192)-TnC S69D/D73N Tn_{IAANS}^{T53C} (▲), and TnC S69D/D73N Tn_{IAANS}^{T53C} (◇) reconstituted thin filaments as a function of *p*Ca.

from the control Tn_{IAANS}^{T53C} but with a slightly reduced cooperativity (Fig. 5 and Table 2). Thus, an engineered Ca^{2+}-desensitizing TnC was able to functionally correct the disease-associated increased Ca^{2+} sensitivity of the actomyosin ATPase activity. Unfortunately, the DCM mutation TnT ΔK210 exhibited a diminished maximal ATPase activity, whereas the ischemic TnI-(1–192) exhibited an increased basal ATPase activity (data not shown). As a result, the Ca^{2+}-regulated ATPase activity for these mutations could not be measured due to compromised signal amplitudes.

Skinned Trabecula Force Measurement—Due to the technical limitations of the actomyosin ATPase assay, force-*p*Ca measurements were performed to assess the physiological relevance of correcting TnT ΔK210 and TnI-(1–192) thin filament Ca^{2+} binding. Recombinant Tn_{IAANS}^{T53C} complexes were exchanged into rat skinned trabecula to measure the force-pCa^{2+} relationship. As shown in Fig. 6*A* and Table 3, the force-pCa_{50} occurred at 5.67 ± 0.06, 5.76 ± 0.07, and 5.74 ± 0.06 for endogenous, mock-exchanged and control Tn_{IAANS}^{T53C}-exchanged skinned trabecula, respectively. Thus, the exchange protocol, the IAANS probe, and the mutations associated with the labeling of TnC did not significantly affect the Ca^{2+} sensitivity of skinned trabecula force generation. However, the Tn exchange protocol appears to reduce both the cooperativity and the maximal force generated (Table 3). Fig. 6*B* shows that rat TnI and human TnI migrate differently in a SDS acrylamide gel and can be used to determine the efficiency of Tn exchange. Quantification of the Tn exchange by Western blot of TnI demonstrated that ~76% of the endogenous Tn was replaced by the exogenous Tn (Fig. 6*B*).

Consistent with the thin filament Ca^{2+} binding studies, DCM TnT ΔK210 desensitized skinned trabecula force generation to Ca^{2+} ~2.5-fold (Fig. 7*A* and Table 3). When combined with its correcting TnC M45Q/S69D, the Tn-exchanged trabecula exhibited a Ca^{2+} sensitivity and cooperativity indistinguishable from that of the control, with a pCa_{50} of 5.74 ± 0.04

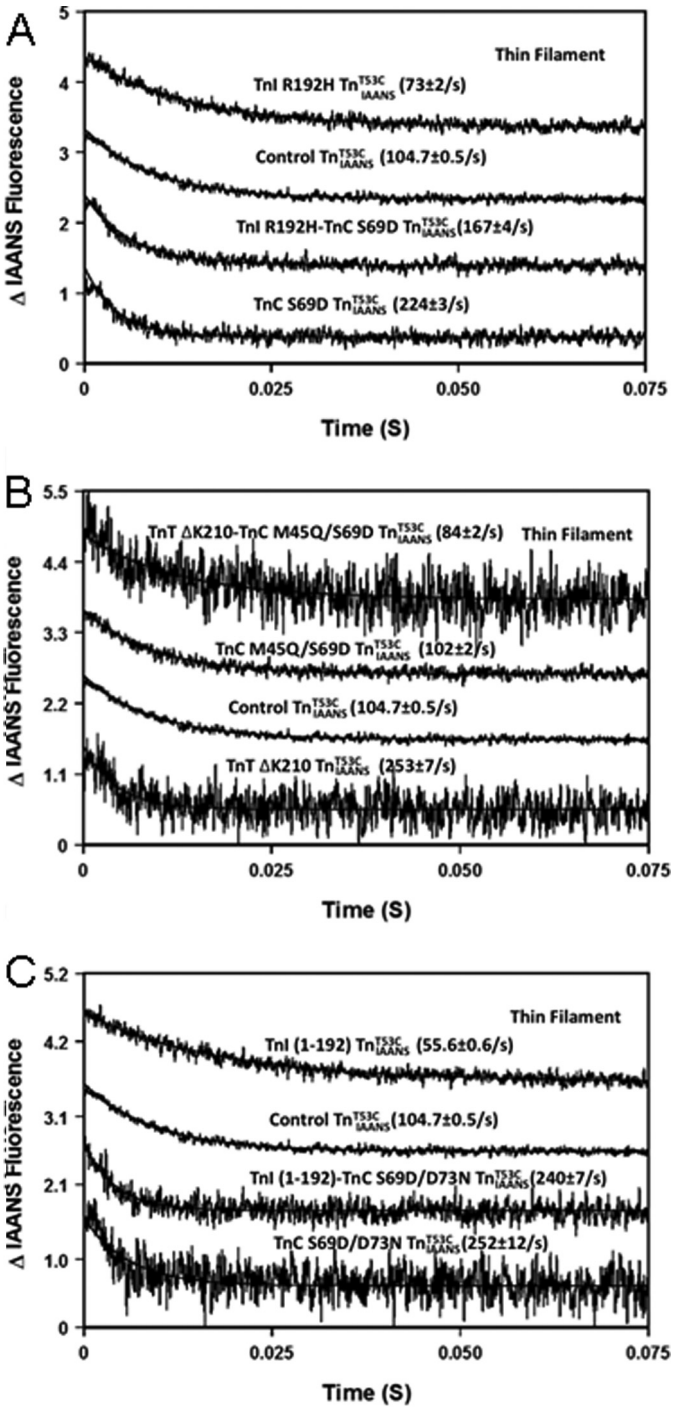

FIGURE 4. **Effect of disease-related protein modifications, correcting TnC and their combinations on rate of Ca^{2+} dissociation from thin filament.** *A*, the time courses of the decrease in IAANS fluorescence as Ca^{2+} was removed by EGTA from thin filament-bound control Tn_{IAANS}^{T53C}, TnI R192H Tn_{IAANS}^{T53C}, TnC S69D Tn_{IAANS}^{T53C}, and TnI R192H-TnC S69D Tn_{IAANS}^{T53C}. *B*, the time courses of the decrease in IAANS fluorescence as Ca^{2+} was removed by EGTA from thin filament-bound control Tn_{IAANS}^{T53C}, TnT ΔK210 Tn_{IAANS}^{T53C}, TnC M45Q/S69D Tn_{IAANS}^{T53C}, and TnT ΔK210-TnC M45Q/S69D Tn_{IAANS}^{T53C}. *C*, the time courses of the decrease in IAANS fluorescence as Ca^{2+} was removed by EGTA from thin filament-bound control Tn_{IAANS}^{T53C}, ischemic TnI(1–192) Tn_{IAANS}^{T53C}, TnC S69D/D73N Tn_{IAANS}^{T53C}, and TnI(1–192)-TnC S69D/D73N Tn_{IAANS}^{T53C}. The data traces have been staggered and normalized for clarity.

(Fig. 7*A* and Table 3). On the other hand, ischemia-induced truncated TnI-(1–192) considerably sensitized skinned trabecula force generation to Ca^{2+} ~8-fold (Fig. 7*B* and Table 3).

Additionally, truncated TnI-(1–192) uniquely raised the Ca^{2+}-independent force at *p*Ca 9.0 by ~21% (Table 3). When combined with its correcting TnC S69D/D73N, Tn-exchanged trabeculae exhibited a substantially improved Ca^{2+} sensitivity but not cooperativity, with a pCa_{50} of 5.88 ± 0.05 (Fig. 7*B* and Table 3). Furthermore, TnC S69D/D73N was able to ameliorate the elevated Ca^{2+}-independent force at *p*Ca 9.0 caused by TnI-(1–192) (Table 3). Thus, disease-related Ca^{2+} sensitivity of force generation can be corrected through engineering TnC, too.

DISCUSSION

The goal of the current study was to test the hypothesis that disease-related myofilament Ca^{2+} sensitivity can be corrected by rationally engineered TnC constructs. RCM TnI R192H, DCM TnT ΔK210, and ischemia-induced truncated TnI-(1–192) were chosen to test the hypothesis because they represent different protein (TnI or TnT) modifications (missense mutation, deletion, or truncation) and disease subtypes (familial or acquired) that can afflict both mouse and humans (27, 47, 48). By engineering TnC with a wide, yet fine-tunable, range of Ca^{2+} sensitivities, abnormally increased or decreased Ca^{2+} binding associated with different cardiac dysfunctions can be corrected, and their altered Ca^{2+} dissociation rates can be reversed. More significantly, disease-associated myofilament function can also be improved by the engineered TnC constructs.

Cardiovascular diseases are diverse and complex. Within the heart, numerous systems can fail, such as cellular coupling (electrical and mechanical), energetics, Ca^{2+} handling, and Ca^{2+} sensitivity (26, 31). Furthermore, the cooperativity and kinetics of Ca^{2+} exchange may be as significant to proper cardiac function as the overall Ca^{2+} sensitivity itself (5). Thus, targeting a single problem may not be sufficient to cure or even curb heart disease. Rather, an integrative approach may ultimately be necessary (26), of which TnC is one potential target. Although the cooperativity of Ca^{2+} binding was not always corrected by the engineered TnC constructs, improving the Ca^{2+} sensitivity may still improve outcome.

Aberrant myofilament Ca^{2+} sensitivity is commonly observed with multiple cardiac diseases, especially familial cardiomyopathies (22). Although the etiology of the cardiomyopathies remains unclear, experimental evidence shows promise that improving cardiac muscle Ca^{2+} sensitivity through either pharmacological or genetic approaches can relieve disease-related symptoms (27, 28, 30, 49). Ca^{2+} sensitizers have attracted growing clinical interest for their potential therapeutic value in treating heart failure and cardiomyopathies that desensitize cardiac muscle to Ca^{2+} (31). Although new compounds have been discovered, many of the Ca^{2+} sensitizers typically have deleterious side effects such as inhibiting cAMP phosphodiesterases and ATP-sensitive potassium channels (31). On the other hand, little effort has been put into developing therapeutic compounds that desensitize cardiac muscle to Ca^{2+}. As an alternative to pharmaceuticals, genetic approaches that directly modulate contractile proteins have recently received increasing attention. Excitingly, chimeric tropomyosin, N-terminal truncated TnI, and fetal TnT have all been shown to improve disease-related abnormal cardiac muscle Ca^{2+} sensitivity and *in vivo* function (28, 30, 49). However, it is not clear how applicable these proteins will be to correcting the wide assortment of Ca^{2+}-sensitizing and -desensitizing cardiac diseases because it is unknown how to specifically tune their performance.

For the past several decades, researchers have been discovering the rules that govern the Ca^{2+} binding properties of EF-hand proteins, especially TnC (5, 32, 33, 41, 50–54). By taking advantage of these rules, TnC has been engineered to encompass a wide range of Ca^{2+} sensitivities, which can accommodate a broad spectrum of disease-related Ca^{2+} binding (32, 33). Thus, TnC is a more versatile protein to modulate and reset disease-associated myofilament Ca^{2+} sensitivity. For instance, the ability of TnC to open its buried N-terminal hydrophobic pocket and bind TnI is a major determinant of its apparent Ca^{2+} sensitivity (Fig. 8) (32, 33). Modifying the network of side chain interactions involved with the opening of the TnI-bind-

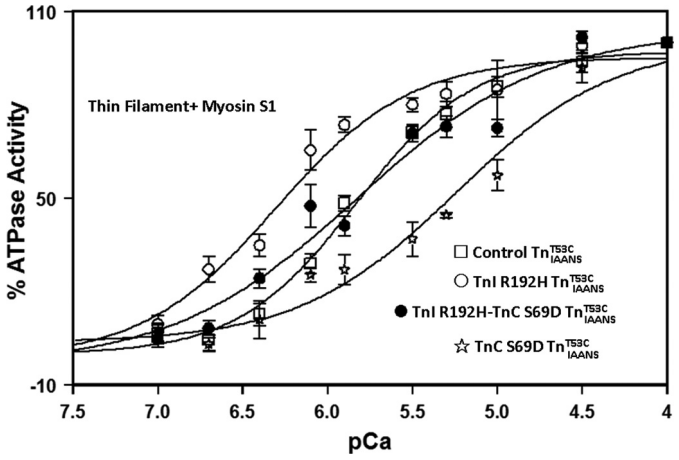

FIGURE 5. **Engineered TnCs functionally correct RCM TnI R192H actomyosin ATPase activity.** Normalized actomyosin S1 ATPase activity for thin filaments containing control Tn^{T53C}_{IAANS} (□), TnI R192H Tn^{T53C}_{IAANS} (○), TnC S69D Tn^{T53C}_{IAANS} (☆), and TnI R192H-TnC S69D Tn^{T53C}_{IAANS} (●) is plotted as a function of *p*Ca in the figure.

TABLE 2

Summary of actomyosin ATPase activity

Values marked with* are significantly different from the control values ($p < 0.05$).

Tn	ATPase pCa_{50}[a]	ATPase Ca^{2+} sensitivity	Hill coefficient	Maximal ATPase activity[b]	Minimal ATPase activity[c]
		μM			
Control	5.81 ± 0.03	1.6 ± 0.1	1.18 ± 0.09	0.037 ± 0.003	0.0072 ± 0.0006
TnI R192H	6.29 ± 0.03*	0.52 ± 0.04*	1.1 ± 0.1	0.029 ± 0.003	0.0110 ± 0.0008*
TnC S69D	5.27 ± 0.07*	5.6 ± 0.9*	1.0 ± 0.2	0.035 ± 0.003	0.010 ± 0.002
TnI R192H-TnC S69D	5.84 ± 0.09	1.5 ± 0.3	0.83 ± 0.08*	0.038 ± 0.002	0.012 ± 0.001*

[a] The Ca^{2+} concentration at half-maximal ATPase activity.
[b] Maximal ATPase activity [mol of phosphate s^{-1} (mol of S1)$^{-1}$].
[c] Minimal ATPase activity [mol of phosphate s^{-1} (mol of S1)$^{-1}$].

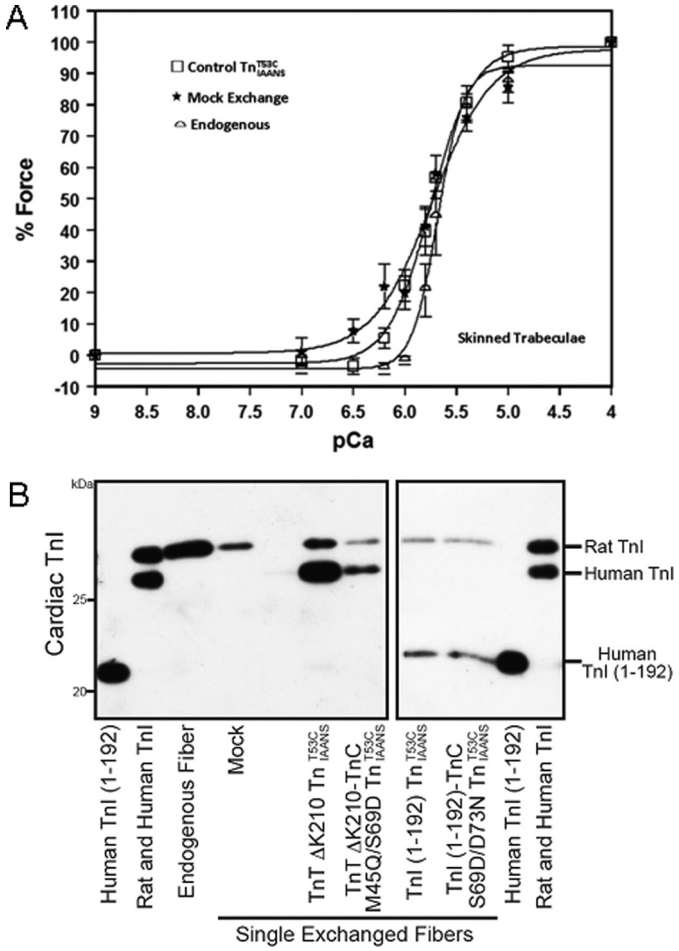

FIGURE 6. **Effect of Tn exchange on skinned cardiac muscle force generation.** *A*, the Ca^{2+}-dependent force generation for skinned trabeculae without Tn exchange (endogenous, □) and without Tn exchange but going through the same exchange protocol (mock exchange, ⋆) and for skinned trabeculae exchanged with control Tn^{T53C}_{IAANS} (□). Data traces were individually normalized. *B*, a Western blot for cardiac TnI demonstrating the presence of experimental human TnI in representative single trabecula from force experiments. Both wild-type and human TnI-(1–192) migrate faster than endogenous rat TnI, allowing for the quantification of human Tn exchanged into trabeculae from functional measurements. *Human TnI (1–192)*, recombinant, purified human TnI-(1–192) fragment; *Rat and Human TnI*, mixed recombinant wild-type rat (50%) and human purified TnI (50%).

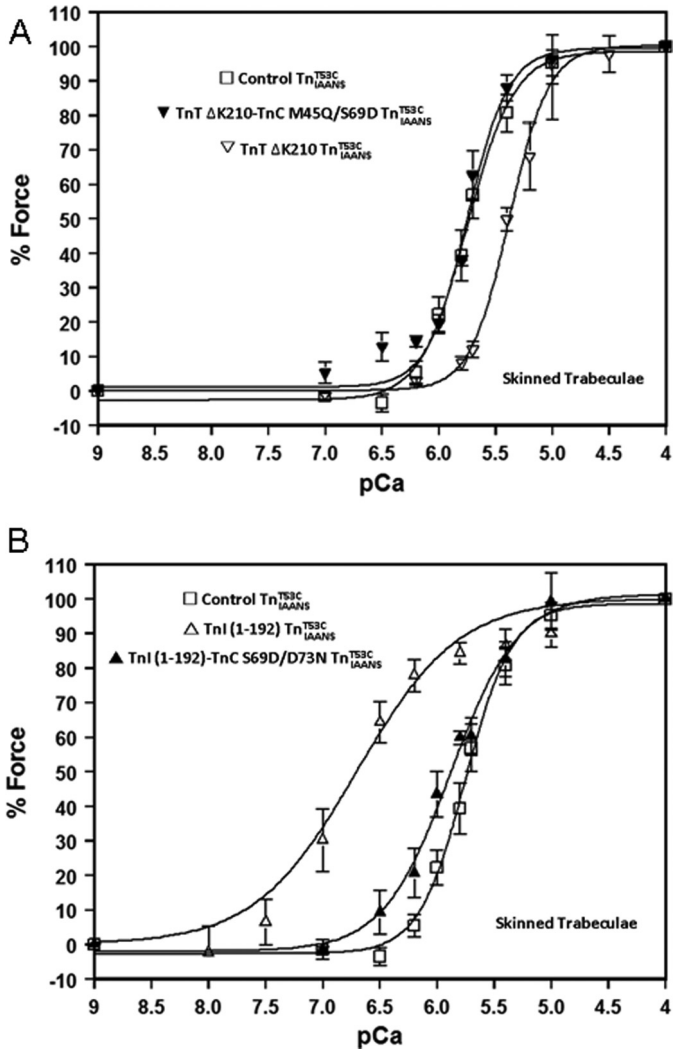

FIGURE 7. **Engineered TnC constructs functionally correct DCM TnT ΔK210 and ischemic TnI-(1–192) skinned cardiac muscle force generation.** *A*, the Ca^{2+}-dependent force generation for skinned trabeculae exchanged with control Tn^{T53C}_{IAANS} (□), TnT ΔK210 Tn^{T53C}_{IAANS} (▽), and TnT ΔK210-TnC M45Q/S69D Tn^{T53C}_{IAANS} (▼). *B*, the Ca^{2+}-dependent force generation for skinned trabeculae exchanged with control Tn^{T53C}_{IAANS} (□), TnI-(1–192) Tn^{T53C}_{IAANS} (△), and TnI-(1–192)-TnC S69D/D73N Tn^{T53C}_{IAANS} (▲). Data traces were individually normalized.

TABLE 3

Summary of skinned trabecula force generation

Values marked with* are significantly different from the control values ($p < 0.05$). NA denotes a measurement that is not applicable.

Tn	pCa_{50}[a]	Hill Coefficient	% of force recovery	% of active tension at *p*Ca 9.0
Endogenous	5.67 ± 0.06	5 ± 2	NA	9 ± 3
Mock exchange	5.76 ± 0.07	1.8 ± 0.3	68 ± 5	1 ± 1
Control	5.74 ± 0.06	2.2 ± 0.2	55 ± 7	1 ± 2
TnI-(1–192)	6.8 ± 0.1*	1.3 ± 0.1*	86 ± 8*	21 ± 4*
TnI-(1–192)-TnCS69D/D73N	5.88 ± 0.05	1.5 ± 0.1*	70 ± 6	7 ± 4
TnT ΔK210	5.35 ± 0.02*	2.7 ± 1.0	51 ± 4	0 ± 1
TnT ΔK210-TnCM45Q/S69D	5.74 ± 0.04	2.4 ± 0.3	52 ± 3	5 ± 1

[a] The Ca^{2+} concentration at half-maximal force.

ing pocket (such as the M45Q mutation; Fig. 8) can substantially affect the apparent Ca^{2+} sensitivity of TnC. Interestingly, these types of hydrophobic pocket mutations do not directly interact with the ligated Ca^{2+} ion. On the other hand, the Ca^{2+} affinity of TnC can be directly altered by manipulating the charge and position of the Ca^{2+}-chelating residues within its Ca^{2+}-binding loop. It would appear that the first and last chelating residues within the loop must be acidic for an EF-hand to bind Ca^{2+} (50, 51, 55), whereas the internal chelating loop residues can vary substantially and still allow Ca^{2+} binding (41). Altering the internal chelating loop residues can directly tune the Ca^{2+} sensitivity of the EF-hand, as was the case for the S69D and D73N mutations of TnC (Fig. 8).

It is currently unknown how the disease-related proteins alter the Ca^{2+} sensitivity of TnC. However, the C-terminal region of TnI (residues 188–210) is thought to contribute to the inhibition of the actomyosin interactions during diastole by directly binding to actin-Tm, competing for the binding of TnI with TnC (17, 56). Both alterations of TnI (TnI R192H and TnI-(1–192)) are located within this region of TnI. We hypothesize that these disease-related modifications reduce the affin-

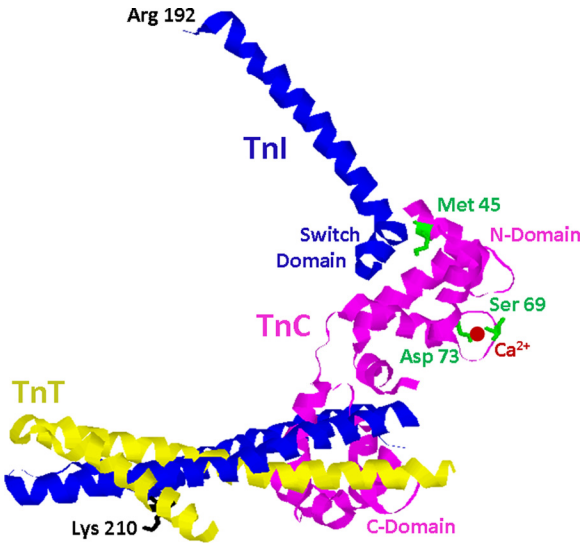

FIGURE 8. **Location of disease-related Tn modifications and engineered TnC mutations in crystal structure of Tn complex.** A ribbon representation of the cardiac Tn core domain is shown (PDB 1J1E). TnC is colored in *magenta*, TnI is colored in *blue*, and TnT is colored in *yellow*. The positions of the disease-related Tn modifications such as Lys-210 of TnT, as well as the TnC mutations including Met-45, Ser-69, and Asp-73 are labeled. The C-terminal end of TnI (residues 192–210) was not included in the crystal structure and is absent from the figure.

ity of TnI for actin-Tm. In this regard, these protein modifications would facilitate the switching of TnI from actin-Tm to TnC and cause an enhanced apparent sensitivity of TnC for Ca^{2+}. In contrast, the TnT mutation ΔK210 may increase the ability of TnI to bind to actin-Tm and decrease the apparent sensitivity of TnC for Ca^{2+}. Because the disease-related protein modifications and the engineered TnC constructs altered thin filament Ca^{2+} sensitivity through potentially different molecular mechanisms, they exerted an additive effect on thin filament Ca^{2+} binding when combined.

The qualitative similarity of the results obtained from the reconstituted thin filaments and skinned trabeculae suggests that the thin filament is a reliable model system to study thin filament Ca^{2+} sensitivity. Thus, the thin filament Ca^{2+} binding and skinned trabecula force-*p*Ca assays are efficient platforms to rapidly screen different thin filament modifications and test the efficiency of engineered TnC constructs. Although numerous disease-related protein modifications have been identified and studied, approximately half of the diagnosed cardiomyopathies remain idiopathic. Even with an unknown genetic background, directly targeting TnC could provide a way to reset the contractile performance back to normal. Ultimately, gene therapy approaches could introduce the correcting TnCs into diseased hearts to evaluate *in vivo* cardiac function. On the other hand, structural studies on the engineered TnC constructs could facilitate more specific pharmaceutical drug design targeted to TnC. The strategies utilized to modify TnC can also be applied to engineer additional EF-hand proteins such as parvalbumin and calmodulin to design potentially more therapeutic proteins for the heart or other organs (57). Thus, in addition to a potential new avenue to correct aberrant cardiac disease-related Ca^{2+} binding, the current study provides a novel perspective for engineering EF-hand Ca^{2+}-binding proteins that are universally involved in cellular signaling cascades. These protein engineering approaches in combination with other therapies may one day improve the function of the diseased heart.

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