THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 22, pp. 15114–15121, May 30, 2008

Increased Cross-bridge Cycling Kinetics after Exchange of C-terminal Truncated Troponin I in Skinned Rat Cardiac Muscle*

Received for publication, February 28, 2008 Published, JBC Papers in Press, March 31, 2008, DOI 10.1074/jbc.M801636200

Kittipong Tachampa, Tomoyoshi Kobayashi, Helen Wang, Anne F. Martin, Brandon J. Biesiadecki, R. John Solaro, and Pieter P. de Tombe[1]

From the Center for Cardiovascular Research and Department of Physiology and Biophysics, University of Illinois, Chicago, Illinois 60612

The precise mechanism of cardiac troponin I (cTnI) proteolysis in myocardial stunning is not fully understood. Accordingly, we determined the effect of cTnI C terminus truncation on chemo-mechanical transduction in isolated skinned rat trabeculae. Recombinant troponin complex (cTn), containing either mouse cTnI-(1–193) or human cTnI-(1–192) was exchanged into skinned cardiac trabeculae; Western blot analysis confirmed that 60–70% of the endogenous cTn was replaced by recombinant Tn. Incorporation of truncated cTnI induced significant reductions (~50%) in maximum force and cooperative activation as well as increases (~50%) in myofilament Ca^{2+} sensitivity and tension cost. Similar results were obtained with either mouse or human truncated cTn. Presence of truncated cTnI increased maximum actin-activated S1 ATPase activity as well as its Ca^{2+} sensitivity *in vitro*. Partial exchange (50%) for truncated cTnI resulted in similar reductions in maximum force and cooperativity; tension cost was increased in proportion to truncated cTnI content. *In vitro*, to determine the molecular mechanism responsible for the enhanced myofilament Ca^{2+} sensitivity, we measured Ca^{2+} binding to cTn as reported using a fluorescent probe. Incorporation of truncated cTnI did not affect Ca^{2+} binding affinity to cTn alone. However, when cTn was incorporated into thin filaments, cTnI truncation induced a significant increase in Ca^{2+} binding affinity to cTn. We conclude that cTnI truncation induces depressed myofilament function. Decreased cardiac function after ischemia/reperfusion injury may directly result, in part, from proteolytic degradation of cTnI, resulting in alterations in cross-bridge cycling kinetics.

In experiments described here, we investigated the functional significance of losing the C terminus region of cardiac TnI as found in myocardial stunning. Myocardial stunning is a form of postischemic dysfunction that persists after restoration of normal coronary flow in the absence of irreversible damage. It has become increasingly evident that myocardial stunning may contribute significantly to the morbidity associated with coronary artery disease (1). As such, elucidation of molecular mechanisms underlying myocardial stunning is critical to aid in the development of novel therapeutic strategies. Proteolytic degradation of cardiac cTn I (cTnI)[2] has emerged has a potential cellular mechanism underlying the depressed contractile function seen in myocardial stunning, possibly as the result of Ca^{2+} activation of the protease calpain-I upon reperfusion (1) and/or increased mechanical load on the heart (2). However, these findings are species-dependent (*i.e.* no degradation of cTnI was observed in larger animal models of myocardial stunning, such as the pig (3) or dog (4)). In addition, although application of activated calpain-I to rodent skinned myocardium results in depressed myofilament function (5, 6), this effect is reported to be correlated with the degradation of cTnI in some (5) but not all studies (6). Importantly, cTnI degradation has been reported in both in human cardiac tissue obtained during coronary artery bypass surgery (7) and in explanted hearts with transplantation (8). Finally, overexpression of truncated rat cTnI in transgenic mice results in a recapitulation of the "stunned phenotype" (*i.e.* reduced cardiac function *in vivo* and reduced myofilament force *in vitro*) (9). Hence, post-translational alterations in myofilament proteins, particularly C-terminal truncations of cTnI, probably play a central role in the depressed myocardial function that is seen in myocardial stunning.

A proteolytic alteration in cTnI has the potential to significantly alter thin filament activation processes and also, as we show here, affect cross-bridge stability and cycling kinetics. The proteolytic damage to cTnI following ischemia/reperfusion has been identified as cleavage of 17 amino acid residues from the C terminus of cTnI. In addition to the inhibitory region of cTnI, biochemical and biophysical studies have identified the C terminus of the molecule as an additional regulatory domain with significant binding affinity to actin (10) and required for the maximum inhibition of the cardiac thin filament. During diastole, several regions of cTnI hold the cardiac sarcomere in the relaxed state by binding to actin and in concert with TNT main-

* This work was supported, in whole or in part, by National Institutes of Health Grants HL62426, HL77195, HL75494, HL082923, HL22231, HL07692, and HL072742. This study was supported in part by American Heart Association Predoctoral Fellowship 0615597Z (to K. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: 835 S. Wolcott, Physiology and Biophysics MC901, Chicago, IL 60612. Tel.: 312-355-0259; Fax: 312-355-0261; E-mail: pdetombe@uic.edu.

[2] The abbreviations used are: cTnI, cardiac troponin I; Tn, troponin; cTn, cardiac troponin; cTnT, cardiac troponin T; cTnC, cardiac troponin C; MOPS, 3-[*N*-morpholino]propane-sulfonic acid; WT, wild type; IAANS, 2-(4′-iodoacetamido)anilinonaphthalene-6-sulfonic acid.

taining the position of tropomyosin on the outer domain of actin blocking the formation of strong binding cross-bridges (blocked state) (11, 12). However, the molecular mechanisms by which truncation of cTnI at its C terminus affect myofilament functions are not well understood. *In vitro* analysis has shown that deletion at the C terminus induces an increase in solution ATPase activity as well as decreased force development, as determined in the motility assay (13, 14). However, in contrast to the effects of stunning on myofilament response to Ca^{2+}, the presence of truncated cTnI in reconstituted preparations induces an increase in apparent myofilament Ca^{2+} sensitivity, both in solution and in the motility assay (14, 15). Increased myofilament Ca^{2+} sensitivity, but not decreased force, has also been demonstrated in human skinned cells and myofibrils exchanged with truncated human cTnI (15).

In experiments reported here, we exchanged endogenous cTn in skinned rat cardiac trabeculae with a recombinant Tn complex containing either wild type cTnI or truncated cTnI to mimic myocardial stunning and determined chemo-mechanical transduction in skinned rat cardiac trabeculae. We found increases in cross-bridge cycle kinetics and myofilament Ca^{2+} sensitivity and reductions in maximum force development and cooperativity of activation. In addition, *in vitro* actin S1-ATPase experiments confirmed increased cross-bridge cycle kinetics and increased Ca^{2+} sensitivity. Interestingly, measurement of Ca^{2+} binding to Tn demonstrated a decrease in the affinity induced by the presence of truncated cTnI but only when Tn was reconstituted into the thin filament. Our data provide novel insights into the molecular mechanisms that underlie the impact of truncated cTnI on myofilament function. In addition, our data suggest that in normal cardiac sarcomeres, the C terminus of cTnI may play an important modulating role in tuning the level of cooperative myofilament activation in the heart through a direct interaction with the thin filament.

EXPERIMENTAL PROCEDURES

cDNA Constructs—C terminus truncated murine and human cardiac cDNA TnI were constructed by site-directed mutagenesis (Stratagene QuikChange™ site-directed mutagenesis kit) from murine and human cTnI cDNA clones according to the manufacturer's protocols. The oligonucleotide primers 5′-GAAGACTGGCGCAAGTAAATCGATGCTGAGTGGC and 3′-GCCACTCAGTGCATCGATTTACTTGCGCCAGTCTCC were used to create a translation stop codon at Arg^{193} in mouse cTnI to create murine cTnI-(1–193). Likewise, human cTnI-(1–192) was obtained using oligonucleotide primers 5′-GTGGGAGACTGGTAAAAGAATATCGATGC and 3′-GCATCGATATTCTTTTACCAGTCTCCCAC. Resultant mutagenesis products were transformed into XL-1 Blue and purified prior to sequence verification of the mutated codons. The single Cys mutants of murine cardiac TnC were constructed as previously described (16).

CTn Expression, Purification, and cTn Complex Reconstitution—The recombinant cTns were expressed and purified as previously described (16–18). The expression and purification of the recombinant human cardiac TNT containing an NH_2-terminal *myc* tag was carried out as previously described with slight modification of the purification protocol (17, 18). Reconstitution and purification of intact Tn complex was carried out by sequential dialysis to remove urea and decrease salt of an equimolar amount of purified cTn components and purification using Resource-Q (1 ml; Amersham Biosciences) (17, 18). Resultant fractions were analyzed by 12% SDS-PAGE. The fractions containing pure cTn were dialyzed three times at 4 °C against exchange buffer: 200 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 20 mM MOPS, pH 6.5. Aliquots were stored at −80 °C until use.

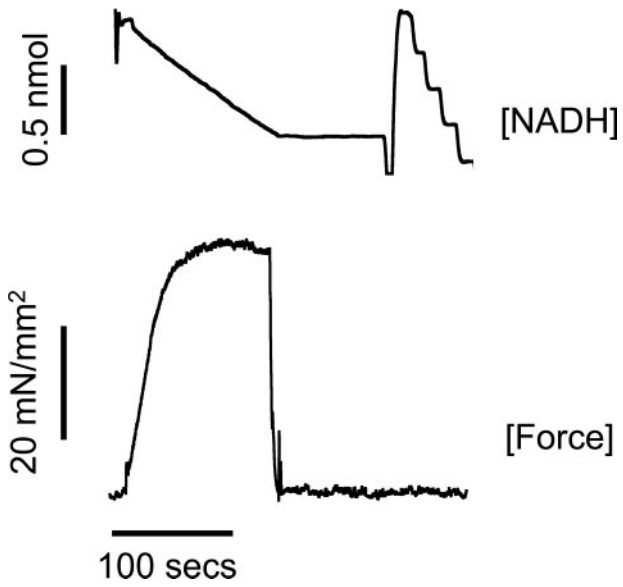

FIGURE 1. **Original recording of NADH (*top*) and force (*bottom*) during maximal Ca^{2+} activation; consumption of NADH was stoichiometrically coupled to ADP production.** Myofilament ATPase activity was determined during steady state activation. Background, nonmuscle ATPase activity was measured upon removal of the muscle from the measurement chamber, followed by calibration of the NADH signal via repeated injections of 250 pmol of ADP into the measurement chamber. Data were obtained from a skinned rat right ventricular trabecula in which troponin was exchanged for murine wild type recombinant troponin.

Exchange of Recombinant Cardiac cTn into Skinned Rat Trabeculae—Free running, unbranched right ventricular trabeculae were dissected from male Lewis Brown Norway-F1 rats (~14 weeks of age; Harlan Laboratories) and, after extraction with Triton X-100, attached to aluminum T-clips (18, 19). Exogenous Tn was exchanged for endogenous Tn by slight modification of previously described methods (18). Skinned trabeculae were transferred to a 96-well microtiter plate and incubated in 13 μM recombinant cardiac Tn in relaxing solution overnight at 4 °C. The composition of the skinned fiber solutions has been described in detail previously (18, 19).

Simultaneous Measurement of Isometric Tension and ATPase Activity—The simultaneous measurement of steady state isometric tension and myofibrillar ATPase activity over a range of free Ca^{2+} concentrations was conducted as previously described (18, 19). Sarcomere length was set at 2.2 μm by laser diffraction. ATP hydrolysis was stoichiometrically coupled to NADH consumption, which was measured in a small cuvette (~25 μl) via UV light absorption (340 nm). As illustrated in Fig. 1, Ca^{2+} activation induced force development and concomitant consumption of NADH in the measurement chamber, the rate

of which became constant during steady state force development. Trabeculae were activated over a range of free [Ca^{2+}] to measure steady-state isometric tension and ATPase activity. In some experiments, the rate of force redevelopment following a release-restretch maneuver, K_{tr}, was measured during a final contraction at maximum Ca^{2+} as previously described (19). Only muscles that maintained greater then 80% maximal tension were included for analysis. Following the mechanical measurements, each trabecula was incubated in sample buffer and stored frozen for biochemical analysis.

Quantification of cTn Exchange by Western Blot—Recombinant TNT in the present study included an NH_2 terminus *myc* tag to allow for quantification of the amount of cTn exchange by Western blotting techniques (17, 18). Previously, our group has demonstrated that the presence of this *myc* tag does not affect myofilament function (17).

Ca^{2+} Binding Measurements—Ca^{2+} binding was measured by fluorescence emission intensity of IAANS attached to Cys-35 of a single Cys mutant cTnC (C84S) for the cTn complex and Cys-84 of a single Cys mutant cTnC (C35S) for reconstituted thin filaments as described previously (16). The solutions contained 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 20 mM MOPS, pH 7.0; titrations with Ca^{2+} were carried out at 25 °C; the measurements were carried out five times for each cTn complex.

Actin-activated Acto-S1 ATPase Measurements—A modified micro-ATPase assay method was used (13, 14). Briefly, the typical reaction conditions were as follows: 6 mM actin, 0.5 mM myosin S1, 0.5 mM tropomyosin, 1.5 mM Tn, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM MOPS, pH 7.0, and various concentrations of $CaCl_2$ (25 °C). The reaction was initiated by the addition of 1.0 mM ATP (final concentration). The ATPase activity was determined from the time course of phosphate liberation, determined using the malachite green method, up to 10 min (every 2 min, a 10-ml aliquot was removed, and the reaction was terminated by the addition of 90 ml of 200 mM perchloric acid). The ATPase rate of S1 alone was subtracted from all measurements.

Data Processing and Statistical Analysis—Sigmoidal relationships were fit to a modified Hill equation (17–19); tension cost was determined by linear fit to the tension-ATPase data. Statistical analyses were performed either as multiple linear regression or one-way or two-way analysis of variance, as appropriate. $p < 0.05$ was considered statistically significant; data are presented mean ± S.E.

RESULTS

Recombinant cTn Exchange—We used the "whole cTn exchange technique" to introduce either wild type or truncated cTnI into the cardiac sarcomere. As we reported previously (17, 18), this procedure caused no major alterations in the structure and properties of the fiber bundles (*i.e.* exchanged skinned muscles retained a clearly detectable laser diffraction pattern). In addition, maximum Ca^{2+} saturated force development was not significantly different between nonexchanged and wild type cTn-exchanged skinned muscles (data not shown). Fig. 2 shows a representative SDS-PAGE and Western blot analysis of the recombinant cardiac cTn exchange procedure. The absence of

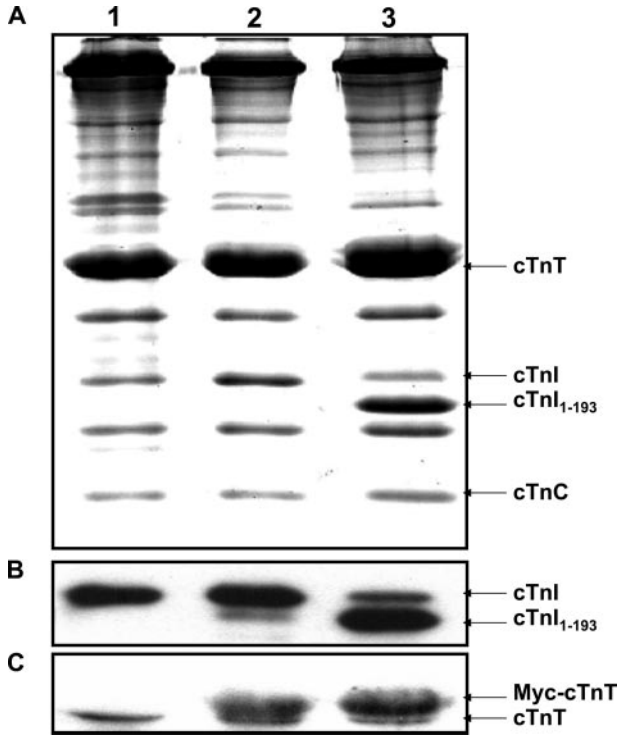

FIGURE 2. *A*, SDS-PAGE (15%) of recombinant cardiac troponin complex and skinned muscles. *Lane 1*, control (nonexchanged skinned rat cardiac trabeculae); *lane 2*, skinned trabeculae exchanged with wild type cardiac troponin; *lane 3*, skinned trabeculae exchanged with cTnI-(1–193) ($cTnI_{1-193}$)-containing troponin. *B*, Western blot probing with anti-TnI (C5) confirmed incorporation of cTnI with faster mobility on in the cTnI-(1–193) group (*lane 3*). *C*, Western blot probing with anti-TNT (CT3), which allows for the quantification of troponin exchange by inclusion of a *myc* tag on the recombinant TNT (*lanes 2* and *3*).

17 amino acids at the C terminus induced a higher mobility of cTnI-(1–193) as compared with $cTnI_{WT}$, as is apparent both in the Coomassie-stained gel shown in *A* and in the cTnI Western blot shown in *B* (*cf. lanes 1* and *2* with *lane 3*). In addition, the presence of a 9-amino acid *myc* tag at the N terminus of recombinant cTnT allowed for the separation of $cTnT_{myc}$ from endogenous cTnT by SDS-PAGE and subsequent quantification by Western blot (17, 18), as illustrated *C*. This analysis revealed that, on average, endogenous cTnI was exchanged for ~70% $cTnI_{WT}$ and ~60% for cTnI-(1–193) in muscles such as those loaded in *lanes 2* and *3*, respectively.

Effect of C-terminal Truncation on Myofilament Function—We evaluated the effect of cTnI truncation on myofilament function by determination of the Ca^{2+}-force and Ca^{2+}-ATP hydrolysis rate relationships in skinned cardiac trabeculae exchanged with Tn containing either wild type or C terminus cTnI. As illustrated in Fig. 3*A*, incorporation of cTnI-(1–193) was associated, on average, with an ~50% reduction of maximum tension development and an ~50% increase in myofilament Ca^{2+} sensitivity, evidenced by the marked leftward shift of the pooled force-Ca^{2+} relationship. In addition, there was a significant decrease in cooperativity of myofilament activation (steepness of the relationship). Maximum ATP hydrolysis rate, on the other hand, was reduced to a lesser extent (~10%) in the cTnI-(1–193) group (Fig. 3*B*). As a result, there was an increase in the ratio of ATP hydrolysis rate to tension, as apparent from

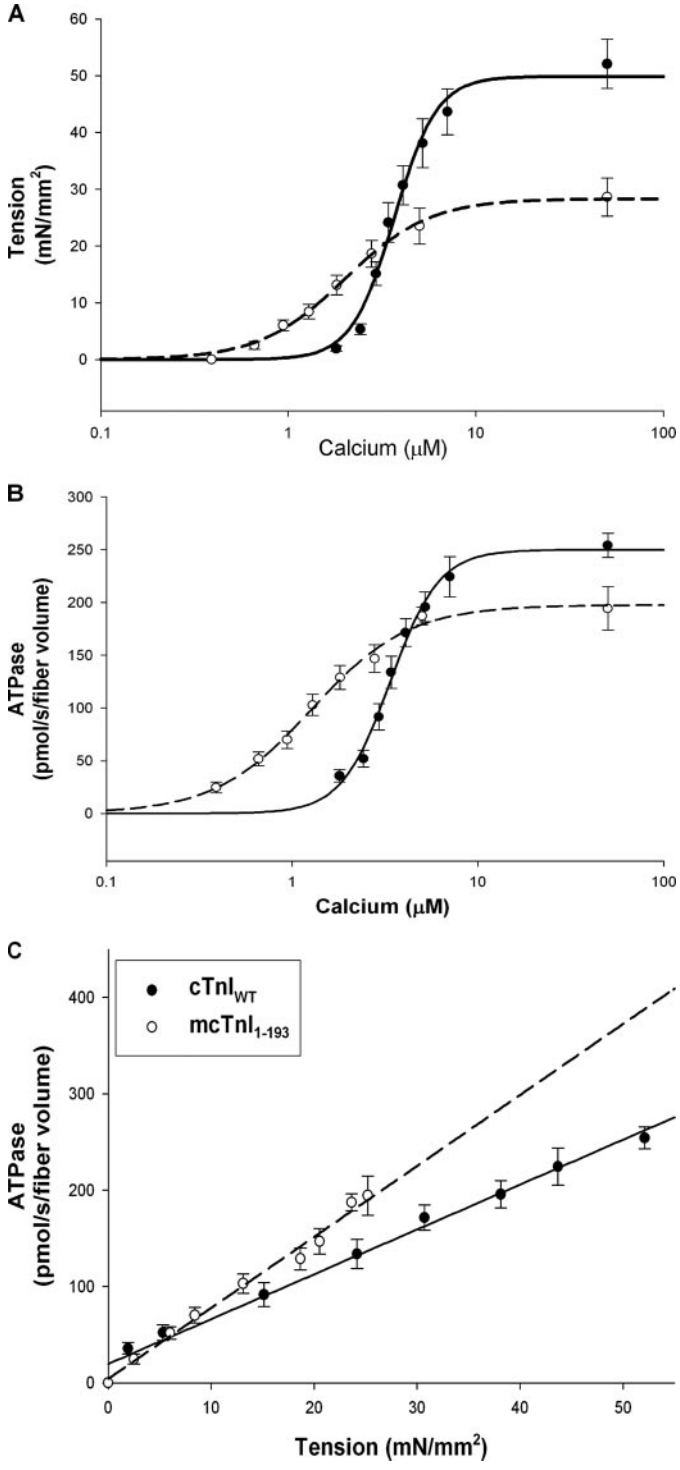

FIGURE 3. **Myofilament chemo-mechanical transduction was measured as tension-Ca^{2+} relationship (*A*), ATPase-Ca^{2+} relationship (*B*), and tension-dependent ATPase activity (*C*) in skinned cardiac trabeculae exchanged with wild type troponin (*closed circles*) and/or C-truncated cTnI troponin (*open circles*).** Tension cost was determined from the slope of the relationship between ATPase activity and tension. Data are presented as means ± S.E. The average fit parameters are summarized in Table 1. C terminus truncation of cTnI was associated with depressed force and cooperativity but enhanced cross-bridge cycling and myofilament Ca^{2+} sensitivity.

the increase in the slope of the pooled tension-ATPase activity relationship (Fig. 3*C*). Thus, the presence of cTnI-(1–193) in the sarcomere is associated with a significant increased tension cost. The average Hill fit and tension cost parameters obtained in individual muscles are summarized in Table 1.

In myocardial stunning, proteolytic degradation of TnI at the C terminus affects only a fraction of the total TnI in the cardiac sarcomere. Therefore, to determine whether the extent of myofilament dysfunction depended on the amount of cTnI-(1–193) exchanged into the sarcomere, we performed exchange experiments with a mixture of 50% wild type and 50% cTnI-(1–193) cTn. As shown in Fig. 4, incorporation of cTnI-(1–193) did not affect all parameters of myofilament Ca^{2+} activation proportionally (*i.e.* 50% cTnI-(1–193) reduced maximum tension and cooperativity to a level similar to that seen with exchange with cTn containing 100% cTnI-(1–193)). On the other hand, the increase in myofilament Ca^{2+} sensitivity was observed only in the group exchanged with cTn containing 100% cTnI-(1–193). Tension cost was the only parameter that increased in proportion to cTnI-(1–193) content.

In addition to measurements of tension cost, we also determined cross-bridge kinetics employing a protocol to determine the rate (K_{tr}) of force redevelopment following a quick release-restretch maneuver. As illustrated by the normalized force traces obtained in a typical experiment shown in Fig. 5, in which the data were fit to an exponential function, the K_{tr} parameter was significantly enhanced in the presence of cTnI-(1–193). On average, K_{tr} was ~30% higher in the cTnI-(1–193) group (*cf. inset*). These data are consistent with the increase in tension cost as determined from the simultaneous measurement of force and ATPase activity (*cf.* Figs. 3 and 4). We next determined whether the effects of truncation of cTnI depend on the particular species or the heterologous cTn incorporation into the rat cardiac sarcomere. The data presented in Fig. 6 show that, as was the case with all mouse cTn, the presence of human cTnI-(1–192) was associated with a significant increases in myofilament Ca^{2+} sensitivity (*A*) and tension-dependent ATP hydrolysis rate (*B*). Likewise, compared with controls, maximum Ca^{2+}-saturated tension development and Hill *n* values were significantly reduced in the group exchanged with human cTn containing cTnI-(1–192), albeit to a lesser extent than that observed with mouse cTn. Thus, exchange with cTn containing either mouse or human truncated TnI demonstrated similar effects on myofilament function.

Impact of cTnI Truncation on Reconstituted Actin-activated S1-ATPase Activity—The presence of truncated cTnI resulted in significant increases in maximum actin-activated S1-ATPase activity and apparent Ca^{2+} sensitivity (Fig. 7). In contrast to the skinned fiber results (Figs. 3 and 4), however, the level cooperativity was not affected by cTnI truncation.

Impact of cTnI Truncation on cTn Ca^{2+} Binding—To gain further insights into the molecular mechanisms underlying the effect of C terminus-truncated cTnI on myofilament function, we measured the affinity of Ca^{2+} binding to cTn. Fig. 7 shows representative Ca^{2+} titration curves of either cTn in isolation (*circles*), or cTn reconstituted thin filaments (*triangles*). Ca^{2+} binding was assessed using a fluorescence probe attached to TnC. The mere presence of cTnI-(1–193) in cardiac cTn did not appreciably affect Ca^{2+} binding affinity. However, when cTn interacted with its neighbors in reconstituted thin filaments (*i.e.* actin + tropomyosin), cTnI-(1–193) induced a

TABLE 1

Chemo-mechanical transduction fit parameters obtained in skinned cardiac trabeculae exchanged with cardiac troponin containing wild type full-length cTnI ($cTnI_{WT}$), truncated cTnI (cTnI-(1–193)), a 50% mixture ($cTnI_{WT}$ + mcTnI-(1–193)), or human truncated cTnI (hcTnI-(1–192))

See "Results" for details; values are means ± S.E.

Parameter	$cTnI_{WT}$	cTnI-(1–193)	$cTnI_{WT}$ + mcTnI-(1–193)	hcTnI-(1–192)
F_{max} (millinewtons mm^{-2})	54.0 ± 4.2	25.2 ± 2.5[a]	29.3 ± 1.9[a]	40.4 ± 3.4[a]
ATPase (pmol ml^{-1} s^{-1})	254 ± 11	194 ± 21[a]	198 ± 24[a]	381 ± 43[a]
EC_{50} (μM)	3.56 ± 0.1	1.92 ± 0.2[a]	3.25 ± 0.3	1.01 ± 0.2[a]
Hill coefficient	3.4 ± 0.3	1.6 ± 0.2[a]	1.6 ± 0.4[a]	1.5 ± 0.1[a]
Tension cost (ATPase·force^{-1})	5.0 ± 0.4	8.6 ± 0.3[a]	6.9 ± 0.6[a]	8.2 ± 0.5[a]
K_{tr} (s^{-1})	11.7 ± 0.7	15.9 ± 0.6[a]		
n	9	9	8	5

[a] $p < 0.05$.

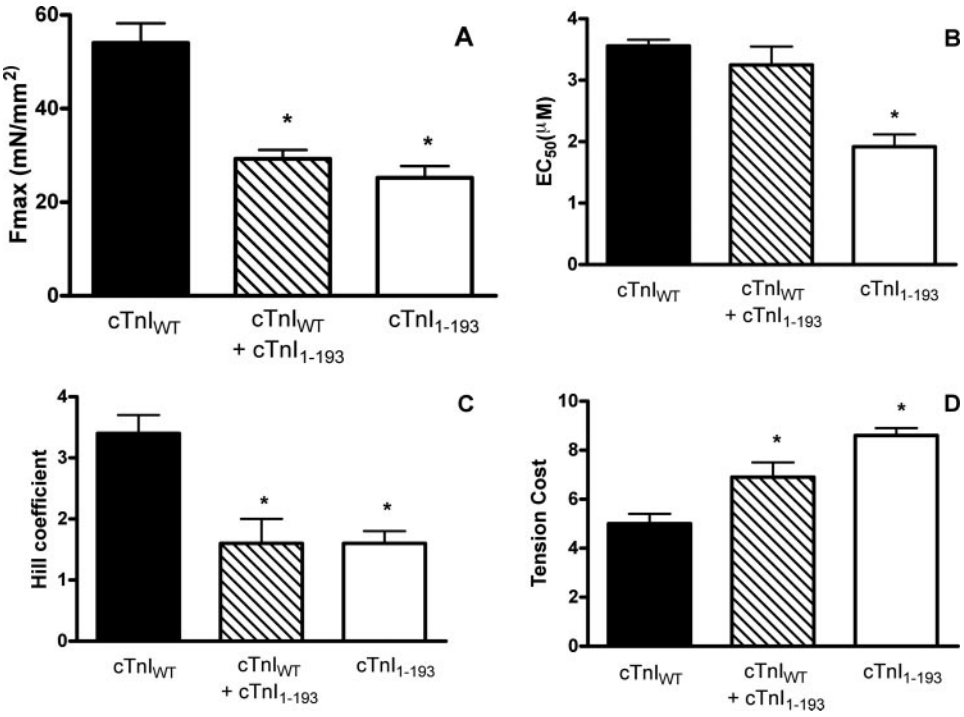

FIGURE 4. **The impact of varying amounts of truncated cTnI in the sarcomere was tested by exchange for either 100% wild type cTnI ($cTnI_{WT}$), 100% truncated TnI ($cTnI_{1–193}$), or for a mixture of 50% wild type and 50% truncated cTnI ($cTnI_{WT}$ + $cTnI_{1–193}$).** Force-Ca^{2+} relationships were fit to a modified Hill equation, yielding F_{max} (*A*), EC_{50} (*B*), and Hill coefficient (*C*). The tension-ATPase relationship was fit by linear regression to yield the tension cost parameter (*D*). *, $p < 0.05$ *versus* $cTnI_{WT}$ (see also Table 1).

marked increase in Ca^{2+} binding affinity. Similar results were obtained in five separate experiments; average fit parameters are summarized in the legend to Fig. 7.

DISCUSSION

Our data provide novel insights into the molecular mechanisms underlying the role of TnI truncation in the contractile dysfunction seen in myocardial stunning. The important and novel findings of our study were that (i) the presence of both low and high amounts of truncated cTnI in the cardiac sarcomere resulted in decreased myofilament force, decreased cooperativity of Ca^{2+} activation, and increased myofilament Ca^{2+} sensitivity; (ii) truncated cTnI induced an increased rate of cross-bridge detachment in direct proportion to the amount of mutant cTnI; (iii) *in vitro* reconstituted actin-activated S1-ATPase displayed increased maximum activity and Ca^{2+} sensitivity but unaltered cooperativity; and (iv) *in vitro* Ca^{2+} binding affinity measurements suggest that these effects require a direct interaction between troponin and the thin filament. Our data are consistent with a molecular model of cardiac muscle activation by Ca^{2+} ions in which an additional interaction between the C terminus of cTnI and actin functions to stabilize the attached cross-bridge. This mechanism may result in both reduced myofilament Ca^{2+} sensitivity and an increase in the fraction of attached cross-bridges and enhanced cooperative activation.

The results of the present study are consistent with previous studies examining the impact of C terminus truncation of cTnI on myofilament function. The removal of amino acid residues from the C terminus of cTnI is reported to result in increased Ca^{2+} sensitivity in solution ATPase activity (13, 14). In addition, reduced force development has been suggested previously, but this measurement was based on an indirect assessment using a modified *in vitro* motility assay (14). Here, using a more direct measurement of chemo-mechanical transduction in skinned cardiac trabeculae, we confirm that the presence of truncated cTnI in the cardiac sarcomere induces reduced maximum myofilament force development (Figs. 3 and 4). Reduced force was not reported by Narolska *et al.* (15). In that study, recombinant human cTn was exchanged for endogenous cTn in human myocyte fragments or bundles of myofibrils. Consistent with that finding, exchange with cTn containing human truncated cTnI in the present study was also associated with a much lower depression of maximum force (~20% human *versus* 50% mouse), which suggests that the impact of cTnI truncation on maximum force depends on residues within cTnI other than those located at the C terminus. In addition, difficulties associated with measurement of size-normalized force in myocyte fragments or myofibril bundles in the study by Narolska *et al.* (15) may have precluded detection of a relatively small (~20%) loss of maximum force. Finally, the use of human myocardium obtained in the setting of cardiac trans-

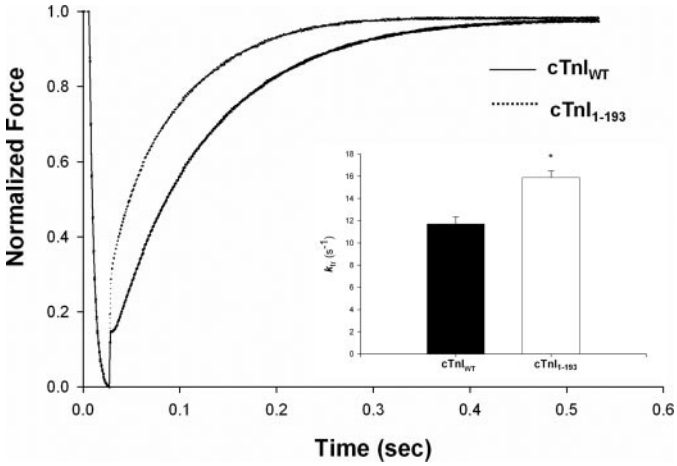

FIGURE 5. **Original recording of force redevelopment following a release-restretch maneuver used to assess K_{tr} in wild type-exchanged (*solid line*) and cTnI-(1–193) (*cTnI$_{1-193}$*)-exchanged skinned trabeculae (*dotted line*); force is normalized to prerelease force.** Exponential fit of the force record during the recovery phase yields K_{tr}. Average K_{tr} values are summarized by the *bar graph* (*inset*). The presence of cTnI-(1–193) in the sarcomere is associated with a significant increase in K_{tr} (*, $p < 0.05$ *versus* cTnI$_{WT}$; $n = 5$).

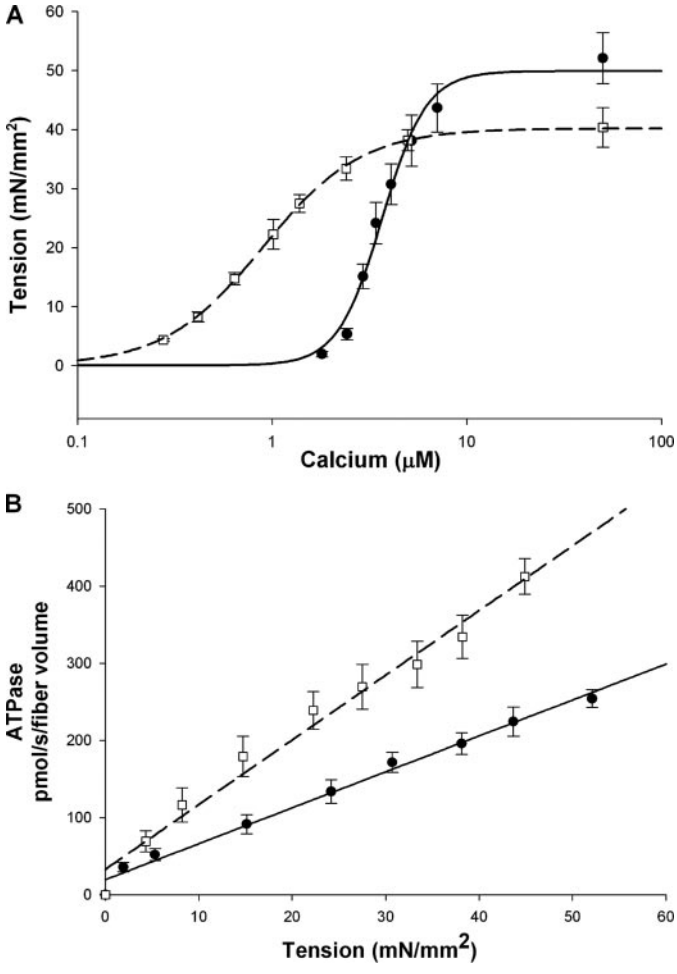

FIGURE 6. **Myofilament chemo-mechanical transduction was also depressed following exchange with human truncated cardiac TnI (hcTnI-(1–192); *open squares*) as compared with wild type murine cardiac troponin exchange (*filled squares*).** *A*, tension-Ca^{+} relationship; *B*, ATPase-tension relationship. Data are presented as mean ± S.E.; see also Table 1.

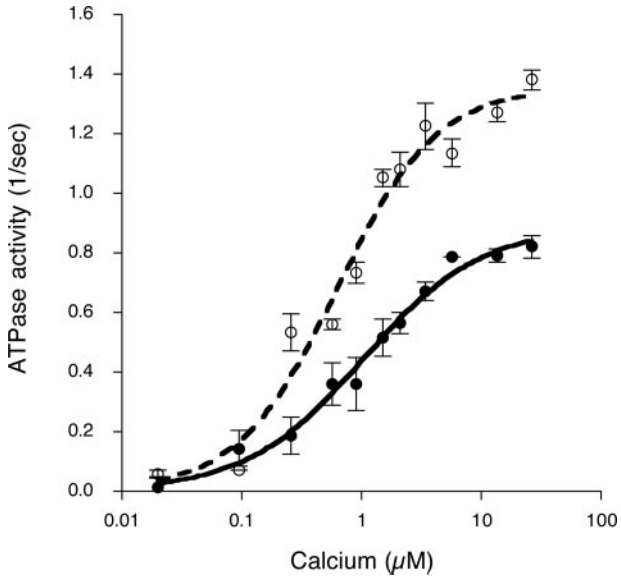

FIGURE 7. **The Ca^{2+}-dependent actin-activated acto-S1-ATPase activity in solution using a reconstituted system with either wild type cTnI (*closed circles*) or cTnI-(1–193) (*open circles*) as a function of [Ca^{2+}].** Each data point represents the mean ± S.E. of 5–7 measurements. The ATPase rate for S1 alone has been subtracted from all measurements. The average Hill fit (*solid* and *dashed lines*) parameters were as follows: maximum actin-activated acto-S1-ATPase activity = 1.36 ± 0.08 s^{-1} (cTnI-(1–193)) *versus* 0.89 ± 0.05 s^{-1} (cTnI$_{WT}$); EC$_{50}$ = 0.62 ± 0.12 μM (cTnI-(1–193)) *versus* 1.04 ± 0.18 μM (cTnI$_{WT}$); Hill coefficient = 0.9 ± 0.1 (cTnI-(1–193)) *versus* 1.0 ± 0.2 (cTnI$_{WT}$).

plantation adds experimental uncertainties that are not encountered when using myocardium isolated from experimental animals (20). Nevertheless, there appear to be genuine differences between the impact of cTnI truncation on maximum force between small rodent cardiac sarcomeres and human cardiac sarcomeres, and this issue will require additional studies.

As in experimental myocardial stunning (21), truncation of cTnI resulted in increased tension cost, a parameter that is directly proportional to the detachment rate (g) of myosin from actin (22). This result implies that truncation of the C terminus of cTnI causes destabilization of the attached cross-bridge by promoting the transition of the myosin head toward the detached state. In a simple two-state cross-bridge model, the fraction of myosin heads attached to acting and, thus, the amount of myofilament force development depends on the ratio of the attachment rate (f) to the sum of the attachment rate f and the detachment rate g. Similarly, the rate of force development, K_{tr}, is proportional to $f + g$. Hence, the increase in tension cost (g) observed in the present study is consistent with both the increase in K_{tr} and the decrease in maximum force (*cf.* Fig. 4). In addition, at the molecular scale, this effect of the C terminus of cTnI to stabilize the attached cross-bridge appears to be a local phenomenon (*i.e.* within a single regulatory unit), since the increase in tension cost was directly proportional to the amount of cTnI present in the sarcomere (Fig. 4). Narolska *et al.* (15) did not report an increase in cross-bridge cycle kinetics. However, cross-bridge kinetics in that study was assessed by the rate of Ca^{2+} activation and deactivation using rapid solution switching, whereas in the current study, we measured the tension-dependent rate of ATP hydrolysis. Incorporation of truncated cTnI into the sarcomere

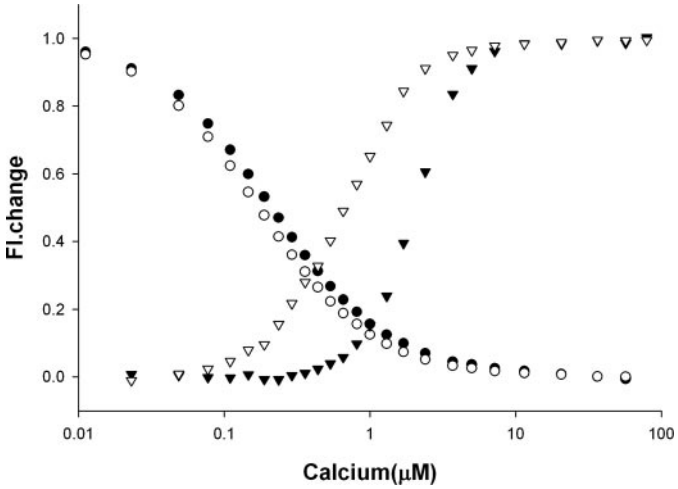

FIGURE 8. **Ca^{2+} binding to troponin was measured in solution by IAANS fluorescence (*Fl.*) conjugated either to cTnC-Cys-35 (*circles*; troponin complex in isolation) or cTnc-Cys-84 (*triangles*; troponin complex interacting with reconstituted thin filaments containing actin and tropomyosin).** Recombinant cardiac troponin either contained full-length wild type cTnI (*closed symbols*) or C terminus truncated cTnI (cTnI-(1–193); *open circles*). Ca^{2+} binding affinity was significantly enhanced by cTnI-(1–193) only when troponin was allowed to interact with the thin filament (2.5 ± 0.06 *versus* 1.7 ± 0.06 μM; $p < 0.01$; $n = 4$).

induces a marked increase in myofilament Ca^{2+} sensitivity. Under those conditions, myofibril Ca^{2+} activation parameters may become more determined by thin filament activation/relaxation kinetics than cross-bridge cycle kinetics. Arguing against this hypothesis is the recent observation that alterations in cTn Ca^{2+} binding affinity do not affect myofibril activation/relaxation kinetics (23). Another possibility is that the effect of C terminus truncation of cTnI on cross-bridge cycle kinetics differs between human and small rodent myocardium.

Truncation of cTnI was associated with a marked increase in myofilament Ca^{2+} sensitivity, consistent with previous *in vitro* studies (13–15). In contrast to the results in skinned fibers, cooperative activation in the reconstituted *in vitro* system was not affected by cTnI truncation (Fig. 7). A significant difference between the two measurement systems is a geometrical constraint in the intact sarcomere lattice that may limit cross-bridge formation to a single seven-actin regulatory unit; consistent with this notion is the low level of cooperativity that we observed in the reconstituted system. Alternatively, truncated cTnI may only affect cooperative activation properties under conditions of significant S1 mechanical strain, such as occurs both in the motility assay (14) and in the skinned fiber system (15) (*cf.* Figs. 3, 4, and 6).

Until now, it has not been known whether the increased myofilament Ca^{2+} sensitivity was the result of a direct effect of the truncated cTnI on cTn Ca^{2+} binding affinity. This, however, does not appear to be the mechanism, since we show for the first time in Fig. 8 that Ca^{2+} binding affinity to cTn in solution was not affected by cTnI truncation. Rather, this required incorporation of cTn into the thin filament lattice composed of actin and tropomyosin. Hence, it is the direct interaction between the C terminus of cTnI and the thin filament that modulates myofilament Ca^{2+} activation. Our data support the hypothesis that the physiological role of the C terminus of cTnI is inhibitory in nature. By direct binding to actin, the C terminus of actin may lock the thin filament in the open state (12), thereby stabilizing the attached cross-bridge state. At the same time, this interaction with actin would reduce the affinity of TnI for TnC (14). As a result, a stronger cooperative Ca^{2+} signal may be required to activate cardiac cTn in the presence of the thin filament. Loss of the C terminus of cTnI, therefore, transforms the Ca^{2+} binding affinity to cardiac cTn in the sarcomere back to that of cTnC alone, a reaction that by itself is not cooperative (*cf.* Fig. 7 and Ref. 10). The net result of this interplay between these two molecular mechanisms may be a high level of cooperative activation in the heart that assures both a rapid systolic pressure development, rapid "avalanche-like" ventricular relaxation (24, 25), and low diastolic levels of contractile activation. From a clinical point of view, increased myofilament Ca^{2+} sensitivity would be beneficial to help maintain contractile strength in the face of reduced maximum force development. However, the reduction in cooperative activation (Hill coefficient) will result in maintained contractile activation over a much wider range of cytosolic Ca^{2+}, potentially resulting in cross-bridge cycling that persists well into and during diastole (22). This comes at the price of an enhanced rate of ATP utilization under conditions of reduced cellular levels of [ATP], particularly such as those that occur immediately following the ischemic episode that triggers myocardial stunning. It should be noted that other cTnI domains besides the C terminus region significantly affect Ca^{2+} sensitivity. For example, it has been well documented that protein kinase A-mediated phosphorylation of the serines 22 and 23 results in marked Ca^{2+} desensitization.

Myocardial stunning is a multifactorial process that involves complex sequences of cellular perturbations, alterations to multiple protein functional groups, and the interaction of multiple mechanisms. Degradation of cTnI is but one of the alterations found in stunning. Alterations of other sarcomeric proteins (*e.g.* TnC, TNT, and MLC-2), proteins involved in energy metabolism (*e.g.* creatine kinase), and proteins regulating redox homeostasis (*e.g.* NADH-Uq) have also been reported to be altered in myocardial stunning (26). Whether these pathways affect myofilament chemo-mechanical transduction in myocardial stunning cannot be determined from the current study. The use of recombinant cTn exchange provides the opportunity to study the direct effect of a single protein mutation on myofilament chemo-mechanical transduction.

In summary, truncation of cardiac TnI at the C terminus by 17 amino acid residues, such as may occur in myocardial stunning, induces altered myofilament function in the form of depressed force development and cooperative activation and increased cross-bridge cycle kinetics and myofilament Ca^{2+} sensitivity. The increased Ca^{2+} affinity requires an interaction between cardiac cTn and the thin filament; the locus of this inhibitory interaction appears to reside in the C terminus of cardiac TnI and may help to decrease the range of cytosolic Ca^{2+} required to activate the cardiac sarcomere. Elucidation of the molecular pathways involved in depressed chemo-mechanical transduction in myocardial stunning will aid in the development of novel therapeutic strategies aimed to improve cardiac pump function in this syndrome.

REFERENCES

1. Bolli, R., and Marban, E. (1999) *Physiol. Rev.* **79,** 609–634
2. Feng, J., Schaus, B. J., Fallavollita, J. A., Lee, T. C., and Canty, J. M., Jr. (2001) *Circulation* **103,** 2035–2037
3. Thomas, S. A., Fallavollita, J. A., Lee, T. C., Feng, J., and Canty, J. M., Jr. (1999) *Circ. Res.* **85,** 446–456
4. Colantonio, D. A., Van Eyk, J. E., and Przyklenk, K. (2004) *Cardiovasc. Res.* **63,** 217–225
5. Gao, W. D., Atar, D., Liu, Y., Perez, N. G., Murphy, A. M., and Marban, E. (1997) *Cir. Res.* **80,** 393–399
6. Papp, Z., van der Velden, J., and Stienen, G. J. (2000) *Cardiovasc. Res.* **45,** 981–993
7. McDonough, J. L., Labugger, R., Pickett, W., Tse, M. Y., MacKenzie, S., Pang, S. C., Atar, D., Ropchan, G., and Van Eyk, J. E. (2001) *Circulation* **103,** 58–64
8. Neagoe, C., Kulke, M., del Monte, F., Gwathmey, J. K., de Tombe, P. P., Hajjar, R. J., and Linke, W. A. (2002) *Circulation* **106,** 1333–1341
9. Murphy, A. M., Kogler, H., Georgakopoulos, D., McDonough, J. L., Kass, D. A., Van Eyk, J. E., and Marban, E. (2000) *Science* **287,** 488–491
10. Kobayashi, T., and Solaro, R. J. (2005) *Annu. Rev. Physiol.* **67,** 39–67
11. Kobayashi, T., Yang, X., Walker, L. A., Van Breemen, R. B., and Solaro, R. J. (2005) *J. Mol. Cell. Cardiol.* **38,** 213–218
12. Geeves, M. A., and Holmes, K. C. (2005) *Adv. Protein Chem.* **71,** 161–193
13. Rarick, H. M., Tu, X. H., Solaro, R. J., and Martin, A. F. (1997) *J. Biol. Chem.* **272,** 26887–26892
14. Foster, D. B., Noguchi, T., VanBuren, P., Murphy, A. M., and Van Eyk, J. E. (2003) *Circ. Res.* **93,** 917–924
15. Narolska, N. A., Piroddi, N., Belus, A., Boontje, N. M., Scellini, B., Deppermann, S., Zaremba, R., Musters, R. J., dos Remedios, C., Jaquet, K., Foster, D. B., Murphy, A. M., van Eyk, J. E., Tesi, C., Poggesi, C., van der Velden, J., and Stienen, G. J. (2006) *Circ. Res.* **99,** 1012–1020
16. Kobayashi, T., and Solaro, R. J. (2006) *J. Biol. Chem.* **281,** 13471–13477
17. Chandra, M., Rundell, V. L., Tardiff, J. C., Leinwand, L. A., De Tombe, P. P., and Solaro, R. J. (2001) *Am. J. Physiol.* **280,** H705–H713
18. Sumandea, M. P., Pyle, W. G., Kobayashi, T., de Tombe, P. P., and Solaro, R. J. (2003) *J. Biol. Chem.* **278,** 35135–35144
19. Rundell, V. L., Manaves, V., Martin, A. F., and de Tombe, P. P. (2005) *Am. J. Physiol.* **288,** H896–H903
20. Jweied, E., Detombe, P., and Buttrick, P. M. (2007) *J. Mol. Cell. Cardiol.* **42,** 722–726
21. Gao, W. D., Dai, T., and Nyhan, D. (2006) *Am. J. Physiol.* **290,** H886–H893
22. de Tombe, P. P. (2003) *J. Biomech.* **36,** 721–730
23. de Tombe, P. P., Belus, A., Piroddi, N., Scellini, B., Walker, J. S., Martin, A. F., Tesi, C., and Poggesi, C. (2007) *Am. J. Physiol.* **292,** R1129–R1136
24. Stehle, R., Kruger, M., and Pfitzer, G. (2002) *Biophys. J.* **83,** 2152–2161
25. Brutsaert, D. L., and Sys, S. U. (1989) *Physiol. Rev.* **69,** 1228–1315
26. White, M. Y., Cordwell, S. J., McCarron, H. C., Prasan, A. M., Craft, G., Hambly, B. D., and Jeremy, R. W. (2005) *Proteomics* **5,** 1395–1410