Effects of Thin and Thick Filament Proteins on Calcium Binding and Exchange with Cardiac Troponin C

Jonathan P. Davis,* Catalina Norman,* Tomoyoshi Kobayashi,† R. John Solaro,† Darl R. Swartz,‡ and Svetlana B. Tikunova*

*Department of Physiology and Cell Biology, The Ohio State University, Columbus, Ohio; †Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois; and ‡Department of Animal Sciences, Purdue University, West Lafayette, Indiana

ABSTRACT Understanding the effects of thin and thick filament proteins on the kinetics of Ca^{2+} exchange with cardiac troponin C is essential to elucidating the Ca^{2+}-dependent mechanisms controlling cardiac muscle contraction and relaxation. Unlike labeling of the endogenous Cys-84, labeling of cardiac troponin C at a novel engineered Cys-53 with 2-(4′-iodoacetamidoanilo)-napthalene-6-sulfonic acid allowed us to accurately measure the rate of calcium dissociation from the regulatory domain of troponin C upon incorporation into the troponin complex. Neither tropomyosin nor actin alone affected the Ca^{2+} binding properties of the troponin complex. However, addition of actin-tropomyosin to the troponin complex decreased the Ca^{2+} sensitivity (~7.4-fold) and accelerated the rate of Ca^{2+} dissociation from the regulatory domain of troponin C (~2.5-fold). Subsequent addition of myosin S1 to the reconstituted thin filaments (actin-tropomyosin-troponin) increased the Ca^{2+} sensitivity (~6.2-fold) and decreased the rate of Ca^{2+} dissociation from the regulatory domain of troponin C (~8.1-fold), which was completely reversed by ATP. Consistent with physiological data, replacement of cardiac troponin I with slow skeletal troponin I led to higher Ca^{2+} sensitivities and slower Ca^{2+} dissociation rates from troponin C in all the systems studied. Thus, both thin and thick filament proteins influence the ability of cardiac troponin C to sense and respond to Ca^{2+}. These results imply that both cross-bridge kinetics and Ca^{2+} dissociation from troponin C work together to modulate the rate of cardiac muscle relaxation.

INTRODUCTION

Cardiac muscle utilizes troponin C (cTnC), an EF-hand Ca^{2+} binding protein, to sense and translate the myoplasmic Ca^{2+} signal into the cascade of events that ultimately leads to force production and/or shortening (for review see Kobayashi and Solaro (1)). In the muscle, cTnC is a subunit of the troponin (cTn) complex, consisting also of cTnI (inhibitory subunit) and cTnT (tropomyosin (cTm) binding subunit). The cTn complex is anchored to the thin filament through multiple protein interactions with actin and cTm. It is the dynamic, Ca^{2+}-dependent interplay between these thin filament proteins that permits myosin to strongly bind to actin and produce force.

It is generally assumed that the thin filament system equilibrates with Ca^{2+} very rapidly (much faster than the mechanical events of contraction and relaxation) and thus must possess fast Ca^{2+} association and dissociation rates (for review Gordon et al. (2)). This definitely is the case for the Ca^{2+} binding properties of isolated cTnC (3). However, the Ca^{2+} sensitivity of the regulatory domain of cTnC is increased at least an order of magnitude by the binding of cTnI and cTnT (4,5). The increase in Ca^{2+} sensitivity of cTnC is primarily due to a slowing of the Ca^{2+} dissociation rate from cTnC as cTnI and then cTnT is added to cTnC (from >1000/s to ~120/s then ~35/s, respectively, at 15°C (6,7)). Thus, both cTnI and cTnT influence the Ca^{2+} binding properties of cTn in solution, by decreasing the rate of Ca^{2+} dissociation from cTnC. Furthermore, in the heart the cTn complex does not function in isolation but as part of the thin filament system.

It is unclear if cTm or actin ± myosin can further affect the kinetics of Ca^{2+} dissociation from cTnC. There are conflicting reports on the effects of cTm and actin-cTm on the steady-state Ca^{2+} binding properties of cTnC (8,9). The most common approach to follow Ca^{2+} binding to cTnC has been to label one or both endogenous Cys residues with a fluorescent probe. Part of the confusion in the literature may arise from the fact that fluorescent labeling of the endogenous Cys residues in cTnC alters the biochemical properties of cTn (8,10,11). Thus, to adequately address these questions, a more accurate reporter of Ca^{2+} binding and dissociation from cTn must be developed.

Consistent with the strong influence of cTnI on the Ca^{2+} binding properties of cTnC, developmental, transgenic, viral mediated, and possibly disease related incorporation of slow skeletal TnI (ssTnI) into cardiac muscle substantially affects cardiac muscle performance (12–22). For instance, in the presence of ssTnI, cardiac muscle force is: 1), more sensitive to Ca^{2+}; 2), less sensitive to β-adrenergic stimulation; 3), more resistant to pH changes; and 4), slower to relax. These physiological phenomena can occur in the presence of cTnC and cTnT, and can be attributed almost entirely to the incorporation of ssTnI into the myofilaments. The biochemical mechanisms behind the Ca^{2+} sensitizing effects of ssTnI have not been previously investigated.

Submitted August 16, 2006, and accepted for publication January 17, 2007.

Address reprint requests to Jonathan P. Davis, Dept. of Physiology and Cell Biology, The Ohio State University, 209 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210. Tel.: 614-688-4467; Fax: 614-292-4888; E-mail: davis.812@osu.edu.

0006-3495/07/05/3195/12 $2.00

doi: 10.1529/biophysj.106.095406

In this article we report the design of a cTnC, fluorescently labeled at a novel position, that minimally affects the biological activity or the Ca^{2+} binding properties of cTnC in isolated cTn and ssTn. The fluorescent cTnC also reports the Ca^{2+} binding properties of the Tn complexes incorporated into a more physiologically relevant biochemical system, reconstituted thin filaments, in the absence and presence of myosin S1.

The objective of these studies was to systematically determine the effects of ssTnI, cTm, actin, and myosin S1 on Ca^{2+} binding and dissociation from the regulatory domain of cTnC in the Tn complex. The ultimate goal of these studies is to be able to better relate the biochemical behavior of cTnC to its physiological function.

MATERIALS AND METHODS

Materials

Phenyl-sepharose CL-4B, Bradford reagent, Tween-20, and EGTA were purchased from Sigma Chemical (St. Louis, MO). Quin-2 was purchased from Calbiochem (La Jolla, CA). 2-(4′-Iodoacetamidoanilo)napthalene-6-sulfonic acid (IAANS) and phalloidin were purchased from Invitrogen (Carlsbad, CA). Affi-Gel 15 affinity media was purchased from Bio-Rad (Hercules, CA).

Protein isolation/purification and mutagenesis

Recombinant human cTnC, cTnI, and cTnT3 (the most abundant cTnT isoform in adult cardiac muscle (23)); recombinant rat ssTnI; rabbit fast skeletal actin and myosin S1; and bovine cTm were isolated, purified, and quantified by standard laboratory procedures (7,8,24,25). The mutations C35S, T53C, and C84S in cTnC (herein denoted $cTnC^{T53C}$) were constructed from the cTnC pET3a plasmid utilizing techniques previously described (7).

Fluorescent labeling of $cTnC^{T53C}$

$cTnC^{T53C}$ was dialyzed against 50 mM Tris, 6 M urea, 90 mM KCl, 1 mM EGTA, pH 7.5. Labeling was initiated by the addition of three- to fivefold molar excess IAANS to $cTnC^{T53C}$. The labeling reaction was allowed to proceed in the dark for 5–8 h with gentle rocking at 4°C. The reaction was stopped by addition of 2 mM DTT, and unreacted IAANS was removed by exhaustive dialysis against 10 mM MOPS, 90 mM KCl, pH 7.0. $cTnC^{T53C}$ was determined to be 88 ± 7% (two different batches; all data are shown as a mean ± SE of the mean) labeled with IAANS (herein denoted $cTnC^{T53C}_{IAANS}$) utilizing the Bradford assay and the extinction coefficient for IAANS at 325nm of 24,900 $M^{-1}cm^{-1}$ (5).

Reconstitution of the Tn complexes

The Tn complexes were prepared following a modified protocol previously described by Tobacman and Lee (26). Briefly, the Tn subunits (TnC, TnI, and TnT) were first dialyzed separately against 10 mM MOPS (pH 7.0), 4.6 M urea, 1 mM DTT, and 0.01% NaN_3. After dialysis, the subunits were mixed at a molar ratio of 1:1.5:1.5 (TnC/TnI/TnT) and incubated at room temperature for 20 min. The complexes were subsequently dialyzed in sequential steps against 4 L of 10 mm MOPS (pH 7.0), 0.5 mM DTT, 3 mM $MgCl_2$, 0.01% NaN_3, and 1), 2 M urea, 1 M KCl; 2), 1 M KCl; and 3), 0.15 M KCl (this final buffer was repeated three times) at 4°C. Excess TnI and TnT that precipitated during dialysis were removed by centrifugation at 14,000 rpm for 20 min at 4°C (27).

Reconstitution of thin filaments

Purified actin filaments, myosin S1, and cTm were exhaustively dialyzed separately in 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT at pH 7.0. After dialysis actin was mixed with an equal molar ratio of phalloidin to stabilize the actin filaments. Control experiments determined that the addition of phalloidin to actin only marginally decreased the rates of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} ($cTnC^{T53C}_{IAANS}$-cTnI-cTnT; ≤1.3-fold) under the experimental conditions described below (data not shown). Actin-phalloidin (4 μM) and cTm (0.57 μM) were mixed in 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT at pH 7.0 and kept on ice for ≥20 min. Tn complexes (0.5 μM) were subsequently added to the actin-cTm mixture and kept on ice for an additional ≥15 min before use. Slightly substoichiometric Tn (7:1:0.88 (actin/cTm/Tn)) was utilized in the experiments to avoid the possibility of contaminating free Tn. Control experiments verified that the Ca^{2+} dissociation rates from the reconstituted thin filaments were not affected by increasing the actin/Tn ratios (ranging from 7:1:0.88 to 7:1:0.26 actin/cTm/Tn). Because the rates at the different actin/Tn ratios were indistinguishable, all the data were pooled and included in the average value (see Table 1). To address the effects of myosin binding on the Ca^{2+} binding properties of Tn, myosin S1 (1.14 μM) was added and allowed to equilibrate with the reconstituted thin filaments for ≥3 min. For all the data shown in the figures, the stoichiometry for the reconstituted thin filaments was 7:1:0.88:2 (actin/cTm/Tn/Mysoin S1). Initial experiments were performed utilizing bovine cardiac actin. However, due to contamination of cardiac actin with endogenous cTn and cTm, only 0.3 μM cTn^{T53C}_{IAANS} could be added to 4 μM of cardiac actin before unbound cTn^{T53C}_{IAANS} was observed via stopped-flow kinetics. This problem was not observed with rabbit fast skeletal actin. Moreover, incorporation of cTn^{T53C}_{IAANS} into reconstituted thin filaments containing cardiac or skeletal actin led to indistinguishable Ca^{2+} sensitivities and dissociation rates (data not shown). Thus, skeletal actin was utilized for the collection of data, because similar results were obtained and near stoichiometric concentrations of proteins could be used. Nonetheless, potential problems could also arise from using unmatched filament proteins (human cTn subunits, rat ssTnI, bovine cTm, and rabbit skeletal myosin S1). However, the biochemical results obtained in this work are consistent with previously observed physiological outcomes (14,28).

Steady-state fluorescence

All steady-state fluorescence measurements were performed using a Perkin-Elmer LS 55 spectrofluorimeter at 15°C. IAANS fluorescence was excited at 330 nm and monitored at 450 nm (both bandwidths set at 10 nm) as microliter amounts of $CaCl_2$ were added to 2 mL of each Tn complex (0.15 μM) in a titration buffer containing 200 mM MOPS (pH 7.0), 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.02% Tween-20, and 2 mM EGTA with constant stirring. Reconstituted thin filaments ± myosin S1 were prepared as described above, and diluted in half with an appropriate solution to achieve the same titration buffer composition (excluding Tween-20). For the myosin S1 experiments, the change in area under the emission spectra from 370 to 440 nm was utilized to plot the acquired data. The $[Ca^{2+}]_{free}$ was calculated using the computer program EGCA02 developed by Robertson and Potter (29). The Ca^{2+} sensitivities of conformational changes were reported as a dissociation constant K_d, representing a mean of three to five separate titrations ± SE. The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described (3).

Determination of Ca^{2+} dissociation kinetics (k_{off})

Ca^{2+} dissociation rates were measured using an Applied Photophysics (Leatherhead, UK) model SX.18 MV stopped-flow instrument at 15°C. The Ca^{2+} dissociation rates from the unlabeled Tn complexes were directly measured using the fluorescent Ca^{2+} chelator Quin-2. Quin-2 (150 μM before mixing) was excited using a 150-W xenon arc source at 330 nm with

TABLE 1 Ca^{2+} binding properties of the Tn^{T53C} complexes in the various systems

System	K_d (nM)	Hill coefficient	k_{off} (/s)
cTn^{T53C}	NA	NA	42.5 ± 0.3
cTn^{T53C}_{IAANS}	649 ± 20	0.90 ± 0.02	41.9 ± 0.4
cTn^{T53C}_{IAANS} + cTm	702 ± 10	0.90 ± 0.01	36 .0 ± 0.8
cTn^{T53C}_{IAANS} + Thin filament	4810 ± 300	1.65 ± 0.04	105 ± 1
cTn^{T53C}_{IAANS} + Thin filament + myosin S1	777 ± 30	0.81 ± 0.02	13.0 ± 0.1
cTn^{T53C}_{IAANS} + Thin filament + myosin S1 + ATP	5040 ± 400	1.65 ± 0.08	110 ± 1
$ssTn^{T53C}$	NA	NA	7.8 ± 0.1
$ssTn^{T53C}_{IAANS}$	248 ± 20	0.94 ± 0.03	8.4 ± 0.1
$ssTn^{T53C}_{IAANS}$ + cTm	186 ± 10	0.89 ± 0.01	7.8 ± 0.1
$ssTn^{T53C}_{IAANS}$ + Thin filament	3530 ± 200	1.52 ± 0.07	85 ± 1
$ssTn^{T53C}_{IAANS}$ + Thin filament + myosin S1	120 ± 10	0.95 ± 0.02	9.6 ± 0.1
$ssTn^{T53C}_{IAANS}$ + Thin filament + myosin S1 + ATP	2870 ± 500	1.62 ± 0.06	79 ± 1

NA represents measurements that are not applicable to the system studied.

emission monitored through a 510-nm broad band-pass interference filter (Oriel, Stratford, CT). As in the steady-state measurements, IAANS fluorescence was excited at 330 nm. IAANS emission for the labeled Tn complexes was monitored through a 420–470 nm band-pass interference filter (Oriel) or the 510-nm broad band-pass interference filter. IAANS emission for the reconstituted thin filaments ± myosin S1 was monitored through the 510-nm broad band-pass interference filter, excluding myosin S1 experiments with $ssTn^{T53C}_{IAANS}$ in which the emission was monitored through a 415–490 nm band-pass interference filter (Newport, Irvine, CA). Each k_{off} represents an average of at least five traces, fit with a single exponential and repeated ≥14 times. The buffer used for the stopped-flow experiments was 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.0. To obtain the data traces, 200 μM Ca^{2+} was added to the Tn complexes (0.3 μM), or to the reconstituted thin filaments ± S1 and rapidly mixed with buffer containing 10 mM EGTA in the stopped-flow apparatus. Control experiments were also conducted, in which the reconstituted thin filaments ± myosin S1 with Ca^{2+} were rapidly mixed with buffer containing Ca^{2+}, or reconstituted thin filaments ± myosin S1 in the presence of EGTA were rapidly mixed with buffer containing EGTA. These control traces were subtracted from the data traces to correct for scattering artifacts. The relative amplitudes of the scattering artifacts to the measured signals were 6.9 ± 0.6%, 16 ± 1%, and 6 ± 1% for the reconstituted thin filaments, in the presence of S1 and the subsequent addition of Mg-ATP, respectively ($n \geq 11$).

Skinned cardiac trabecula experiments

Solutions for the trabecula force measurements were prepared as previously described (30). All experiments were performed at 15°C. Unbranched trabecula were dissected from the right ventricle of male Lewis-Brown Norway F1 rats and placed overnight at 4°C in relaxing solution containing 1% Triton-X. All trabeculae were used within 48 h of harvest. A reticule on the eyepiece of the dissecting microscope was used to measure the width and depth of the trabecula to calculate the cross-sectional area of the muscle by assuming an elliptical circumference. Aluminum T-clips were used to mount the trabecula between the arms of a high-speed length controller (model 322C, Aurora Scientific, Ontario, Canada) and an isometric force transducer (model 403A, Aurora Scientific) in relaxing solution. The resting sarcomere length was adjusted to ~2.2 μm utilizing the first-order diffraction pattern from a HeNe laser. The trabecula was then activated in a pCa 4.0 solution and rapidly slackened after isometric force reached a plateau. The position of the high-speed length controller and the analog output of the force transducer were monitored by LabView 7.0 software (National Instruments, Austin, TX). The total force was measured between the plateau and baseline levels. The same procedure was utilized to obtain the resting force level of the trabecula in a pCa 9.0 solution. The active force generated by the trabecula in various pCa solutions was calculated as the total force minus the resting force. Average maximal active force per cross-sectional area before extraction of endogenous TnC was calculated to be 43 ± 7 kN/m^2, in which resting force accounted for 9 ± 1% of the total force ($n = 21$).

Endogenous cTnC was extracted by soaking the trabecula for 30 min in an extraction solution containing 10 mM HEPES, 5 mM EDTA, and 0.5 mM trifluoperazine dihydrochloride (TFP) at pH 7.0. The trabecula was then washed extensively with a pCa 9.0 solution to remove residual TFP. The average postextraction force was 2 ± 1% ($N = 11$) of the maximal preextraction force. cTnC was reconstituted into the trabecula by soaking the extracted trabecula for 30 min in a pCa 9.0 solution containing 16.7 μM wild-type TnC or $cTnC^{T53C}_{IAANS}$. The reconstituted trabecula was then exposed to a series of pCa solutions ranging from pCa 9.0 to pCa 4.0, to measure the Ca^{2+} sensitivity of force development. The maximally activated tension of the trabecula was obtained at pCa 4.0 before the extraction. pCa 4.0 activations were performed at the beginning, middle, and end of each force versus pCa experiment, averaged, and used to normalize the submaximal activations. During the course of the force versus pCa experiments, the rundown in force (% reduction in force of the last pCa 4.0 contraction versus the initial pCa 4.0 contraction) was 3 ± 1% ($N = 21$). For each protein, the force versus pCa experiment was repeated five to nine times.

RESULTS

Characterization of the biochemical and physiological properties of $cTnC^{T53C}_{IAANS}$

To study thin and thick filament effects on Ca^{2+} binding and kinetics with cTnC, we developed a novel fluorescent cTnC, $cTnC^{T53C}_{IAANS}$. Thr-53 was mutated to Cys for the following reasons: 1), a Thr to Cys mutation is relatively conservative; 2), Cys can be selectively labeled with environmentally sensitive fluorophores, such as IAANS; 3), Thr-53, within the BC subdomain (consisting of helices B and C), moves away from the NAD subdomain (consisting of helices N, A, and D) as the regulatory domain of cTnC binds Ca^{2+} and interacts with cTnI, potentially changing its environment for spectroscopic analysis (Fig. 1, *A* and *B*); 4), Thr-53 does not directly ligate Ca^{2+} and is spatially separated from the Ca^{2+} binding loop, thus upon mutation should not directly interfere with Ca^{2+} binding; 5), Thr-53 does not interact with the residues within cTnC that contribute to the core of the hydrophobic pocket utilized to bind cTnI and that have been shown to modulate Ca^{2+} sensitivity of cTnC (7); and 6), Thr-53 itself does not interact with cTnI or cTnT (as can be observed from the Ca^{2+} saturated cTn structures (31)). Thus, substitution of Thr-53 with Cys and subsequent labeling with IAANS was expected to minimally affect cTnC function, and report the structural changes that occur in the regulatory domain of cTnC upon Ca^{2+} binding and dissociation.

As predicted, Fig. 1 *B* (*B1*), shows that the fluorescence emission intensity of cTn^{T53C}_{IAANS} was sensitive to Ca^{2+} binding, and decreased ~43% upon Ca^{2+} saturation at 450 nm. The Ca^{2+}-dependent decrease in fluorescence intensity

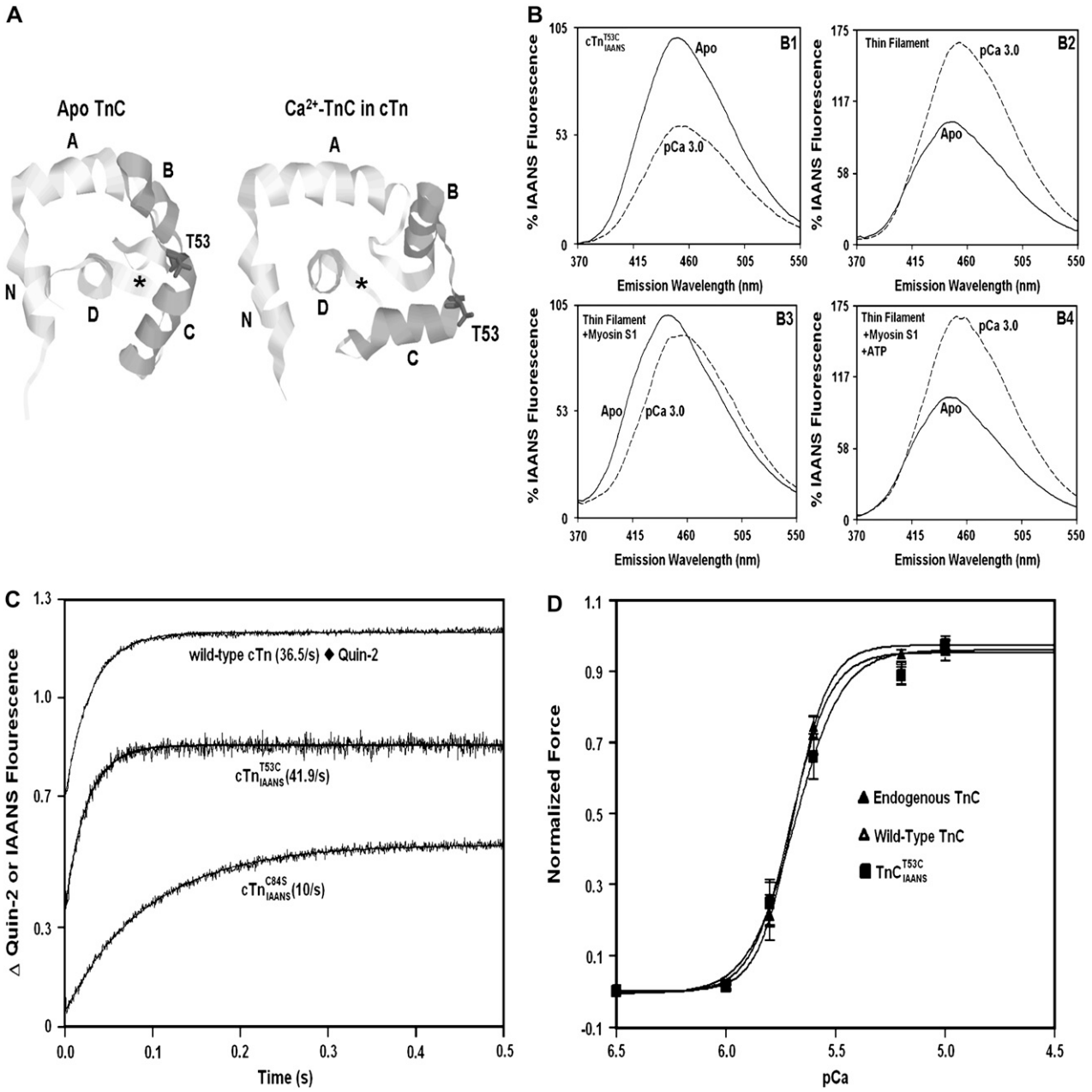

FIGURE 1 $cTnC^{T53C}_{IAANS}$ behaves biochemically and physiologically similar to wild-type and endogenous cTnC. Panel *A* shows a ribbon representation of the regulatory domain of cTnC in the apo state (1SPY (53)) and in Ca^{2+} saturated cTn (1J1E; TnI, and TnT have been omitted for clarity (31)) utilizing the software Rasmol (54). The regulatory domain of cTnC contains five helices denoted as N, A, B, C, and D. As shown in panel *A*, helices B and C (BC subdomain) move away from the N, A, and D helices (NAD subdomain) upon Ca^{2+} and TnI binding. The D-helix is pointing out of the page, with the NAD subdomain colored light gray, the BC subdomain colored dark gray, and the Ca^{2+} binding loop indicated by an asterisk (*). Thr-53 is depicted in a stick representation. Panel *B* shows the IAANS emission spectra of the apo state (*solid line*) and Ca^{2+} saturated state (*dashed line*, pCa 3.0) for $cTnC^{T53C}_{IAANS}$ in the cTn complex (*B1*), in reconstituted thin filaments (*B2*), plus myosin S1 (*B3*), and plus ATP (*B4*). The emission fluorescence of the spectra was calculated relative to the peak fluorescence of each respective apo state, which was considered 100%. Panel *C* shows the time course of Ca^{2+} dissociation from wild-type cTn directly followed by an increase in quin-2 fluorescence. Panel *C* also shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and cTn^{C84S}_{IAANS} reported by an increase in IAANS fluorescence. Overlaid with the kinetic traces are the fitted exponential curves to the data (*smooth curves*, which may be difficult to discern). Panel *D* shows the Ca^{2+}-dependent increase in force development in skinned rat trabeculae containing endogenous cTnC (▲), wild-type cTnC (△), and $cTnC^{T53C}_{IAANS}$(■).

of cTn_{IAANS}^{T53C} was only marginally affected by addition of cTm (data not shown). However, upon incorporation of cTn_{IAANS}^{T53C} into reconstituted actin-cTm (thin filament), the fluorescence intensity of cTn_{IAANS}^{T53C} increased ~63% at 450 nm upon Ca^{2+} saturation (Fig. 1 *B* (*B2*)). Thus, the Ca^{2+}-induced change in the fluorescence of free cTn_{IAANS}^{T53C} was spectroscopically distinct from that of cTn_{IAANS}^{T53C} bound to the thin filament. In the presence of myosin S1, Ca^{2+} saturation of the reconstituted thin filaments induced a red shift in the maximal emission fluorescence and ~35% decrease in the area under the emission spectra between 370 and 440 nm (Fig. 1 *B* (*B3*)). Addition of Mg-ATP reversed the effects of rigor myosin S1, causing the reconstituted thin filament system to appear as if there was no myosin S1 in the solution (Fig. 1 *B* (*B2* and *B4*)). Thus, the fluorescence of cTn_{IAANS}^{T53C} reports the Ca^{2+}-dependent structural changes that occur in the regulatory domain of cTnC in the cTn complex, and in reconstituted thin filaments in the absence and presence of myosin S1.

To determine whether the fluorescence of cTn_{IAANS}^{T53C} reported the Ca^{2+}-dependent structural changes associated with the regulatory domain of cTnC, stopped-flow studies were performed. Ca^{2+} dissociated from the regulatory domain of wild-type human cTn at a rate of 36.5 ± 0.4/s utilizing the fluorescent Ca^{2+} chelator quin-2 (Fig. 1 *C*), which was similar to what we have previously reported for the regulatory domain of wild-type human cTn under slightly different environmental conditions (6). As the $[Ca^{2+}]_{free}$ was rapidly chelated by EGTA, subsequently causing Ca^{2+} to dissociate from cTn_{IAANS}^{T53C}, the IAANS fluorescence increased at a rate of 41.9 ± 0.4/s (Fig. 1 *C* and Table 1). Over a longer time (200 s) there was an additional ~14% increase in fluorescence associated with the C-terminal domain of $cTnC_{IAANS}^{T53C}$ that occurred at 0.04 ± 0.01/s (data not shown). Thus, the decrease in IAANS fluorescence that occurs as cTn_{IAANS}^{T53C} binds Ca^{2+} can be largely attributed to the regulatory N-terminal domain of cTnC. Furthermore, it appears that the initial and major IAANS fluorescence increase occurs at the actual Ca^{2+} dissociation rate from the regulatory domain of cTn. This was not the case for the rate of IAANS fluorescence increase that occurred with the endogenously labeled Cys-35 of cTn_{IAANS}^{C84S} (10 ± 0.3/s; Fig. 1 *C*). Thus, as predicted and unlike cTn_{IAANS}^{C84S}, it appears that the fluorescence of cTn_{IAANS}^{T53C} follows and minimally affects the Ca^{2+} binding properties of the regulatory domain of cTn. Consistent with this idea, skinned trabeculae reconstituted with $cTnC_{IAANS}^{T53C}$ developed force with nearly identical Ca^{2+} sensitivity (pCa 5.68 ± 0.03) and Hill coefficient (4.7 ± 0.5) values as wild-type cTnC (pCa 5.70 ± 0.02 and 5.5 ± 0.8 Hill coefficient) and endogenous cTnC (pCa 5.70 ± 0.02 and 6.5 ± 0.5 Hill coefficient; Fig. 1 *D*). Furthermore, $cTnC_{IAANS}^{T53C}$ was able to substantially recover force (79 ± 4%) to a similar extent as wild-type cTnC (79 ± 7%). Thus, $cTnC_{IAANS}^{T53C}$ behaved biochemically and physiologically similar to wild-type and endogenous cTnC, at 15°C.

Effects of ssTnI on the emission spectra of $cTnC_{IAANS}^{T53C}$ in systems of increasing complexity

We also wanted to test whether $cTnC_{IAANS}^{T53C}$ could be utilized to follow Ca^{2+} binding and dissociation from ssTn. Similar to cTn_{IAANS}^{T53C}, Fig. 2 *A* shows that the fluorescence emission intensity of $ssTn_{IAANS}^{T53C}$ ($cTnC_{IAANS}^{T53C}$-ssTnI-cTnT) was sensitive to Ca^{2+} binding, and decreased ~41% upon Ca^{2+} saturation at 450 nm. The Ca^{2+}-dependent decrease in fluorescence intensity of $ssTn_{IAANS}^{T53C}$ was also only marginally affected by addition of cTm (data not shown). Again similar to cTn_{IAANS}^{T53C}, Ca^{2+} saturation of $ssTn_{IAANS}^{T53C}$ incorporated into reconstituted actin-cTm increased the fluorescence intensity ~39% at 450 nm (Fig. 2 *B*). In the presence of myosin S1, Ca^{2+} saturation of the reconstituted thin filaments induced a red shift in the maximal emission fluorescence and decreased the area under the emission spectra between 370 and 440 nm by ~25% (Fig. 2 *C*), which could be reversed to the thin filament state by addition of Mg-ATP (Fig. 2 *D*). Thus, the Ca^{2+}-dependent structural changes that occur in the regulatory domain of ssTn can be followed by the fluorescence of $cTnC_{IAANS}^{T53C}$ in the ssTn complex, and in reconstituted thin filaments in the absence and presence of myosin S1.

Ca^{2+} binding properties and Ca^{2+} dissociation rates from the Tn complexes

Fig. 3 *A* shows the Ca^{2+}-dependent decreases in IAANS fluorescence of cTn_{IAANS}^{T53C} and $ssTn_{IAANS}^{T53C}$. Consistent with physiological observations that ssTnI increases the Ca^{2+} sensitivity of cardiac muscle force development (14), the Ca^{2+} sensitivity of $ssTn_{IAANS}^{T53C}$ was ~2.6-fold higher than that of cTn_{IAANS}^{T53C} (comparison of K_d values shown in Table 1). The apparent Hill coefficients for both Tn complexes were less than one, indicative of a negative cooperative Ca^{2+} binding process (Table 1).

Consistent with $ssTn_{IAANS}^{T53C}$ displaying a higher Ca^{2+} sensitivity than cTn_{IAANS}^{T53C}, the rate of Ca^{2+} dissociation from $ssTn_{IAANS}^{T53C}$ was approximately fivefold slower than that reported by cTn_{IAANS}^{T53C} (Fig. 3 *B*). Fig. 3 *B* also demonstrates that the rate of structural change reported by IAANS for both Tn complexes occurs at the actual rate of Ca^{2+} dissociation from the unlabeled Tn complexes. Thus, $cTnC_{IAANS}^{T53C}$ is an excellent probe to follow the Ca^{2+} binding and kinetic properties of cardiac and slow skeletal Tn.

Ca^{2+} binding properties and Ca^{2+} dissociation rates from the Tn complexes in the presence of cTm, or actin ± myosin S1 without cTm

Because the Tn complex does not function in muscle in isolation but as an integral part of the thin filament, it is essential to understand the effects of actin and other relevant proteins on the behavior of cTn and ssTn. Fig. 4 *A* and

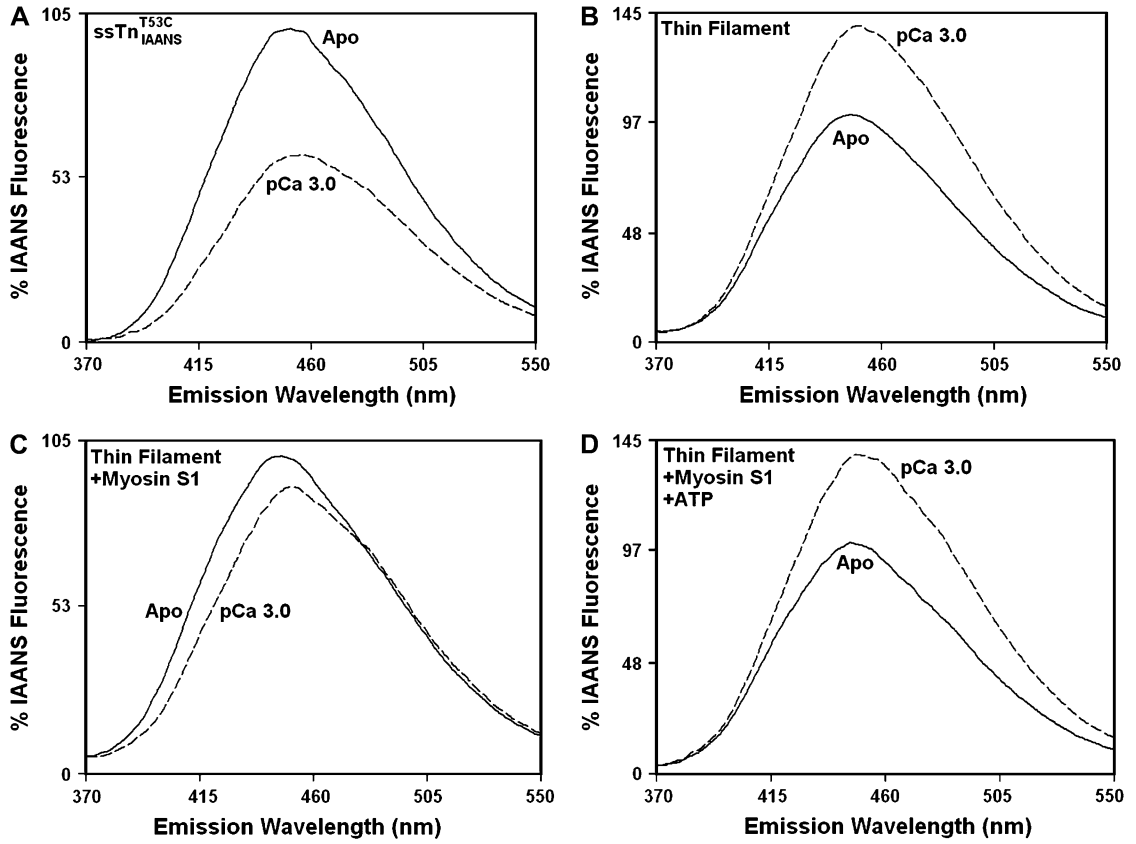

FIGURE 2 Effects of ssTnI on the IAANS emission spectra of $cTnC^{T53C}_{IAANS}$ in systems of increasing complexity. Panels *A–D* show the IAANS emission spectra of the apo state (*solid line*) and Ca^{2+}-saturated state (*dashed line*, pCa 3.0) for $cTnC^{T53C}_{IAANS}$ in the ssTn complex (*A*), in reconstituted thin filaments (*B*), plus myosin S1 (*C*), and plus ATP (*D*). The emission fluorescence of the spectra was calculated relative to the peak fluorescence of each respective apo state, which was considered 100%.

Table 1 show that the addition of cTm to either cTn^{T53C}_{IAANS} or $ssTn^{T53C}_{IAANS}$ had only marginal effects on their Ca^{2+} sensitivities and apparent cooperativities. Fig. 4, *B* and *C*, also show that the addition of cTm had little effect on the rate of structural change in the fluorescent Tn complexes as Ca^{2+} was rapidly chelated by EGTA (for comparison see Table 1). Additionally, in the absence of cTm, neither actin alone nor actin in the presence or absence of myosin S1 affected the apparent rate of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} (Fig. 4 *B*) or $ssTn^{T53C}_{IAANS}$ (Fig. 4 *C*). Thus, at the molar ratios used, neither cTm, actin, nor myosin S1 have nonspecific effects on the Tn complexes.

Ca^{2+} binding properties and Ca^{2+} dissociation rates from the Tn complexes in the presence of cTm-actin

Although cTm or actin alone had little effect on the Ca^{2+} binding properties of the Tn complexes, the combination of the two proteins drastically affected the Tn complexes. Fig. 5 *A* shows the Ca^{2+}-dependent increase in cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ fluorescence in reconstituted thin filaments. Incorporation of cTn^{T53C}_{IAANS} into reconstituted thin filaments caused an ~7.4-fold decrease in the Ca^{2+} sensitivity and ~1.8-fold increase in the Hill coefficient of cTn^{T53C}_{IAANS} (Table 1). Similarly, incorporation of $ssTn^{T53C}_{IAANS}$ into the reconstituted thin filaments caused an ~14-fold decrease in the Ca^{2+} sensitivity and ~1.6-fold increase in the Hill coefficient of $ssTn^{T53C}_{IAANS}$ (Table 1). Furthermore, thin filaments reconstituted with $ssTn^{T53C}_{IAANS}$ were ~1.4-fold more Ca^{2+} sensitive than those reconstituted with cTn^{T53C}_{IAANS}. Thus, incorporation of the Tn complexes into the thin filaments decreased the Ca^{2+} sensitivity of cTnC, but led to a positive cooperative Ca^{2+} binding process.

Consistent with the lower Ca^{2+} sensitivities of the Tn complexes on the thin filaments, Fig. 5 *B* shows that compared to the isolated Tn complexes, the rates of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments increased ~2.5-fold and ~10.1-fold, respectively (Table 1). Furthermore, in the reconstituted thin filaments, Ca^{2+} dissociated from $ssTn^{T53C}_{IAANS}$ only ~1.2-fold more slowly than from cTn^{T53C}_{IAANS}. Thus, actin-cTm attenuates the Ca^{2+} sensitizing effects of ssTnI on cTnC.

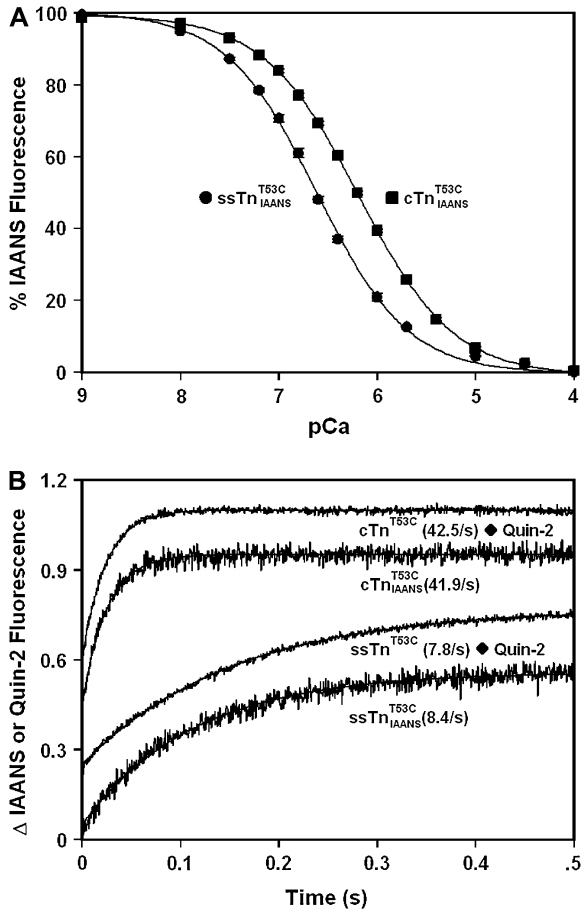

FIGURE 3 Ca^{2+} sensitivities and dissociation rates from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$. Panel *A* shows the Ca^{2+}-dependent decrease in IAANS fluorescence of cTn^{T53C}_{IAANS} (■) and $ssTn^{T53C}_{IAANS}$ (●). Panel *B* shows the time courses of Ca^{2+} dissociation from unlabeled cTn^{T53C} and $ssTn^{T53C}$ directly followed by an increase in quin-2 fluorescence. Panel *B* also shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reported by an increase in IAANS fluorescence.

Effects of myosin S1 on the Ca^{2+} binding properties and Ca^{2+} dissociation rates from the Tn complexes in reconstituted thin filaments

It has long been known that strong cross-bridge binding increases the apparent Ca^{2+} sensitivity of cTnC in muscle (28). Consistent with this effect, Fig. 6 *A* shows that addition of myosin S1 to the reconstituted thin filaments increased the Ca^{2+} sensitivity of cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ ~6.2-fold and 29-fold, respectively (Table 1). Similar to that of the isolated Tn complexes, the apparent Hill coefficients of the reconstituted thin filaments bound with myosin S1 indicated a negative cooperative Ca^{2+} binding process (Table 1). The effect of myosin S1 could be completely reversed by the addition of Mg-ATP, causing the Ca^{2+} sensitivity of the Tn complexes to be similar to that of the reconstituted thin filaments in the absence of myosin S1 (Fig. 6 *A*; Table 1). Furthermore, dissociation of attached myosin S1 from actin by addition of Mg-ATP increased the Hill coefficients back

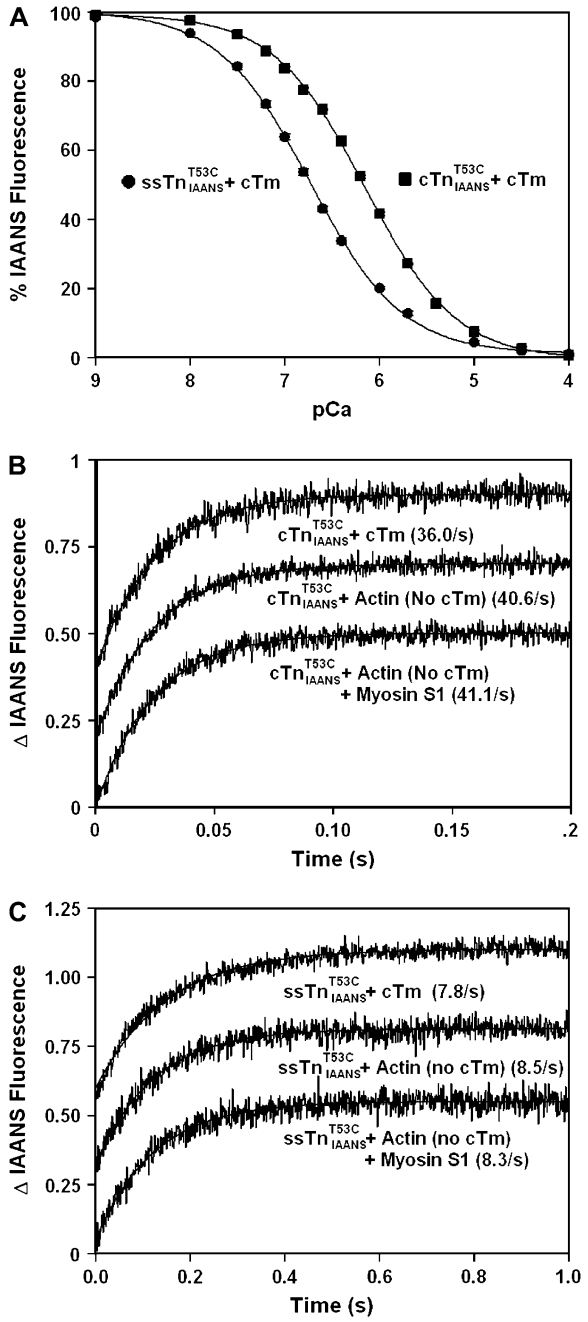

FIGURE 4 Ca^{2+} binding properties and Ca^{2+} dissociation rates from the Tn complexes in the presence of cTm, or actin ± myosin S1 without cTm. Panel *A* shows the Ca^{2+}-dependent decrease in IAANS fluorescence of cTn^{T53C}_{IAANS} (■) and $ssTn^{T53C}_{IAANS}$ (●) in the presence of cTm. Panel *B* shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} (0.3 μM) in the presence of cTm (0.9 μM) or actin (4 μM) ± myosin S1 (0.57 μM) reported by an increase in IAANS fluorescence. Panel *C* shows the EGTA-induced time courses of Ca^{2+} dissociation from $ssTn^{T53C}_{IAANS}$ (0.3 μM) in the presence of cTm (0.9 μM) or actin (4 μM) ± myosin S1 (0.57 μM) reported by an increase in IAANS fluorescence. Increasing the concentration of cTn^{T53C}_{IAANS} or $ssTn^{T53C}_{IAANS}$ to 0.5 μM did not alter the results (data not shown).

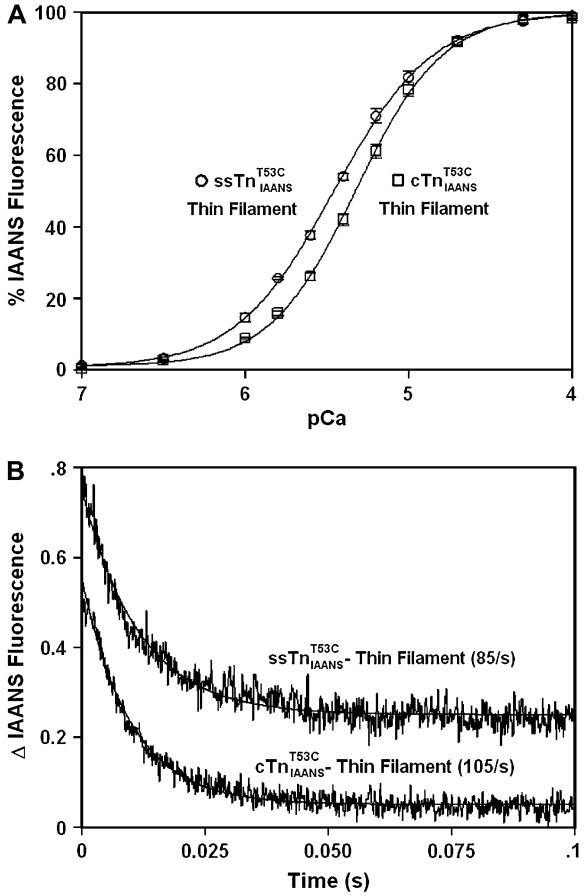

FIGURE 5 Ca^{2+} sensitivities and dissociation rates from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments. Panel *A* shows the Ca^{2+}-dependent increase in IAANS fluorescence of cTn^{T53C}_{IAANS} (□) and $ssTn^{T53C}_{IAANS}$ (○) reconstituted thin filaments. Panel *B* shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments reported by a decrease in IAANS fluorescence.

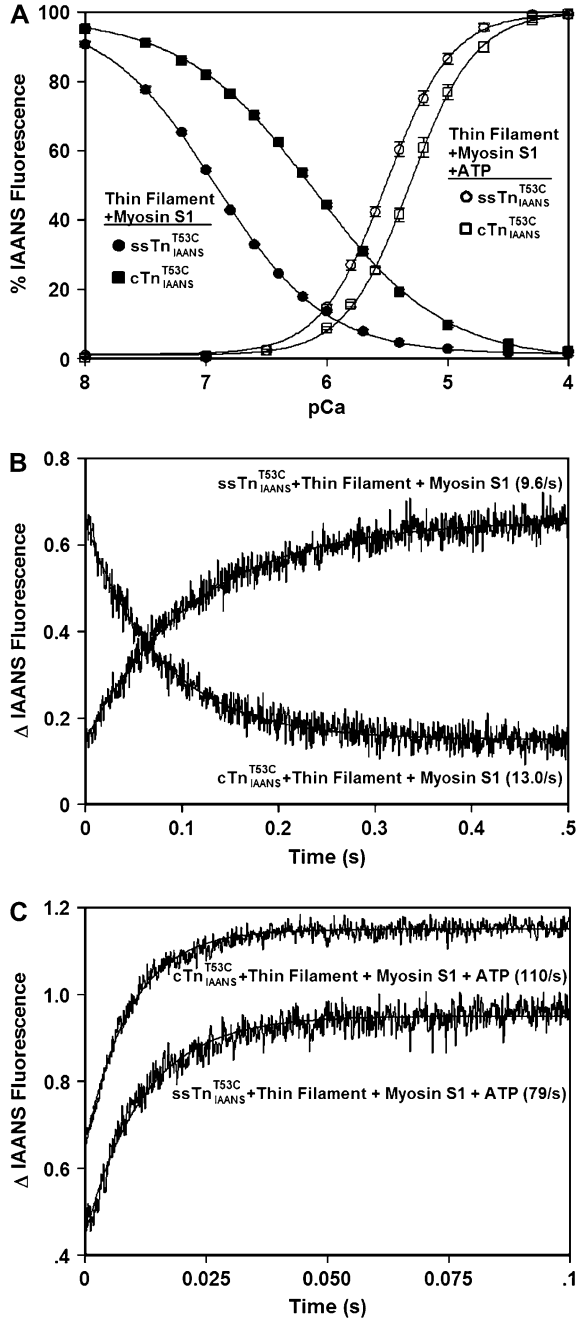

FIGURE 6 Effects of myosin S1 on the Ca^{2+} sensitivities and dissociation rates from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments. Panel *A* shows the Ca^{2+}-dependent decrease in IAANS fluorescence of cTn^{T53C}_{IAANS} (■) and $ssTn^{T53C}_{IAANS}$ (●) reconstituted thin filaments in the presence of myosin S1. Panel *A* also shows the Ca^{2+}-dependent increase in IAANS fluorescence of cTn^{T53C}_{IAANS} (□) and $ssTn^{T53C}_{IAANS}$ (○) reconstituted thin filaments in the presence of myosin S1 and ATP. Panel *B* shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments in the presence of myosin S1 reported by changes in IAANS fluorescence. The EGTA-induced decrease in cTn^{T53C}_{IAANS} fluorescence as opposed to the increase in $ssTn^{T53C}_{IAANS}$ fluorescence is due to the different emission filters used to capture the signals. Panel *C* shows the EGTA-induced time courses of Ca^{2+} dissociation from cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$ reconstituted thin filaments in the presence of myosin S1 and ATP reported by an increase in IAANS fluorescence.

to that of the thin filament alone, which again indicated a positive cooperative Ca^{2+} binding process (Table 1). Additionally, in the presence of rigor myosin S1, $ssTn^{T53C}_{IAANS}$ displayed ~6.5-fold higher Ca^{2+} sensitivity than cTn^{T53C}_{IAANS}. However, the Ca^{2+} sensitivity of $ssTn^{T53C}_{IAANS}$ was only ~1.8-fold higher than that of cTn^{T53C}_{IAANS} after addition of Mg-ATP (Table 1).

Consistent with the increased Ca^{2+} sensitivity of the Tn complexes upon addition of myosin S1 to the reconstituted thin filaments, the rates of Ca^{2+} dissociation reported by a change in IAANS fluorescence decreased ~8.1-fold and ~8.9-fold for cTn^{T53C}_{IAANS} and $ssTn^{T53C}_{IAANS}$, respectively (Fig. 6 *B* and Table 1). Fig. 6 *C* shows that the decrease in the rate of Ca^{2+} dissociation from the Tn complexes caused by myosin S1 binding could be completely reversed by the addition of Mg-ATP (Table 1). The qualitative effects on the Ca^{2+} sensitivity and Ca^{2+} dissociation rate from cTnC in the reconstituted thin filaments by actin-cTm and myosin S1 were similar to that observed for skeletal TnC in reconstituted thin

filaments (32). In the presence of myosin S1 (without Mg-ATP), the rate of Ca^{2+} dissociation from the thin filaments reconstituted with $ssTn_{IAANS}^{T53C}$ was only ~1.4-fold slower than from thin filaments containing cTn_{IAANS}^{T53C} (Fig. 6 *B* and Table 1). Thus, myosin S1 binding to actin-cTm potentiates the Ca^{2+} sensitizing effects of ssTnI on cTnC without proportionally slowing the rate of Ca^{2+} dissociation.

DISCUSSION

To determine the effects of myofilament proteins on cTnC function, we have fluorescently labeled cTnC at a novel position. This was accomplished by mutating the endogenous Cys residues to Ser and Thr-53 to Cys, which was then labeled with the environmentally sensitive fluorophore IAANS, resulting in $cTnC_{IAANS}^{T53C}$. When incorporated into the cTn complex, $cTnC_{IAANS}^{T53C}$ biochemically and physiologically responded to changes in [Ca^{2+}] similarly to wild-type and endogenous cTnC. This cannot be stated for the functional behavior of cTnC labeled with IAANS on either endogenous Cys-35 or 84. Labeling of the endogenous Cys residues on cTnC has been shown to modify the Ca^{2+} binding properties of cTn and the Ca^{2+}-dependent ATPase activity of cardiac reconstituted thin filaments (Fig. 1 *C* and (8,10,11)). Furthermore, the ability of $cTnC_{IAANS}^{T53C}$ to remain spectroscopically sensitive to Ca^{2+} binding in the Tn complex and on the thin filament is unique and different from that observed with single labeling of either endogenous Cys residue of cTnC (8,11). Thus, $cTnC_{IAANS}^{T53C}$ is a very useful and novel sensor to test the effects of thin and thick filament proteins on the Ca^{2+} binding properties of the cTn and ssTn complexes.

Neither cTm nor actin (± S1) affected the Ca^{2+} binding properties of the Tn complexes. However, reconstitution of the thin filaments decreased the Ca^{2+} sensitivity and increased the rate of Ca^{2+} dissociation from both Tn complexes. Thus, actin-cTm drastically attenuated the Ca^{2+} sensitizing effects of cTnI and cTnT on cTnC. This observation is consistent with cTnC and actin competing for binding to the C-terminal domain of cTnI (for review see Kobayashi and Solaro (1)). This competition for TnI binding may be the reason for the reduced Ca^{2+} sensitivity of the troponin complexes upon incorporation into the reconstituted thin filaments.

The positive cooperativity of Ca^{2+} binding observed in the reconstituted thin filaments may arise from Ca^{2+} binding to one stoichiometric unit (seven actins, one cTm dimer, and one cTn complex), allowing cTnC to out compete actin for cTnI, resulting in cTm repositioning on actin. This cTm movement is thought to span over a distance greater than a single stoichiometric unit (for review see Solaro and Rarick (33)) and may weaken the cTnI-actin interactions in neighboring stoichiometric units. These weakened cTnI-actin interactions will effectively increase the probability of cTnC binding to cTnI, increasing the apparent Ca^{2+} sensitivity of the neighboring cTnCs and thus cooperativity. Consistent with this hypothesis, positive cooperativity of Ca^{2+} binding was not observed with the Tn complexes alone or with ''mini''-thin filaments that contain only a single stoichiometric unit (8,9,34).

If cTnC and actin truly act as competitive antagonists for cTnI binding, and this was the sole mechanism determining the Ca^{2+} sensitivity of cTnC on the thin filament, then the rate of Ca^{2+} dissociation from cTnC bound to cTnI in the different systems should not change. Alternatively or additionally, the Ca^{2+} sensitivity, cooperativity, and dissociation rate from the Tn complexes on the thin filaments may be modulated by cTnT-cTm interactions or direct cTnT-cTnC interactions (for review see Farah and Reinach (35)), which may change depending on the state of the thin filament. Consistent with cTnT and cTm influencing the Ca^{2+} sensitivity of cTn on the thin filament, isoform variations and disease-related mutations in cTnT and cTm have been shown to affect the Ca^{2+} sensitivity and cooperativity of cardiac muscle force development, even in the presence of ssTnI (6,36,37).

The myosin S1 effects on the reconstituted thin filaments were studied with two myosin S1 per stoichiometric unit. This concentration of myosin S1 is at the upper limit for the physiological range of myosin strongly bound to a stoichiometric unit during maximal contraction in muscle (for review (2,38)). The effect of myosin S1 on the Ca^{2+} sensitivity of the reconstituted thin filaments is consistent with strong binding cross-bridges increasing the Ca^{2+} sensitivity of cTnC in cardiac muscle (11,28,39,40). For cTn, the myosin S1 induced increase in the Ca^{2+} sensitivity of the thin filaments (approximately sixfold) could be explained by a proportionally similar decrease in the rate of Ca^{2+} dissociation from cTnC (approximately eightfold). The effects of myosin S1 binding to the thin filaments could be completely reversed by the addition of Mg-ATP. The molecular mechanism(s) behind these S1 effects on the thin filament are unknown. However, myosin binding to actin moves Tm further on actin than does Ca^{2+} binding alone (for review see Gordon et al. (38)) and drastically increases the affinity of cTm for actin (41). Thus, myosin binding to actin may cause a structural change in actin and move cTm to ultimately increase the probability of cTnI-cTnC binding. Furthermore, the positive cooperativity of Ca^{2+} binding to the thin filaments was abolished in the presence of myosin S1. Because our Ca^{2+} binding studies were performed after the addition of myosin S1, it is likely that the rigor binding of myosin S1 already displaced cTm so that the binding of Ca^{2+} to the Tn complexes had no effect on repositioning cTm (the proposed mechanism for the cooperativity). Consistent with the effects of myosin S1 on the thin filaments, the addition of NEM-S1 (an irreversible rigor like myosin S1) to cardiac muscle increased the Ca^{2+} sensitivity of force development with a reduced cooperativity (for review see Moss et al. (42)).

It is generally assumed that Ca^{2+} dissociation from cTnC in muscle is very rapid and that the rate of cross-bridge detachment is the primary determinant of cardiac muscle relaxation (for review see Gordon et al. (2)). Our data clearly demonstrate that myosin binding to actin drastically slows the rate of Ca^{2+} dissociation from cTn to a rate comparable to that of cardiac muscle relaxation (~5–11/s at similar ionic strength and the same temperature used in this article (15°C); (43–45)). The fact that myosin binding slows the Ca^{2+} dissociation rate from cTnC incorporated into the thin filament fits well with recent mathematical models predicting this phenomenon to describe steady-state (46) and kinetic (47) muscle data. Assuming the reconstituted thin filament is a reasonable model system for studying the kinetics of Ca^{2+} dissociation from cTnC in muscle, then it may be that both cross-bridge kinetics and Ca^{2+} dissociation from cTn work together to modulate the rate of cardiac muscle relaxation. However, the reconstituted thin filaments represent only two extreme conditions, unbound myosin and rigor bound myosin. At the physiological salt conditions used in this work it is not possible to address the effects of cycling cross-bridges on cTnC.

Consistent with the fact that ssTnI increases the Ca^{2+} sensitivity of force development and slows the rate of cardiac muscle relaxation and relengthening (14–17,21), we have demonstrated that biochemically ssTn displayed a higher Ca^{2+} sensitivity and slower Ca^{2+} dissociation rate than cTn, under all experimental conditions. These data further support the hypothesis that the Ca^{2+} dissociation rate from Tn may influence the rate of cardiac muscle relaxation. Interestingly, there are numerous cTnI mutations linked to hypertrophic cardiomyopathy that also display increased myofilament and force Ca^{2+} sensitivities (8,48,49). However, transgenic expression of ssTnI in an adult heart does not trigger a hypertrophic response and actually improves cardiac muscle performance under various pathophysiological conditions (14,50–52). It may be that the hypertrophic cardiomyopathy related cTnI mutants increase the myofilament Ca^{2+} sensitivity through different mechanism(s) from that of ssTnI. For instance, unlike ssTnI, several hypertrophic cardiomyopathy linked cTnI mutants did not directly increase the Ca^{2+} sensitivity of cTnC in the Tn complex (8).

Steady-state affinities are set by the rates of association and dissociation. It is clear from the data that the relative changes in the Ca^{2+} sensitivity of the Tn complexes that occur upon incorporation into the reconstituted thin filaments in the absence and presence of myosin S1 are not always associated with proportional changes in the respective Ca^{2+} dissociation rates. Contrary to dogma, these results imply that the Ca^{2+} association rates to cTn and ssTn in the various biochemical systems must also change. This hypothesis is consistent with the fact that several Ca^{2+} sensitizing mutations in $cTnC^{F27W}$ increased the Ca^{2+} association rates to the regulatory domain of isolated cTnC (7). As a consequence of this phenomenon, ssTnI has a much larger effect on the Ca^{2+} sensitivity of the Tn complex incorporated into the reconstituted thin filaments in the presence of myosin S1 than on the rate of Ca^{2+} dissociation, compared to that of cTnI. This mechanism would allow cardiac muscle expressing ssTnI to be substantially more sensitive to Ca^{2+}, while only marginally affecting the rate of cardiac muscle relaxation. Consistent with our results, cardiac muscle expressing ssTnI exhibits a much larger increase in the Ca^{2+} sensitivity of force development than a decrease in the rate of muscle relaxation or relengthening (14,17,21).

In summary we developed a novel fluorescent cTnC that accurately reports the rate of Ca^{2+} dissociation from cTnC incorporated into cTn and ssTn. Furthermore, the fluorescent cTnC also reports the Ca^{2+} binding properties of the Tn complexes reconstituted into thin filaments in the absence and presence of myosin S1. This study demonstrated: 1), reconstitution of cTnC into the thin filament dramatically increases the Ca^{2+} dissociation rate from cTn; 2), myosin S1 substantially decreases the rate of Ca^{2+} dissociation from cTn reconstituted into thin filaments to a rate similar to reported rates of cardiac muscle relaxation; 3), ssTn possesses a higher Ca^{2+} sensitivity than cTn in all systems studied; and 4), ssTn possesses a slower Ca^{2+} dissociation rate than cTn reconstituted into thin filaments in the absence and presence of myosin S1. Thus, all the major thin filament proteins (TnI, TnT, Tm, and actin) have direct or indirect effects on the Ca^{2+} sensitivity and dissociation rate from cTnC. Even the thick filament protein myosin can drastically alter the sensitivity and kinetics of Ca^{2+} dissociation from cTnC. All these proteins influence the ability of cTnC to sense and respond to the Ca^{2+} signal, allowing cardiac muscle to be finely or grossly tuned to perform mechanically on a beat-to-beat basis.

We thank Dr. Lawrence Smillie for the generous gift of the human cTnC and cTnI plasmids, and Dr. James D. Potter and Dr. Aldrin V. Gomes for the generous gift of human cTnT3 protein. We thank Dr. Jack Rall for critical reading of the manuscript and financial support. We also thank Bin Liu, Laszlo Sarkozy, and Chad Warren for technical assistance.

This research was funded, in part, by National Institutes of Health grants HL073828 (D.R.S.), HL022231 (R.J.S.), HL073600 (S.B.T.), and AR020792 (Jack A. Rall), and awards from the American Heart Association (to J.P.D., C.N., S.B.T., and T.K. (0230038N));

REFERENCES

1. Kobayashi, T., and R. J. Solaro. 2005. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu. Rev. Physiol.* 67:39–67.
2. Gordon, A. M., E. Homsher, and M. Regnier. 2000. Regulation of contraction in striated muscle. *Physiol. Rev.* 80:853–924.
3. Tikunova, S. B., J. A. Rall, and J. P. Davis. 2002. Effect of hydrophobic residue substitutions with glutamine on Ca(2+) binding and exchange with the N-domain of troponin C. *Biochemistry.* 41:6697–6705.
4. Dong, W. J., J. M. Robinson, J. Xing, and H. C. Cheung. 2003. Kinetics of conformational transitions in cardiac troponin induced by

Ca2+ dissociation determined by Forster resonance energy transfer. *J. Biol. Chem.* 278:42394–42402.

5. Johnson, J. D., J. H. Collins, S. P. Robertson, and J. D. Potter. 1980. A fluorescent probe study of Ca2+ binding to the Ca2+-specific sites of cardiac troponin and troponin C. *J. Biol. Chem.* 255:9635–9640.
6. Gomes, A. V., G. Venkatraman, J. P. Davis, S. B. Tikunova, P. Engel, R. J. Solaro, and J. D. Potter. 2004. Cardiac troponin T isoforms affect the Ca(2+) sensitivity of force development in the presence of slow skeletal troponin I: insights into the role of troponin T isoforms in the fetal heart. *J. Biol. Chem.* 279:49579–49587.
7. Tikunova, S. B., and J. P. Davis. 2004. Designing calcium-sensitizing mutations in the regulatory domain of cardiac troponin C. *J. Biol. Chem.* 279:35341–35352.
8. Kobayashi, T., and R. J. Solaro. 2006. Increased Ca2+ affinity of cardiac thin filaments reconstituted with cardiomyopathy-related mutant cardiac troponin I. *J. Biol. Chem.* 281:13471–13477.
9. Robinson, J. M., W. J. Dong, J. Xing, and H. C. Cheung. 2004. Switching of troponin I: Ca(2+) and myosin-induced activation of heart muscle. *J. Mol. Biol.* 340:295–305.
10. Tobacman, L. S., and D. Sawyer. 1990. Calcium binds cooperatively to the regulatory sites of the cardiac thin filament. *J. Biol. Chem.* 265:931–939.
11. Putkey, J. A., W. Liu, X. Lin, S. Ahmed, M. Zhang, J. D. Potter, and W. G. Kerrick. 1997. Fluorescent probes attached to Cys 35 or Cys 84 in cardiac troponin C are differentially sensitive to Ca(2+)-dependent events in vitro and in situ. *Biochemistry.* 36:970–978.
12. Wolska, B. M., K. Vijayan, G. M. Arteaga, J. P. Konhilas, R. M. Phillips, R. Kim, T. Naya, J. M. Leiden, A. F. Martin, P. P. de Tombe, and R. J. Solaro. 2001. Expression of slow skeletal troponin I in adult transgenic mouse heart muscle reduces the force decline observed during acidic conditions. *J. Physiol.* 536:863–870.
13. Wolska, B. M., G. M. Arteaga, J. R. Pena, G. Nowak, R. M. Phillips, S. Sahai, P. P. de Tombe, A. F. Martin, E. G. Kranias, and R. J. Solaro. 2002. Expression of slow skeletal troponin I in hearts of phospholamban knockout mice alters the relaxant effect of beta-adrenergic stimulation. *Circ. Res.* 90:882–888.
14. Fentzke, R. C., S. H. Buck, J. R. Patel, H. Lin, B. M. Wolska, M. O. Stojanovic, A. F. Martin, R. J. Solaro, R. L. Moss, and J. M. Leiden. 1999. Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. *J. Physiol.* 517:143–157.
15. Metzger, J. M., D. E. Michele, E. M. Rust, A. R. Borton, and M. V. Westfall. 2003. Sarcomere thin filament regulatory isoforms. Evidence of a dominant effect of slow skeletal troponin I on cardiac contraction. *J. Biol. Chem.* 278:13118–13123.
16. Westfall, M. V., E. M. Rust, and J. M. Metzger. 1997. Slow skeletal troponin I gene transfer, expression, and myofilament incorporation enhances adult cardiac myocyte contractile function. *Proc. Natl. Acad. Sci. USA.* 94:5444–5449.
17. Pena, J. R., and B. M. Wolska. 2004. Troponin I phosphorylation plays an important role in the relaxant effect of beta-adrenergic stimulation in mouse hearts. *Cardiovasc. Res.* 61:756–763.
18. Dieckman, L. J., and R. J. Solaro. 1990. Effect of thyroid status on thin-filament Ca2+ regulation and expression of troponin I in perinatal and adult rat hearts. *Circ. Res.* 67:344–351.
19. Metzger, J. M., W. I. Lin, and L. C. Samuelson. 1994. Transition in cardiac contractile sensitivity to calcium during the in vitro differentiation of mouse embryonic stem cells. *J. Cell Biol.* 126:701–711.
20. Thijssen, V. L., J. Ausma, L. Gorza, H. M. van der Velden, M. A. Allessie, I. C. Van Gelder, M. Borgers, and G. J. van Eys. 2004. Troponin I isoform expression in human and experimental atrial fibrillation. *Circulation.* 110:770–775.
21. Layland, J., D. J. Grieve, A. C. Cave, E. Sparks, R. J. Solaro, and A. M. Shah. 2004. Essential role of troponin I in the positive inotropic response to isoprenaline in mouse hearts contracting auxotonically. *J. Physiol.* 556:835–847.
22. Reiser, P. J., M. V. Westfall, S. Schiaffino, and R. J. Solaro. 1994. Tension production and thin-filament protein isoforms in developing rat myocardium. *Am. J. Physiol.* 267:H1589–H1596.
23. Gomes, A. V., G. Guzman, J. Zhao, and J. D. Potter. 2002. Cardiac troponin T isoforms affect the Ca2+ sensitivity and inhibition of force development. Insights into the role of troponin T isoforms in the heart. *J. Biol. Chem.* 277:35341–35349.
24. Swartz, D. R., M. L. Greaser, and B. B. Marsh. 1990. Regulation of binding of subfragment 1 in isolated rigor myofibrils. *J. Cell Biol.* 111:2989–3001.
25. Swartz, D. R., and R. L. Moss. 1992. Influence of a strong-binding myosin analogue on calcium-sensitive mechanical properties of skinned skeletal muscle fibers. *J. Biol. Chem.* 267:20497–20506.
26. Tobacman, L. S., and R. Lee. 1987. Isolation and functional comparison of bovine cardiac troponin T isoforms. *J. Biol. Chem.* 262:4059–4064.
27. Szczesna, D., R. Zhang, J. Zhao, M. Jones, G. Guzman, and J. D. Potter. 2000. Altered regulation of cardiac muscle contraction by troponin T mutations that cause familial hypertrophic cardiomyopathy. *J. Biol. Chem.* 275:624–630.
28. Pan, B. S., and R. J. Solaro. 1987. Calcium-binding properties of troponin C in detergent-skinned heart muscle fibers. *J. Biol. Chem.* 262:7839–7849.
29. Robertson, S., and J. D. Potter. 1984. The regulation of free Ca2+ ion concentration by metal chelators. *Methods in Pharmacology.* 5:63–75.
30. Greaser, M. L., R. L. Moss, and P. J. Reiser. 1988. Variations in contractile properties of rabbit single muscle fibres in relation to troponin T isoforms and myosin light chains. *J. Physiol.* 406:85–98.
31. Takeda, S., A. Yamashita, K. Maeda, and Y. Maeda. 2003. Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form. *Nature.* 424:35–41.
32. Rosenfeld, S. S., and E. W. Taylor. 1985. Kinetic studies of calcium binding to regulatory complexes from skeletal muscle. *J. Biol. Chem.* 260:252–261.
33. Solaro, R. J., and H. M. Rarick. 1998. Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. *Circ. Res.* 83:471–480.
34. Gong, H., V. Hatch, L. Ali, W. Lehman, R. Craig, and L. S. Tobacman. 2005. Mini-thin filaments regulated by troponin-tropomyosin. *Proc. Natl. Acad. Sci. USA.* 102:656–661.
35. Farah, C. S., and F. C. Reinach. 1995. The troponin complex and regulation of muscle contraction. *FASEB J.* 9:755–767.
36. Gomes, A. V., J. A. Barnes, K. Harada, and J. D. Potter. 2004. Role of troponin T in disease. *Mol. Cell. Biochem.* 263:115–129.
37. Wolska, B. M., and D. M. Wieczorek. 2003. The role of tropomyosin in the regulation of myocardial contraction and relaxation. *Pflugers Arch.* 446:1–8.
38. Gordon, A. M., M. Regnier, and E. Homsher. 2001. Skeletal and cardiac muscle contractile activation: tropomyosin ''rocks and rolls''. *News Physiol. Sci.* 16:49–55.
39. Wang, Y. P., and F. Fuchs. 1994. Length, force, and Ca(2+)-troponin C affinity in cardiac and slow skeletal muscle. *Am. J. Physiol.* 266:C1077–C1082.
40. Bell, M. G., E. B. Lankford, G. E. Gonye, G. C. Ellis-Davies, D. A. Martyn, M. Regnier, and R. J. Barsotti. 2006. Kinetics of cardiac thin-filament activation probed by fluorescence polarization of rhodamine-labeled troponin C in skinned guinea pig trabeculae. *Biophys. J.* 90: 531–543.
41. Cassell, M., and L. S. Tobacman. 1996. Opposite effects of myosin subfragment 1 on binding of cardiac troponin and tropomyosin to the thin filament. *J. Biol. Chem.* 271:12867–12872.
42. Moss, R. L., M. Razumova, and D. P. Fitzsimons. 2004. Myosin crossbridge activation of cardiac thin filaments: implications for myocardial function in health and disease. *Circ. Res.* 94:1290–1300.
43. Saeki, Y., K. Takigiku, H. Iwamoto, S. Yasuda, H. Yamashita, S. Sugiura, and H. Sugi. 2001. Protein kinase A increases the rate of

relaxation but not the rate of tension development in skinned rat cardiac muscle. *Jpn. J. Physiol.* 51:427–433.

44. Fitzsimons, D. P., J. R. Patel, and R. L. Moss. 1998. Role of myosin heavy chain composition in kinetics of force development and relaxation in rat myocardium. *J. Physiol.* 513:171–183.
45. Fitzsimons, D. P., J. R. Patel, and R. L. Moss. 2001. Cross-bridge interaction kinetics in rat myocardium are accelerated by strong binding of myosin to the thin filament. *J. Physiol.* 530:263–272.
46. Robinson, J. M., Y. Wang, W. G. Kerrick, R. Kawai, and H. C. Cheung. 2002. Activation of striated muscle: nearest-neighbor regulatory-unit and cross-bridge influence on myofilament kinetics. *J. Mol. Biol.* 322:1065–1088.
47. Ter Keurs, H. E., Y. Wakayama, M. Miura, T. Shinozaki, B. D. Stuyvers, P. A. Boyden, and A. Landesberg. 2006. Arrhythmogenic Ca(2+) release from cardiac myofilaments. *Prog. Biophys. Mol. Biol.* 90:151–171.
48. Lang, R., A. V. Gomes, J. Zhao, P. R. Housmans, T. Miller, and J. D. Potter. 2002. Functional analysis of a troponin I (R145G) mutation associated with familial hypertrophic cardiomyopathy. *J. Biol. Chem.* 277:11670–11678.
49. Gomes, A. V., J. Liang, and J. D. Potter. 2005. Mutations in human cardiac troponin I that are associated with restrictive cardiomyopathy affect basal ATPase activity and the calcium sensitivity of force development. *J. Biol. Chem.* 280:30909–30915.
50. Arteaga, G. M., C. M. Warren, S. Milutinovic, A. F. Martin, and R. J. Solaro. 2005. Specific enhancement of sarcomeric response to Ca2+ protects murine myocardium against ischemia-reperfusion dysfunction. *Am. J. Physiol. Heart Circ. Physiol.* 289:H2183–H2192.
51. Layland, J., A. C. Cave, C. Warren, D. J. Grieve, E. Sparks, J. C. Kentish, R. J. Solaro, and A. M. Shah. 2005. Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. *FASEB J.* 19:1137–1139.
52. Urboniene, D., F. A. Dias, J. R. Pena, L. A. Walker, R. J. Solaro, and B. M. Wolska. 2005. Expression of slow skeletal troponin I in adult mouse heart helps to maintain the left ventricular systolic function during respiratory hypercapnia. *Circ. Res.* 97:70–77.
53. Spyracopoulos, L., M. X. Li, S. K. Sia, S. M. Gagne, M. Chandra, R. J. Solaro, and B. D. Sykes. 1997. Calcium-induced structural transition in the regulatory domain of human cardiac troponin C. *Biochemistry.* 36:12138–12146.
54. Sayle, R. A., and E. J. Milner-White. 1995. RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* 20:374–376.