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ARTICLE *in* JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY · NOVEMBER 2003

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Original Article

Altered signaling surrounding the C-lobe of cardiac troponin C in myofilaments containing an α-tropomyosin mutation linked to familial hypertrophic cardiomyopathy

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Abstract

A region of interaction between the near N-terminal of cardiac troponin I (cTnI) and the C-lobe of troponin C (cTnC), where troponin T (cTnT) binds, appears to be critical in regulation of myofilament Ca^{2+}-activation. We probed whether functional consequences of modulation of this interface influence the function of tropomyosin (Tm) in thin filament activation. We modified the C-lobe of cTnC directly by addition of the Ca^{2+}-sensitizer, EMD 57033, and indirectly by replacing native cTnI with cTnI-containing Glu residues at Ser-43 and Ser-45 (cTnI-S43E/S45E) in myofilaments from hearts of non-transgenic (NTG) and transgenic (TG) mice expressing a point mutation on α-Tm (E180G) linked to familial hypertrophic cardiomyopathy. Introduction of cTnI-S43E/S45E induced a significantly greater reduction in tension in TG myofilaments compared to NTG controls. Furthermore, the effect of EMD 57033 to restore Ca^{2+}-sensitivity was higher in TG compared to NTG fiber bundles containing cTnI-S43E/S45E and compared to TG or NTG fiber bundles containing native TnI. Our results indicate that alterations in regions of interaction among the N-terminal of cTnI, the C-lobe of cTnC, and the C-terminus of cTnT are important in the regulation of myofilament activity. Although levels of phosphorylation at protein kinase C-dependent sites were the same in TG and NTG myofilaments, our data indicate that the effects of phosphorylation were more depressive in TG hearts.

Keywords: Troponin; Tropomyosin; Hypertrophy; Protein kinase C; Ca^{2+}-sensitizers

1. Introduction

Cardiac muscle contraction is triggered when Ca^{2+} binds to the C-lobe of troponin C (cTnC) and initiates a sequence of protein–protein interactions that signals changes in the interaction of tropomyosin (Tm) with actin and promotes a force-generating interaction between cross-bridges and the thin filament [1]. This trigger is located in an N-terminal domain (N-lobe) of cTnC, which contains a single Ca^{2+}-binding site that exchanges Ca^{2+} fast enough to regulate the transition between systole and diastole [2]. This signaling through the N-lobe promotes binding of a C-terminal domain and inhibitory peptide (Ip) of cardiac troponin I (cTnI) to cTnC. Movement of the Ip away from its actin-binding site, together with altered interactions of cardiac troponin T (cTnT) with Tm, induces a release of the myofilaments from an inhibited state. The C-lobe of cTnC contains two additional Ca^{2+}-binding sites, but exchange of Ca^{2+} with these sites is too slow to occur within the contraction–relaxation cycle of the heart [3,4]. Moreover, removal of Ca^{2+} from these sites releases cTnC from the thin filament by disengaging a tight interaction with a near N-terminal region of cTnI (residues 39–58). Thus, in contrast to the N-lobe, which is generally considered to be a regulatory domain of cTnC, the C-lobe of cTnC has been considered to be a structural domain. Yet, there are data indicating that tension can be modulated by signaling through the C-lobe of cTnC and its interface at a near

Abbreviations: cTnC, cardiac troponin C; Tm, tropomyosin; Ip, inhibitory peptide of cTnI; cTnI, cardiac troponin I; cTnT, cardiac troponin T; PKC, protein kinase C; PKA, protein kinase A; NTG, non-transgenic; TG, transgenic; FHC, familial hypertrophic cardiomyopathy; WT, wild type; T1, N-terminal fragment of TnT; T2, C-terminal fragment of TnT; NMR, nuclear magnetic resonance.

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doi:10.1016/S0022-2828(03)00240-2

N-terminal region of cTnI. These data indicate that EMD may increase Ca^{2+}-sensitivity and tension by competing with cTnI for binding to the C-lobe of cTnC. Interaction of the near N-terminal region of cTnI with the C-lobe of cTnC and/or the C-terminal region of cTnT may also be altered by charge changes induced by protein kinase C (PKC)-mediated phosphorylation of Ser-43 and Ser-45 of cTnI [5].

In experiments reported here, we have tested whether modifications at the interface of the C-lobe of cTnC and the near N-terminal region of cTnI influence the function of Tm in thin filament activation. We employed myofilaments from non-transgenic (NTG) mice or from transgenic mice (TG) expressing a mutant Tm (Tm-E180G) linked to familial hypertrophic cardiomyopathy (FHC). This point mutation on α-Tm has been shown to induce disorganization of myocytes, possible sarcomeric disarray, and to trigger a hypertrophic response that culminates in death of these mice by 5 months of age [6]. To test whether signaling through the C-lobe of cTnC is altered in skinned fiber bundles containing α-Tm (E180G), we modified the cTnC C-lobe directly by addition of EMD 57033. We also modified the region surrounding the C-lobe by replacing native cTnI with a variant into which we had introduced Glu residues (which mimic the phosphorylation [5]) at positions 43 and 45 of cTnI. Our data indicate that alterations in signaling through the C-lobe of cTnC may be an important mechanism in the pathogenesis and pharmacology of FHC and emphasize the functional significance of protein–protein interactions at the C-lobe of cTnC.

2. Materials and methods

2.1. Materials

TG mouse lines that encode an FHC mutation in α-Tm (E180G) were generated as previously described [6]. The Ca^{2+}-sensitizer EMD 57033 (active enantiomer) was synthesized at the Chemical Department of the Pharmaceutical Research Division of E. Merck, Darmstadt, Germany. Stock solutions of 3 mM EMD 57033 were diluted in 100% dimethylsulfoxide (DMSO). The final volume of DMSO constituted only 0.1% of the total volume of the bathing solutions for fiber bundles.

2.2. Protein purification

Expression and purification of recombinant human cTnC, mouse cTnI (wild type (WT) and mutant), and mouse cTnT was previously described [7]. cTnT was modified with a myc tag at its N-terminus. Recombinant PKC-ε was prepared in our laboratory using a recombinant baculovirus system as previously described [8].

2.3. Preparation of detergent-skinned fiber bundles

Male and female FVBN mice (age 3–4 months) were anesthetized by injection with pentobarbital sodium (50 mg/kg body weight) into the peritoneal cavity. The hearts were quickly excised and left ventricular papillary muscle fiber bundles (approximately 3–4 mm long, 150–250 μm wide) were dissected and detergent-skinned overnight as previously described [5,9] in a high relaxing (HR) solution containing 20 mM 3-morpholinopropane-sulfonic acid (MOPS) (pH 7.0), 53.5 mM KCl, 10 mM ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.025 mM $CaCl_2$, 1 mM free Mg^{2+}, 5 mM $MgATP^{2-}$, 12 mM creatine phosphate, 10 IU/ml creatine kinase (bovine heart, Sigma), 1 mM 1,4-dithiothreitol (DTT), and 1% Triton X-100. A cocktail of protease inhibitors was included in all buffers (1 μg/ml pepstatin, 5 μg/ml leupeptin, and 0.2 mM phenylmethyl-sulfonyl fluoride (PMSF)).

2.4. Treatment of skinned fiber bundles

Fiber bundles were mounted between a force transducer and a micro-manipulator, and the sarcomere length was adjusted to 2.3 μm. Maximum force was initially measured in activating solution (pCa 4.5) and then the fiber was relaxed in HR solution. Force was then measured while the fiber bundles were bathed in sequentially increasing Ca^{2+} concentrations (pCa 8–4.5; pCa values calculated using binding constants reported by Godt and Lindley [10]). The fiber bundles were then treated for 90 min with an extraction solution containing TnT (1.5 mg/ml) and TnI (1.0 mg/ml), and then maximum Ca^{2+}-activated force was measured in pCa 4.5 solution to determine the extent of endogenous troponin (Tn) removed. Next, the fiber bundles were treated for 90 min with a TnC reconstitution solution (4.0 mg/ml), followed by measurement of maximum Ca^{2+}-activated force in pCa 4.5 solution. Fiber bundles were then relaxed in HR solution before subsequent force measurements in sequentially increasing Ca^{2+} concentrations. Cross-sectional area was determined by measuring the width of the fiber bundle at three different points along the fiber bundle and averaging this value (which we called '*a*'), followed by measuring the width of the fiber bundle from a different viewpoint at three different points along the fiber bundle and averaging this value (which we called '*b*'). Cross-sectional area was then calculated by fitting these parameters to the equation: area = πr^2, where *r* is the radius, or in our case, area = πab (assuming elliptical cross-sectional area). Cross-sectional area was determined twice during the exchange experiment (before treatment with the TnT–TnI extraction solution and after total reconstitution by treatment with TnC solution) and was not observed to change throughout the course of each experiment.

2.5. Treatment of skinned fiber bundles with myofilament Ca^{2+}-sensitizers

The effect of 3 μM EMD 57033 was determined in NTG and TG α-Tm (E180G) skinned fiber bundles containing either native Tn components or skinned fiber bundles containing cTnI-containing Glu residues at Ser-43 and Ser-45

(cTnI-S43E/S45E). EMD or vehicle was added directly to pCa solutions and force measurements were determined in sequentially increasing Ca^{2+} concentrations (pCa 8–4.5).

2.6. PKC-site phosphorylation levels

Endogenous levels of PKC-ε phosphorylation were determined using a back-phosphorylation technique modified from Karczewski et al. [11]. Purified myofibrils from NTG or TG α-Tm (E180G) FHC mouse hearts were prepared according to the method of Pagani and Solaro [12]. Myofibrillar protein concentration was determined by the Lowry assay [13]. Forty micrograms of myofibrillar protein were used for each phosphorylation reaction. Myofibrils were centrifuged briefly (14,000 rpm, less than 1 min) and the pellet was resuspended in 5 mM histidine–HCl (pH 7.4), 0.75 M KCl, 0.2 mM DTT, 0.1 mM PMSF, 50 mM inorganic phosphate, 25 mM NaF, and 10 mM EDTA. The mixture was sonicated for 1 min and then centrifuged (14,000 rpm, 5 min). The supernatant fraction was resuspended in a final solution containing 40 mM histidine–HCl (pH 6.8), 10 mM $MgCl_2$, 15 mM NaF, 1 mM EGTA, 0.3 mM phosphatidylserine, 0.2 mM diacylglycerol, and 0.1 % Triton X-100. This lysate was phosphorylated in the presence of approximately 2 μg PKC-ε. The phosphorylation reaction was initiated by the addition of 50 μM [γ-^{32}P] ATP and carried out for 1 h at 30 °C. Maximum phosphorylation levels were seen to occur after approximately 30 min and did not increase with incubations up to 2 h (data not shown). The reaction was stopped by addition of an equal volume of gel-loading buffer (125 mM Tris–HCl (pH 6.8), 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% bromphenol blue, and 50 mM β-mercaptoethanol). The samples were immediately boiled in a waterbath for 5 min and processed for gel electrophoresis.

2.7. Polyacrylamide gel electrophoresis and autoradiography

SDS-polyacrylamide gels (12.5%) used for autoradiography were run as previously described [14]. Myofilament proteins from phosphorylation experiments were initially visualized by Coomassie brilliant blue staining. Destained gels were then exposed to a phosphor screen overnight for quantification of ^{32}P incorporated into myofilament protein bands using a phosphorimager (Molecular Dynamics, STORM). The comparison of ^{32}P-incorporation across each lane for a gel was determined after background correction using ImageQuant software. Phosphorylation of cTnI in TG α-Tm (E180G) samples was expressed as a percentage of ^{32}P-incorporation into cTnI from NTG control samples. Myofilament proteins in fiber bundle preparations were also visualized by SDS-polyacrylamide gels (12.5%).

2.8. Western blot analysis

Extent of endogenous Tn removal by treatment with cTnT–cTnI extraction solution was confirmed by western blot analysis. Myofilament proteins in skinned fiber bundle preparations were visualized by SDS-polyacrylamide gels (15%), which were then probed for the presence of endogenous vs. recombinant mouse cTnT using a primary anti-cTnT antibody (Sigma, clone # JLT-12) and secondary goat anti-mouse IgG antibody conjugated to horseradish-peroxidase (Sigma). The signal was visualized using an ECL-plus western blotting detection system (Amersham Biosciences).

2.9. Statistical analysis

Data from the tension measurements in skinned fiber bundles were normalized and fitted to the Hill equation as previously described [15] by using a non-linear least-square regression procedure to obtain the pCa_{50} (–log of free Ca^{2+}-concentration required for half-maximal activation) and the Hill coefficient (n). Statistical differences were analyzed by an unpaired t-test and two-way ANOVA with the criteria for significance set at $P < 0.05$. Data are expressed as mean ± S.E. Phosphorylation levels of cTnI in TG α-Tm (E180G) samples were expressed as a percentage of ^{32}P-incorporation into cTnI from NTG control samples and averaged for each phosphorylation reaction. Statistical differences were analyzed by an unpaired t-test with the criteria for significance set at $P < 0.05$. Data are expressed as mean ± S.E.

3. Results

3.1. Exchange of cTnI in cardiac-skinned fiber bundles

Our strategy to understand the effects of charge modification that occur at regions of protein–protein interaction surrounding the C-lobe of cTnC involved the exchange of native Tn components in detergent-extracted (skinned) fiber bundles with Tn containing a mutant form of cTnI in which Ser-43 and Ser-45 were replaced with Glu residues (cTnI-S43E/S45E). We used a procedure developed in our laboratory by Chandra et al. [9] in which the tension of the skinned fiber bundle was first measured over a range of pCa values. The fiber bundle was then exposed to a large excess of a cTnT–cTnI after which the tension generated by the fiber bundle at high levels of intracellular Ca^{2+} (pCa 4.5) was a small fraction of the initial value generated before cTnT–cTnI treatment. Then after subsequent incubation of the fiber bundle in a cTnC reconstitution solution, tension was again measured through a series of pCa values. As previously reported [5], the pCa–tension relation determined in fiber bundles before incubation with the exchange solutions gave pCa_{50}values of 5.70 ± 0.01. This pCa–tension relationship did not change after the fiber bundles were incubated with exchange solutions without proteins (TnI–TnT and TnC) and gave pCa_{50} values of 5.72 ± 0.01. Also, the maximum tension after this ‘pseudo’-exchange procedure decreased by approximately 5–10% (data not shown), a result that indicated

the fall-off of maximum tension seen after exchange of native Tn components was time dependent and that the exchange protocol did not result in changes in the myofilament activity due to non-specific protein–protein interactions.

Fig. 1 demonstrates the effective exchange of Tn components in fiber bundles during the course of the extraction and reconstitution treatment. Fig. 1A shows Coomassie-stained SDS-PAGE analysis of myofilament proteins from NTG and TG α-Tm (E180G) skinned fiber bundles with endogenous Tn complex (lanes 2 and 5), and after removal of endogenous Tn complex by extraction with cTnT–cTnI and reconstitution of Tn with cTnC treatment (lanes 3 and 6). Pure cTnT, cTnI, and cTnC standards are shown in lanes 1 and 4. The exogenous, recombinant mouse cTnT was modified at its N-terminus with a myc tag, which slowed its mobility so it runs closer with actin, as seen in lanes 3 and 6. The myc tag has been demonstrated to have no effect on cTnT function [16], but served to alter the mobility of cTnT permitting demonstration of exchange of Tn. Fig. 1B shows western blot analysis of cTnT from NTG and TG α-Tm (E180G) skinned fiber bundles with endogenous Tn complex (lanes 2 and 5), after removal of endogenous Tn complex by extraction with cTnT–cTnI complex and reconstitution of Tn with cTnC treatment (lanes 3 and 6). A pure cTnT standard is shown in lanes 1 and 4. The endogenous cTnT shown in lanes 2 and 5 demonstrates a faster mobility, compared to recombinant cTnT shown in lanes 1, 3, 4, and 6, and the endogenous cTnT band essentially disappears after extraction and reconstitution (lanes 3 and 6) indicating exchange of Tn occurred.

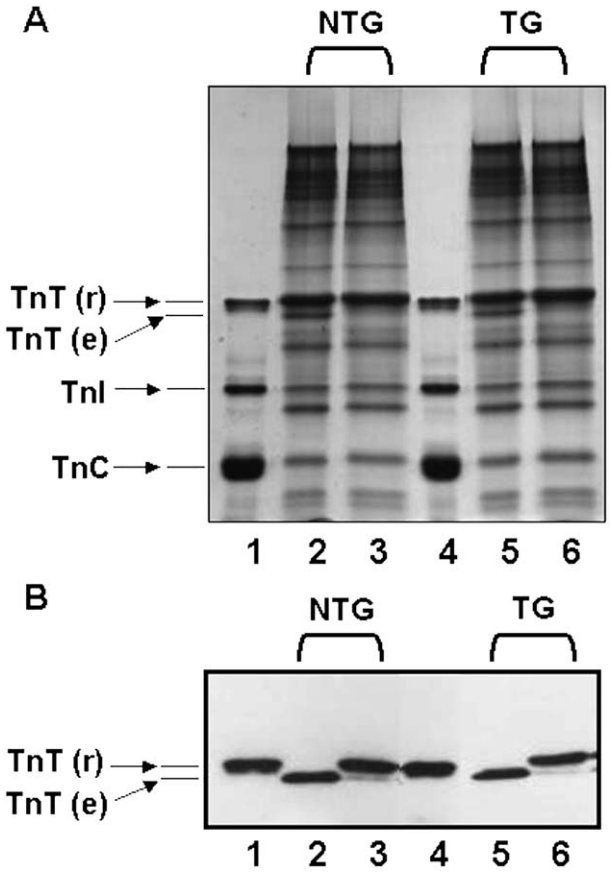

Fig. 1. Procedure for Tn exchange in Triton X-100 skinned mouse cardiac fiber bundles. (A) Coomassie-stained SDS-PAGE analysis of myofilament proteins from NTG and TG- skinned fiber bundles with endogenous Tn complex (lanes 2 and 5), and after removal of endogenous Tn complex by extraction with cTnT–cTnI and reconstitution with cTnC treatment (lanes 3 and 6). Pure cTnT, cTnI, and cTnC standards are shown in lanes 1 and 4. (B) Western blot analysis of cTnT from NTG and TG-skinned fiber bundles with endogenous Tn complex (lanes 2 and 5), after removal of endogenous Tn complex by extraction with cTnT–cTnI complex and reconstitution with cTnC treatment (lanes 3 and 6). A pure cTnT standard is shown in lanes 1 and 4. TnT (r) represents recombinant TnT that was exchanged into fiber bundles, whereas TnT (e) represents endogenous TnT present in fiber bundles before the exchange procedure.

3.2. Differential effects of cTnI-S43E/S45E on tension generated by TG α-Tm (E180G) and NTG myofilaments

To determine whether charge modifications of cTnI exacerbate the alterations in the myofilament response to Ca^{2+} in hearts of TG α-Tm (E180G) mice, we compared NTG and TG myofilament Ca^{2+}-sensitivity and tension generation in detergent-extracted fiber bundles in which native cTnI was exchanged with either cTnI-WT or cTnI-S43E/S45E. Fig. 2 and Table 1 summarize the results of these experiments.

Fig. 2A shows levels of Ca^{2+}-dependent tension generated in NTG or TG α-Tm (E180G) fiber bundles containing cTnI-WT or cTnI-S43E/S45E. The maximum Ca^{2+}-dependent tension developed in TG fiber bundles containing cTnI-WT (32.4 ± 0.3 mN/mm^2) was significantly higher than NTG fiber bundles containing cTnI-WT (27.9 ± 0.3 mN/mm^2) (Fig. 2B, Table 1). Upon substitution of cTnI-WT with cTnI-S43E/S45E, tension developed by TG fiber bundles fell from 32.4 ± 0.3 to 18.6 ± 0.3 mN/mm^2, whereas tension developed by NTG fiber bundles fell from 27.9 ± 0.3 to 18.1 ± 1.0 mN/mm^2. Unlike TG and NTG fibers containing cTnI-WT, the maximum Ca^{2+}-dependent tension developed by TG and NTG fiber bundles containing cTnI-S43E/S45E was not significantly different. However, the decrease in tension upon exchange of cTnI-WT with cTnI-S43E/S45E was significantly greater in TG (tension decreased by approximately 13.8 mN/mm^2) compared to NTG fiber bundles (tension decreased by approximately 9.8 mN/mm^2) (Fig. 2B, Table 1).

Fig. 2C shows differences in the Ca^{2+}-sensitivity (as indicated by the pCa_{50}) of TG and NTG fiber bundles containing cTnI-WT or cTnI-S43E/S45E. As previously reported by Prabhakar et al. [6], fiber bundles from TG α-Tm (E180G) mouse hearts were sensitized to Ca^{2+} compared to the NTG controls (Fig. 2C, Table 1). TG fiber bundles containing cTnI-WT had a pCa_{50} value of 5.78 ± 0.04, whereas NTG fiber bundles had a pCa_{50} value of 5.68 ± 0.04. When cTnI-WT was substituted with cTnI-S43E/S45E, the Ca^{2+}-sensitivity of both TG and NTG fiber bundles was reduced to a similar extent. TG α-Tm (E180G) skinned fiber bundles containing cTnI-S43E/S45E showed a decrease in pCa_{50} to 5.42 ± 0.02, which was 0.36 less than TG fiber bundles containing cTnI-WT. NTG fiber bundles containing cTnI-S43E/S45E showed a decrease in pCa_{50} to 5.29 ± 0.02, which

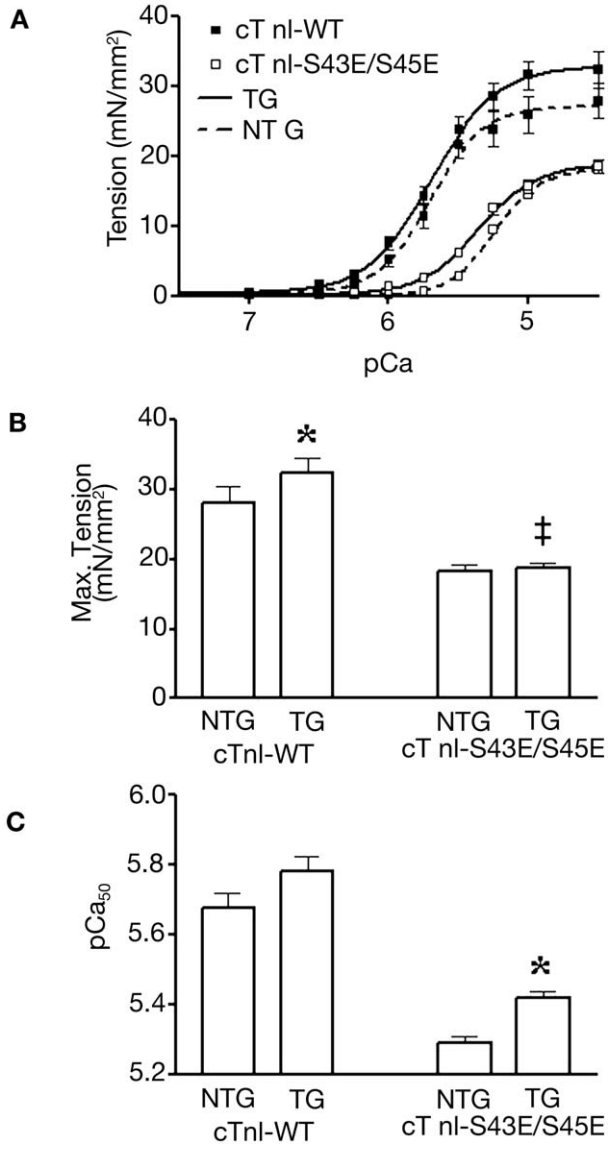

Fig. 2. pCa–tension relations in NTG and TG α-Tm (E180G) skinned fiber bundles containing either cTnI-WT or cTnI-S43E/S45E. (A) The pCa–tension relations for NTG (dotted lines) or TG (solid lines) fiber bundles containing cTnI-WT (filled squares) or cTnI-S43E/S45E (open squares). (B) The maximum Ca^{2+}-dependent tension produced at pCa 4.5 in NTG and TG fiber bundles containing cTnI-WT or cTnI-S43E/S45E. As noted (*) the maximum tension was significantly higher for TG compared to NTG fiber bundles containing cTnI-WT ($P < 0.03$). As noted (‡) substitution of cTnI-WT for cTnI-S43E/S45E produced a significantly larger decrease in maximum tension in TG compared to NTG fiber bundles ($P < 0.01$). (C) Changes in Ca^{2+}-sensitivity of force, as reported by the pCa_{50} values ([Ca^{2+}] required to produce half-maximal force) for NTG and TG fiber bundles containing cTnI-WT or cTnI-S43E/S45E. As noted (*) TG fiber bundles were more sensitive to Ca^{2+} compared to NTG fiber bundles ($P < 0.01$). $n = 11$ each for NTG or TG fiber bundles containing cTnI-WT, and $n = 32$ each for NTG or TG fiber bundles containing cTnI-S43E/S45E.

was 0.39 less than NTG fiber bundles containing cTnI-WT. Fiber bundles from TG α-Tm (E180G) FHC mice containing cTnI-S43E/S45E were significantly more sensitive to Ca^{2+} compared to NTG fiber bundles.

3.3. Differential effects of EMD 57033 on tension generated by TG α-Tm (E180G) and NTG myofilaments containing cTnI-S43E/S45E

To probe potential differences in signaling through the C-lobe of cTnC, we measured changes in myofilament Ca^{2+}-sensitivity and tension generation before and after treatment with 3 μM EMD 57033 in NTG and TG α-Tm (E180G) fiber bundles. Fig. 3 summarizes the results of determinations of the effect of EMD 57033 in fiber bundles containing native Tn components. Fig. 3 shows levels of Ca^{2+}-dependent force generated in NTG or TG α-Tm (E180G) fiber bundles containing native Tn. TG α-Tm (E180G) fiber bundles were significantly more sensitive to Ca^{2+} ($pCa_{50} = 6.03 \pm 0.01$), compared to NTG fiber bundles ($pCa_{50} = 5.79 \pm 0.01$). Treatment of TG α-Tm (E180G) fiber bundles with 3 μM EMD 57033 increased the Ca^{2+}-sensitivity of force (Fig. 3B) by approximately 0.15 ($pCa_{50} = 6.18 \pm 0.02$) and by approximately 0.08 ($pCa_{50} = 5.87 \pm 0.01$) in NTG fiber bundles (Fig. 3B). The ability of EMD 57033 to increase Ca^{2+}-sensitivity was significantly greater in TG fiber bundles compared to NTG fiber bundles.

As summarized in Fig. 4 and Table 1, we also measured changes in myofilament Ca^{2+}-sensitivity and tension generation induced by 3 μM EMD 57033 treatment of NTG and TG α-Tm (E180G) fiber bundles containing cTnI-S43E/S45E. TG fiber bundles containing cTnI-S43E/S45E were significantly more sensitive to Ca^{2+} ($pCa_{50} = 5.42 \pm 0.01$), compared to NTG fiber bundles containing cTnI-S43E/S45E ($pCa_{50} = 5.29 \pm 0.01$) (Fig. 4A). Treatment of TG fiber bundles containing cTnI-S43E/S45E with 3 μM EMD 57033 increased the pCa_{50} value by approximately 0.30 to 5.72 ± 0.03. Treatment of NTG fiber bundles containing cTnI-S43E/S45E with 3 μM EMD 57033 increased the pCa_{50} value by approximately 0.17 to 5.46 ± 0.02. The ability of EMD 57033 to restore Ca^{2+}-sensitivity was significantly greater in TG compared to NTG fiber bundles containing cTnI-S43E/S45E (Fig. 4A,B). However, as shown in Fig. 4B,C, 3 μM EMD 57033 treatment of NTG or TG fiber bundles containing cTnI-S43E/S45E did not significantly increase the maximum Ca^{2+}-dependent tension. After EMD treatment, tension increased by approximately 3.4 mN/mm^2 in TG fiber bundles containing cTnI-S43E/S45E, and by approximately 2.1 mN/mm^2 in NTG fiber bundles containing cTnI-S43E/S45E. The difference between the increase in tension seen in NTG and TG α-Tm (E180G) fiber bundles after EMD treatment was not significant.

3.4. Endogenous levels of PKC-ε-mediated phosphorylation of cTnI in myofilaments from hearts of TG α-Tm (E180G) mice and NTG controls

We used the 'back-phosphorylation' technique to determine endogenous levels of PKC-ε phosphorylation of cTnI in TG α-Tm (E180G) mouse myofibrils (Fig. 5). Shown in Fig. 5A is an autoradiograph demonstrating the level of ^{32}P-incorporation into lysates prepared from TG and NTG

Table 1
Ca^{2+}-sensitivity and maximum tension generated in NTG vs. TG α-Tm (E180G) fiber bundles containing cTnI-WT, cTnI-S43E/S45E, or c TnI-S43E/S45E plus treatment with 3 μM EMD 57033

Modification to fiber	Mice	pCa_{50}	Maximum tension (mN/mm^2)
cTnI-WT	NTG	5.68 ± 0.04	27.9 ± 2.6
	TG	5.78 ± 0.04	32.4 ± 2.6*
cTnI-S43E/S45E	NTG	5.29 ± 0.02	18.1 ± 1.0
	TG	5.42 ± 0.02*	18.6 ± 1.0‡
cTnI-S43E/S45E	NTG	5.46 ± 0.02	20.1 ± 2.1
plus EMD	TG	5.72 ±0.03*†	21.2 ± 2.1

TG fiber bundles demonstrated higher pCa_{50} (*$P < 0.01$ compared to NTG), higher maximum tension (*$P < 0.03$ compared to NTG), larger decrease in tension after cTnI substitution with cTnI-S43E/S45E (‡$P < 0.01$ compared to NTG), and larger increase in pCa_{50} after EMD treatment (†$P < 0.01$ compared to NTG).

myofibrils after they were treated with PKC-ε and phosphorylated in the presence of [γ-^{32}P] ATP. The levels of ^{32}P-incorporation were quantified using a densitometer, and as shown in Fig. 4B the percent of ^{32}P-incorporation into cTnI (or 'back-phosphorylation' of cTnI) seen in TG samples (135 ± 19%) was slightly higher but not statistically significant, compared to NTG controls (normalized to 100%).

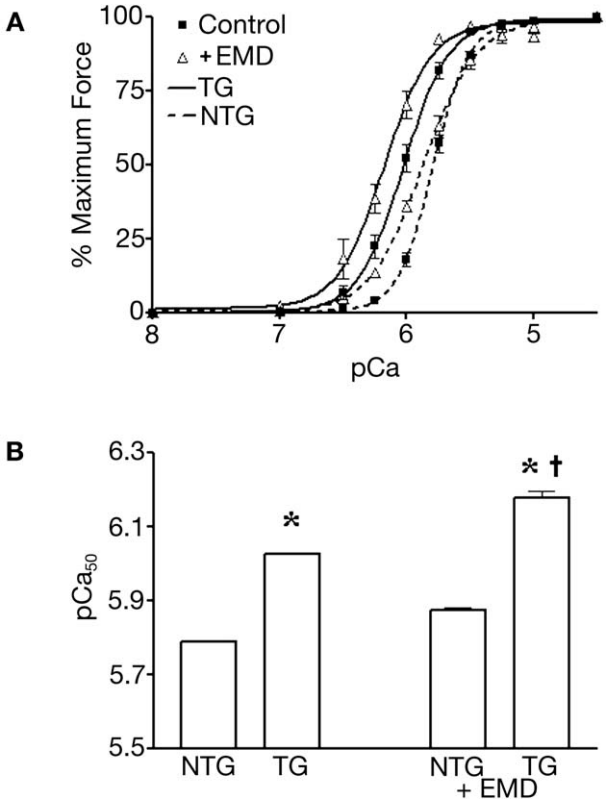

Fig. 3. Effect of 3 μM EMD 57033 on pCa–force relations of NTG and TG α-Tm (E180G) skinned fiber bundles containing native Tn components. (A) The pCa–force relations determined in NTG (dotted lines) and TG (solid lines) fiber bundles containing their native Tn components (filled squares) and after treatment of these fiber bundles with 3 μM EMD 57033 (open triangles). (B) Changes in Ca^{2+}-sensitivity (pCa_{50}) for NTG and TG fiber bundles containing their native Tn components, before and after treatment with 3 μM EMD 57033. As noted (*) fiber bundles from TG mice were significantly more sensitive to Ca^{2+} compared to NTG fiber bundles, both before and after EMD treatment ($P < 0.01$). As noted (†) the ability of EMD 57033 to increase Ca^{2+}-sensitivity was significantly greater in TG fiber bundles compared to NTG fiber bundles ($P < 0.01$). $n = 7$ each for NTG or TG fiber bundles containing native Tn and treated with 3 μM EMD 57033.

4. Discussion

Our data provide new understanding regarding how alterations in signaling through the C-lobe of cTnC can modulate the generation of tension in the myofilament. Moreover, results demonstrating differential effects of altered signaling at this interface in myofilaments containing WT and mutant Tm provide new insights into the pathology of FHC and its treatment with pharmacological agents. Data on the structure of the Tn complex indicate that the C-terminal lobe of cTnC interacts simultaneously with the near N-terminus of cTnI as well as a C-terminal region of cTnT [17]. Although this region is critical for transduction of the Ca^{2+}-binding signal triggered at the cTnC N-lobe to actin–Tm, there is little information on whether modulation of this interface affects this signaling cascade. To investigate the significance of signaling at the interface of the near N-terminal region of cTnI with the C-lobe of cTnC, we employed myofilaments containing a point mutation in Tm (E180G), which has been shown to induce a significant increase in Ca^{2+}-sensitivity. If the modifications we imposed had demonstrated the same or similar effects on the myofilaments containing either Tm (E180G) or Tm (WT), we could conclude that modulation of the protein–protein interactions surrounding the C-lobe does not significantly influence signaling from the C-lobe of cTnC to actin–Tm. However this was not the case. Our data show phosphorylation of cTnI at its interface with the cTnC C-lobe induced a significantly greater decrease in maximum tension in the α-Tm (E180G) myofilaments than in the NTG myofilaments. Moreover, the increase in Ca^{2+}-sensitivity induced by EMD 57033 was substantially greater in the Tm (E180G) myofilaments than in controls.

There is strong evidence supporting our conclusion that the modifications we introduced into cTnI and cTnC specifically involve the interaction of the C-lobe of cTnC with its neighbors on the thin filaments. Using NMR spectroscopy and selective isotope labeling, we previously reported [18] that a region of cTnI comprised of amino acids (residues 33–80) reacts specifically with the C-lobe of cTnC. Thus, introduction of Glu residues at positions 43 and 45, which mimic PKC-mediated phosphorylation [5], would be expected to specifically affect the interface of cTnI and the C-lobe of cTnC. This same near N-terminal region of cTnI competes for binding with EMD 57033 at the C-lobe of

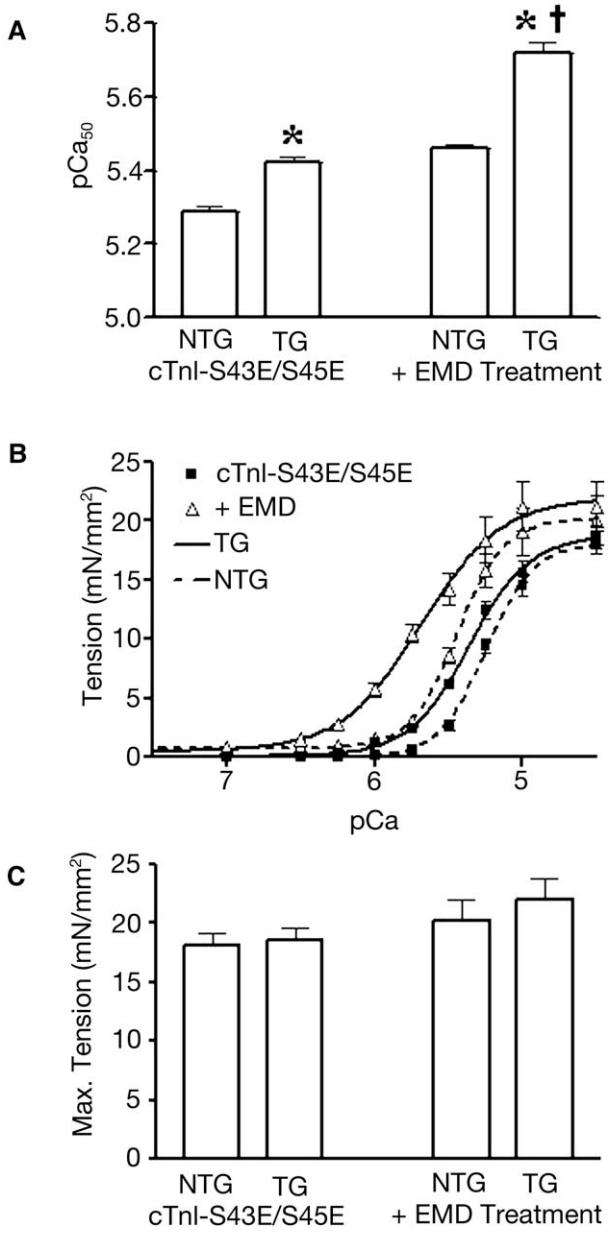

Fig. 4. Effect of 3 μM EMD 57033 on pCa–force and tension relations of NTG and TG-skinned fiber bundles containing cTnI-S43E/S45E. (A) Changes in Ca^{2+}-sensitivity (pCa_{50}) for NTG and TG fiber bundles containing cTnI-S43E/S45E, before and after treatment with 3 μM EMD 57033. As noted (*) fiber bundles from TG mice containing cTnI-S43E/S45E were significantly more sensitive to Ca^{2+} compared to NTG fiber bundles containing cTnI-S43E/S45E, both before and after EMD treatment ($P < 0.01$). As noted (†) the ability of EMD 57033 to increase Ca^{2+}-sensitivity was significantly greater in TG compared to NTG fiber bundles containing cTnI-S43E/S45E ($P < 0.01$). (B) The pCa–tension relations determined in NTG (dotted lines) or TG (solid lines) fiber bundles containing cTnI-S43E/S45E (filled squares), and after treatment of these fiber bundles with EMD 57033 (open triangles). (C) The changes in maximum Ca^{2+}-dependent tension measured at pCa 4.5 in NTG or TG fiber bundles containing cTnI-S43E/S45E were not significantly different, before or after treatment of these fiber bundles with EMD 57033. n = 13–19 each for NTG or TG fiber bundles containing cTnI-S43E/S45E and treated with 3 μM EMD 57033.

cTnC. Our previous study, done in collaboration with Sykes and co-workers [19], reported that EMD 57033 docks at the

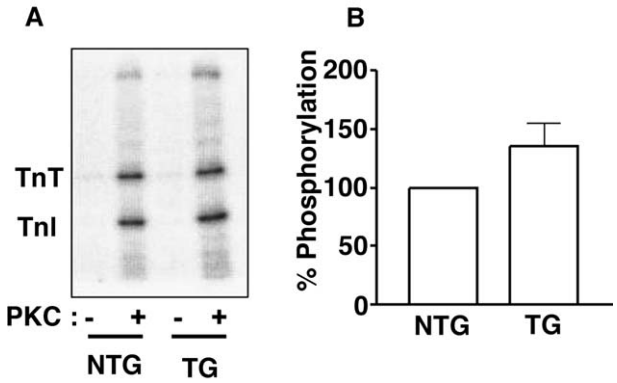

Fig. 5. Endogenous levels of PKC-ε phosphorylation of cTnI in NTG vs. TG-skinned fiber bundles as determined by a back-phosphorylation technique. (A) An autoradiograph showing the level of PKC-ε-mediated ^{32}P-incorporation into NTG and TG myofilaments. (B) Bar graph summary of the quantification of ^{32}P-incorporation (or 'back-phosphorylation) into cTnI bands. The percent of phosphorylation was normalized to the levels of ^{32}P-incorporation into cTnI bands from NTG myofilaments. n = 15 myofibrillar lysates each prepared from NTG or TG hearts for back-phosphorylation.

C-lobe of cTnC and is displaced by a cTnI peptide comprised of amino acids (residues 34–71), but not by a cTnI peptide comprised of amino acids (residues 128–147).

Evidence from other studies also indicates that the C-lobe of cTnC not only plays a structural role, but may also regulate the interactions among cTnI, cTnT, and Tm that promote the force-generating actin–myosin interaction. Studies by Morimoto et al. [20] indicated that the interactions of cTnI with the C-lobe of cTnC are required for a drop in pH to reduce cross-bridge-dependent activation of force in cardiac myofilaments. Their data showed that, whereas removal of cTnC did not inhibit cross-bridge-dependent activation at pCa 9.0, the effect of a fall from pH 7.0 to 6.2 on cross-bridge-dependent activation was no longer evident. Morimoto et al. also demonstrated that switching cTnI with slow skeletal TnI significantly altered the effect of pH on cross-bridge-dependent activation. At pCa 9.0, amino acid residues 39–58 of cTnI have the tightest interaction with the C-lobe of cTnC [21]. Effects of phosphorylation in this region of cTnI also provide evidence for the significance of this interface in regulation of tension. Noland and Kuo [22] reported a decrease in the affinity (K_{app}) of myosin S-1 for regulated thin filaments containing cTnI phosphorylated at Ser-43 and Ser-45 residues, when compared to those containing unphosphorylated residues. We have also demonstrated that replacement of Ser-43 and Ser-45 with Glu in cTnI of skinned fiber bundles induces a depression in maximum tension [5]. Functional consequences of phosphorylation of these sites on maximum tension have also been determined by comparing effects of activation of the PKC pathway on WT cardiac myofilaments and myofilaments from TG mouse hearts in which native cTnI was partially replaced with cTnI-S43A/S45A [14].

Evidence on the differential effects of EMD 57033 on TG α-Tm (E180G) and NTG myofilaments supports the idea that

modifications of the interaction of cTnI with the C-lobe of TnC may ultimately affect the ability of strong cross-bridges to move Tm. We examined whether signaling through the cTnC C-lobe is altered in fiber bundles containing Tm (E180G) by directly modifying the C-lobe with EMD 57033, a myofilament Ca^{2+}-sensitizer that binds stereo-specifically to this region. EMD treatment of Tm (E180G) myofilaments containing cTnI-S43E/S45E increased the Ca^{2+}-sensitivity to a significantly greater extent than in NTG myofilaments containing cTnI-S43E/S45E. Therefore, while the presence of Glu residues at Ser-43 and Ser-45 on cTnI did not appear to alter the ability of Tm (E180G) to receive the Ca^{2+}-activated signal transmitted from cTnC through cTnI and cTnT, the additional binding of EMD 57033 at the cTnC C-lobe appeared to influence on the role of Tm (E180G) in thin filament activation. Studies by Wang et al. [19], which showed that the cTnI peptide, $cTnI_{33–80}$, effectively competes with EMD 57033 for binding to the hydrophobic pocket within the C-lobe of cTnC, strongly support our hypothesis that alterations in this region of cTnI, i.e. phosphorylation by PKC, affects the ability of this N-terminal region of cTnI to bind to the cTnC C-lobe, which could in turn affect EMD 57033 binding to the cTnC C-lobe. Recent studies by Finley et al. [23] support this idea and showed that PKC pseudo-phosphorylation of cTnI (they substituted Ser-43 and Ser-45 with Asp residues) leads to structural modifications of the cTnC C-lobe that might affect the Ca^{2+}–Mg^{2+}-dependent binding region. Therefore, the presence of Glu residues at Ser-43 and Ser-45 are likely to have similar effects on the cTnC C-lobe structure in a manner that may allow EMD 57033 to bind more easily to the C-lobe of cTnC than in the presence of unphosphorylated cTnI.

Previous studies from our laboratory support the idea that the ability of EMD 57033 to significantly increase Ca^{2+}-sensitivity in TG α-Tm (E180G) myofilaments containing cTnI-S43E/S45E may be due to its ability to promote strong cross-bridge movement of Tm, rather than an effect on Ca^{2+} binding to TnC [24]. We previously studied the effects of EMD 57033 on Ca^{2+} signaling in myofilament preparations from canine hearts and demonstrated that EMD 57033 was able to exert significant positive inotropic effects, stimulate maximum MgATPase rate, increase filament-sliding velocity, and increase the actin–myosin interaction. However, in these studies concentrations of EMD 57033 up to 30 μM did not significantly alter the Ca^{2+}-binding properties of TnC in skinned fiber bundle preparations.

Modulation of signaling involving the C-lobe of cTnC and its differential effects in myofilaments containing Tm (WT) and Tm (E180G) are likely to involve altered interactions with C-terminal regions of cTnT. The changes in the state of cTnC and cTnI can be sensed by cTnT in a signaling cascade that is ultimately responsible for the movement of Tm allowing strong cross-bridge binding [25]. The C-terminal head of cTnT interacts with the C-lobe of cTnC and the N-terminal region of cTnI. The N-terminal tail of cTnT interacts with a region of Tm surrounding Cys-190, which is close to the FHC-linked mutation at Tm (E180G). The interaction between TnT and TnC has been shown by Potter et al. [26] to be necessary for activation of Ca^{2+}-dependent actomyosin ATPase. In addition, Blumenschein et al. [17] showed that two fast skeletal (fs) TnT peptides from the C-terminal T2 region, $TnT_{160–193}$ and $TnT_{228–260}$, can bind to the C-lobe of TnC. Their studies also showed that the $TnT_{160–193}$ peptide can simultaneously bind to the C-lobe of TnC along with a peptide of the N-terminal region of fsTnI, $TnI_{1–40}$. This region of fsTnI corresponds to the region of cTnI containing Ser-43 and Ser-45. $TnT_{160–193}$ binding to TnC occurs at a region just N-terminal to the region where $TnI_{1–40}$ binds to TnC. Although these regions of TnT and TnI do not directly compete for binding to the same site on TnC, it is apparent that allosteric effects may occur at this region. Our hypothesis is that regions of similar sequence on cTnT and cTnI bind similarly to the C-lobe of cTnC, and that modulation of this region not only affects activation of the thin filament by Ca^{2+} binding, but also by cross-bridge binding. These observations provide a structural basis for the possibility that the FHC-linked mutation on α-Tm at position 180 is not only affecting the interaction of Tm with TnT, but also causing further secondary alterations because of the close interactions TnT has with TnC and TnI. In addition to alterations on Tm that potentially affect TnT, we have proposed that alterations in the phosphorylation state of Ser-43 and Ser-45 on cTnI might affect the phosphorylation state of cTnT [14]. The fact that these regions of TnT, TnI, TnC, and Tm are so closely regulated could explain why such significant alterations in Ca^{2+}-signaling were seen in our experiments using myofilaments containing alterations on Tm (E180G), cTnI (S43E/S45E), and cTnC (EMD 57033 treatment).

Our experiments examined the possibility that as with the hypertrophy induced by extrinsic factors, such as hypertension, the hypertrophy of FHC induced by Tm (E180G) may signal an increase in PKC activity and phosphorylation of cTnI. Supporting this hypothesis is evidence from studies of human hearts sampled at end-stage heart failure [27]. Compared to controls, these hearts showed an increase in PKC activity and phosphorylation of cTnI. But, upon comparison of the basal levels of cTnI phosphorylation by back-phosphorylation using PKC-ε, the predominant isoform found in adult cardiac myocytes [28], we found no significant difference between the hearts containing Tm (WT) vs. Tm (E180G). This finding was rather interesting, because even though the Tm (E180G) hearts demonstrated considerable amounts of hypertrophy, they did not have increased levels of PKC phosphorylation of cTnI. All of our studies were performed on mice that were approximately 3 months of age, but previous findings by Prabhakar et al. [6] have shown these mice demonstrate a dramatic hypertrophic response that culminates in the death of these mice by around 5 months of age. It is possible that changes in the level of PKC phosphorylation have not yet started to occur in these mice-expressing Tm (E180G) at 3 months of age, and instead, may play a more significant role during the latter transition from hyper-

trophy to heart failure that results in the death of these mice. Whatever the case, our data indicate that the same levels of phosphorylation at PKC sites on cTnI will depress function to a greater extent in myofilaments containing Tm (E180G) than in controls.

Acknowledgements

This work was supported by National Institutes of Health Grants R01 HL 64035 (R.J.S.), P01 HL 62426 (Project 1, R.J.S.), P01 HL 22619 (D.F.W.), T3207692 (E.M.B., M.P.S.), an Individual National Research Service Award F32 HL 10409 (M.P.S.), and an American Heart Association Pre-Doctoral Fellowship, Midwest Affiliate (E.M.B.).

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