Identification of a Functionally Critical Protein Kinase C Phosphorylation Residue of Cardiac Troponin T *

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Cardiac Troponin T (cTnT) is one prominent substrate through which protein kinase C (PKC) exerts its effect on cardiomyocyte function. To determine the specific functional effects of the cTnT PKC-dependent phosphorylation sites (Thr197, Ser201, Thr206, and Thr287) we first mutated these residues to glutamate (E) or alanine (A). cTnT was selectively mutated to generate single, double, triple, and quadruple mutants. Bacterially expressed mutant troponins were replaced with a recombinant cTnT-wt. In experiments reported here we have focused on the functional significance of PKC phosphorylation and specific charge modifications, mimicking phosphorylation, of cardiac cTnT. cTnT is the “lever” that transmits the signal generated by Ca2+-induced conformational changes in cTnC-cTnT structures to the filamentous protein, Tm. Upon Ca2+-activation Tm undergoes both a movement and a rotation on the actin filament to facilitate the binding of myosin heads to actin and to promote the process of contraction (11–13). As a critically positioned molecule in the thin filament, it is clear that modifications of cTnT have the potential for versatile interactions with adjacent proteins and for producing significant functional effects (14). Even so, the specific effects of cTnT phosphorylation on tension and ATP hydrolysis have not been well studied in the myofilament lattice. There is no evidence for phosphorylation of cTnT by PKA. However, in vitro studies demonstrated that there are four main sites for PKC-dependent phosphorylation on cTnT (15), which are at Thr197, Ser201, Thr206, and Thr287 (mouse sequence). These sites are located in the functionally significant C-terminal half of the molecule. It is this region of cTnT that is likely to interact with TnI and TnC and possibly with Tm, and is essential in the transmission of the Ca2+-binding signal to Tn-actin (16). Noland and Kuo (17) reported that exclusive phosphorylation of cTnT results in a ~50% decrease in maximum actomyosin Mg-ATPase activity using in vitro fully reconstituted systems. When cTnT was exclusively phosphorylated under the same conditions, there was a much

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smaller, −20%, decrease. A recent study in our laboratory has indicated that pan-activation of PKC results in phosphorylation of cTnT and cTnI and induces a 30% reduction of maximum force of mouse cardiac myofilaments (18). When cTnT was partially replaced with a fast skeletal (fs) isoform, which is not phosphorylated by PKC, the effect of PKC activation was no longer evident. These results indicated that cTnT phosphorylation might be pivotal for the PKC-induced depression of tension in the myofilament.

However given the large number of PKC isoforms, multiple phosphorylation sites, the lack of homogeneously phosphorylated samples and inability of performing site-directed phosphorylation, the role of each cTnT phosphorylation site remains unknown. In the present study we generated cTnT mutants in which Glu (mimic of phosphorylation) or Ala residues were placed at the PKC phosphorylation sites. cTnT was selectively mutated to generate single, double, triple, and quadruple mutants (see Fig. 3). The wild-type and mutated cTnTs were used to reconstitute a recombinant cTn complex, which was incorporated into detergent extracted left ventricular papillary muscle fiber bundles by replacing the endogenous troponin complex (19, 20). We simultaneously determined tension development and actomyosin Mg-ATPase activity in the reconstituted preparations as a function of Ca\(^{2+}\) concentration (21). Our data provide the first evidence that Thr\(^{206}\) is the functionally critical phosphorylation residue. Its exclusive phosphorylation by PKC-α or replacement by Glu (mimicking phosphorylation) significantly decreased maximum tension, actomyosin Mg-ATPase activity, myofilament Ca\(^{2+}\)-sensitivity and cooperativity. On the other hand the charge modification of the other 3 residues together (T\(^{197}/S\(^{201}/T\(^{206}\)) had no functional effect. Fibers bundles containing cTnT-wt-P (but not T\(^{197}/S\(^{201}/T\(^{206}\)) atg gct gac gtt ttc ccg ggc aac-3') exhibited a significant decrease of tension cost as compared with cTnT-wt implicating cTnT phosphorylation in the myofilament.

To reconstitute a recombinant cTn complex, which was incorporated into detergent extracted left ventricular papillary muscle fiber bundles by replacing the endogenous troponin complex. For protein expression, SF-9 cells were grown at 27°C in suspension culture to 2 × 10\(^6\) cells/ml and infected with a MOI of 10. After 3 days cells were harvested and resuspended in 40 ml of Lysis Buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM DTT, 0.5 mM leupeptin, 1% Triton X-100, and 0.1% phosphatidylinositol fluoride. The cell suspension was lysed in a 50-ml hand-held homogenizer chilled on ice. The lysate was centrifuged at 50,000 × g for 30 min, and the supernatant fraction was subjected to ammonium sulfate fractionation as previously reported (25). The final ammonium sulfate pellet was solubilized in TrnT-Buffer A, and then dialyzed at 4°C against TrnT-Buffer A. The dialyzed sample was applied on a DEAE-Fast Flow Sepharose column (Amersham Biosciences) connected to a FPLC System (Amersham Biosciences). cTnT was eluted with a 0.0–0.4 M KCl gradient in TrnT-Buffer A. The fractions containing cTnT were analyzed by 12% SDS-PAGE and those containing >90% pure cTnT were pooled, extensively dialyzed against 0.1% formic acid, 1 mM DTT in H\(_2\)O, lyophilized, and stored in powder form at −80°C.

**PKC-α Expression and Purification—** Recombinant human PKC-α (26) was purchased from ATCC (American Type Culture Collection, ATCC 80045). The following sense primers (from Operon) were used for the subsequent subcloning into pVL1392 vector (BD Pharmingen): PKCABRI restriction sites by PCR, for the

| Mutated residues are underlined. |
|----------------------------------|
| cTnT-T197A-1                     |
| cTnT-T197E-1                     |
| cTnT-T206A-1                     |
| cTnT-T206E-1                     |
| cTnT-T287A-1                     |
| cTnT-T287E-1                     |
| cTnT-T197S201A                   |
| cTnT-T197E201E                   |
| cTnT-T287A201E                   |
| cTnT-T287E201E                   |
| cTnT-T206A201E                   |
| cTnT-T206E201E                   |
| cTnT-T197S201E/T206A            |
| cTnT-T197E201E/T206E            |
| cTnT-T287A201E/T206E            |
| cTnT-T287E201E/T206E            |
| cTnT-T206A201E/T206E            |
| cTnT-T206E201E/T206E            |

**Materials and Methods**

**Preparation of Site-directed cTnT Mutants—** cDNA of adult mouse cardiac TnC (a generous gift of Dr. Jil Tardiff) was previously cloned into a pSBETA vector (22) and selectively mutated using the Quick-Change Site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Sense primers (from Operon) shown in Table I were used for the respective mutations. Mutated residues are underlined. The identity of constructs was verified by DNA sequencing.

**Contractile Protein Expression and Purification—** Human cardiac TnC and mouse cardiac TnI were expressed and purified as previously described (23). Bovine cardiac Tn for the codelubination assay was purified as previously described by Tobacman and Adelstein (24). Recombinant adult mouse cTnT-wt and mutants were expressed and purified by a modified method of Chandra et al. (25). cTnT was expressed in BL21(DE3) cells using the pSBETa expression plasmid. BL21(DE3) cells grown over night in Luria Broth supplemented with 25 μg/ml kanamycin, were collected by centrifugation at 6000 × g for 10 min. at 4°C. The cell pellet was then resuspended well in a suitable volume (about 200 ml) of TrnT-Buffer A (20 mM Tris, pH 8.0, 6 mM urea, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM DTT) containing 0.5% Triton X-100. The cells were lysed by sonication on ice, followed by 60 min of centrifugation at 48,000 × g, at 4°C. The supernatant fraction was subjected to ammonium sulfate fractionation as previously reported (25). The final ammonium sulfate pellet was solubilized in TrnT-Buffer A, and then dialyzed at 4°C against TrnT-Buffer A. The dialyzed sample was applied on a DEAE-Fast Flow Sepharose column (Amersham Biosciences) connected to a FPLC System (Amersham Biosciences). cTnT was eluted with a 0.0–0.4 M KCl gradient in TrnT-Buffer A. The fractions containing cTnT were analyzed by 12% SDS-PAGE and those containing >90% pure cTnT were pooled, extensively dialyzed against 0.1% formic acid, 1 mM DTT in H\(_2\)O, lyophilized, and stored in powder form at −80°C.

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**TABLE I**

| Oligonucleotide primers |
|-------------------------|
| T\(^{197}\)S201A         |
| T\(^{197}\)E201E         |
| T\(^{206}\)A201E         |
| T\(^{206}\)E201E         |
containing 20 mM MOPS pH 6.5, 200 mM KCl, 5 mM EGTA, 5 mM MgCl2, 1 mM DTT (19, 20). Next, the cTn complex was aliquoted and stored at −80 °C until it was used for exchange experiments.

Exchange of Recombinant cTn in Left Ventricular Fiber Bundles—Fiber bundles were prepared from hearts of 5–6 months old, FVBn mice purchased from Harlan or Charles Rivers laboratories. The mice were anesthetized with ether and hearts were quickly removed and rinsed free of blood in ice-cold saline (0.9% NaCl). Muscle strips (~150–200 μm wide and 3–4 mm long) were dissected from left ventricular papillary muscle. Then the fiber bundles were detergent-treated at 4°C, in a High Relax Buffer containing 20 mM MOPS pH 7.0, 50 mM potassium phosphate, 6.8 mM MgCl2, 10 mM EGTA, 25 μM CaCl2, 12 mM phosphocreatine, 5 mM Na2ATP, 10 μM creatine kinase, 0.5 mM DTT, a mixture of protease inhibitors and 1% Triton-X100 (21). Following the detergent treatment the fiber bundles were transferred to a bath containing a recombinant cTn complex in Exchange Buffer (see above) and incubated overnight at 4°C. The extent of recombinant cTn exchange was determined after isometric tension and actomyosin Mg-ATPase activity measurements (see below) by immunoblot analysis of control versus exchanged fiber bundles using anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology) followed by treatment of the same membrane (after c-Myc removal) with anti-cTnT antibody (clone JLT-12, Sigma). The presence of the 9-amino acid Myc tag at the N terminus of recombinant cTnT (20) allowed us to separate it from the endogenous mouse cTnT on a 15% SDS-PAGE (acylamide:his-acylamide ratio 200:1).

Isometric Tension and Actomyosin Mg-ATPase Activity Measurements—The method of de Tombe and Stienen used in these experiments was described in detail elsewhere (21). The fiber bundles (control or exchanged) were attached to a displacement generator at one end and a force transducer at the other end using aluminum T-clips. The sarcomere length was adjusted to 2.3 μm using a laser defraction pattern and the cross-sectional area was determined based on an elliptical model. Fiber bundles were equilibrated for 5 min in Relaxing Buffer (100 mM BES, pH 7.1, 8.57 mM MgCl2, 5.80 mM Na2ATP, 20 mM EGTA, 42.5 mM potassium phosphate), followed by 2 min in Pre-Activating Buffer (100 mM BES pH 7.1, 7.78 mM MgCl2, 5.80 mM Na2ATP, 0.50 mM EGTA, 19.5 mM HDTA, 43.6 mM potassium phosphate) and then immersed into a bath containing a Maximal Activating Solution (100 mM BES, pH 7.1, 7.63 mM MgCl2, 5.87 mM Na2ATP, 20 mM Ca2+-EGTA, 43.6 mM potassium phosphate). Relaxing, pre-activating, and maximum activating solutions also contained 900 μM NH4Cl, 5 mM sodium azide, 10 mM phosphate/ENPRyuvate, 1 mg/ml pyruvate kinase (500 units/mg), 0.12 mg/ml lactate dehydrogenase (870 units/mg), 10 μM oligomycin B, 20 μM P2P-di(adenosine-5')pentaphosphate, and 10 μM leupeptin, 1 μM pepstatin, 1 mM DTT, and 10 mM phenylmethylsulfonyl fluoride. After each contraction, fiber bundles were incubated for 1 min in Relaxing Buffer, followed by 2 min in Pre-Activating Buffer. The final contraction was induced in Activating solution containing maximally activating [Ca2+]i. Only those fibers able to generate greater than 80% of initial tension in their final contraction were kept for analysis. The isometric tension and actomyosin Mg-ATPase activity were determined simultaneously at 20°C in the presence of variable Ca2+ concentrations as described (30). Data were analyzed using Labview (National Instruments, Austin, Texas). Tension-, actomyosin Mg-ATPase activity-[Ca2+]i relations were fit by a nonlinear fit procedure to a modified Hill equation shown in Equation 1,

\[ P = \text{Max} \times [\text{Ca}^{2+}]^n / (K_{d}^{n} + [\text{Ca}^{2+}]^n) \] (Eq. 1)

where \( P \) is the parameter of interest (isometric tension, actomyosin Mg-ATPase activity); Max is the maximum value at saturating [Ca2+]i; \( K_{d} \) is the [Ca2+]i at which 50% of Max is reached; and \( n \) represents the slope of the relationship (Hill coefficient).

Cosedimentation Assay—The binding affinity of cTn complexes containing cTnT-wt or mutants to bovine cardiac troponymosin (Tm) was assessed using a cosedimentation assay (31). Variable amounts of cTn complex (wt or mutant) in the presence of 1 μM Tm were incubated in 10 mM MOPS pH 7.0, 5 mM MgCl2, 100 mM KCl and of either 1 mM EGTA or 100 μM CaCl2 at 4°C (conditions shown to promote sedimentation of cTn-Tm complex, (31)). The Tm-bound cTn was determined by cosedimentation at 74,000 rpm for 45 min using a TLA rotor. Sedimentation supernatants and pellets were analyzed by 15% SDS-PAGE. Densitometric analysis of the gels was carried out using NIH Image software.

Materials—Triton X-100 was purchased from Pierce. Ammonium sulfate (enzyme grade) and formic acid (sequencing grade) were from Fisher. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (phosphatidyl-serine) and 1,2-sn-dioleoylglycerol (diacylglycerol) were purchased from Avanti Polar-Lipids, Inc. Unless mentioned otherwise all the other chemicals were from Sigma.

Animal Care—All animals were handled in accordance with the guidelines of the Animal Care Committee at the University of Illinois, Chicago.

Statistical Analysis—All values are presented as mean ± S.E., and values of \( p < 0.05 \) were the criteria for statistical significance. Data was analyzed using a one-way ANOVA and post-hoc Dunnett’s t test.

RESULTS

Recombinant cTn Incorporation into the Myofilament Lattice—To assess the functional role of each cTnT phosphorylation site we used a modified version (see “Materials and Methods”) of the technique of whole troponin exchange introduced by Brenner et al. (19). This method allows the gentle replacement of the endogenous cTn with a recombinant cTn in muscle fiber bundles. The exchange procedure introduces no major alteration in the structure and properties of the fiber bundles, since the myofilament is never depleted of cTn. Fig. 1 shows the Western blot analysis of representative fibers used in our measurements (1 fiber bundle per lane). The samples were separated on 15% SDS-PAGE, and after transfer to a nitrocellulose membrane probed with an anti-c-Myc antibody (data not shown) and then with anti-cTnT antibody. Lanes 1, 5, 6, and 10 show representative native (no exchange) fibers bundles that have been used in control measurements, whereas the other lanes contain fibers that had undergone exchange with recombinant cTnT-wt or Tn-T3SE. The middle lanes 3 and 8 serve as standards for recombinant cTn. The endogenous mouse cTnT and recombinant mouse cTnT have different mobilities on the SDS-PAGE due to the presence of a c-Myc tag at the N terminus of recombinant cTnT. The c-Myc tag retards the recombinant cTnT migration on the gel allowing us to assess the exchange efficiency. The Western blot analysis demonstrated that exchange of endogenous cTnT with recombinant cTnT was basically 100% since the fibers bundles treated with the recombinant cTnT showed no band corresponding to endogenous cTnT.

Mechanoenergetic Characteristics of Native and Recombinant cTnT-wt Exchanged Fibers—In a first set of experiments,
we determined the functional effect of introducing recombinant cTn-wt into the myofilament lattice of left ventricular fiber bundles. To determine the effect of cTn-wt incorporation we compared the mechanoenergetic characteristics of native (no exchange) and recombinant cTn-wt exchanged fiber bundles. The data (Fig. 2 and Table II) indicate that the basal mechanics and energetics of native versus cTn-wt were not significantly altered. Native and cTn-wt exchange fibers demonstrated no significant differences in myofilament Ca$^{2+}$ sensitivity or Hill coefficients for any parameter (Table II).

**Effects of Glu Substitution at the cTnT PKC Phosphorylation Sites**—In a second set of experiments, we tested whether glutamate incorporation, mimicking phosphorylation, at the PKC phosphorylation sites of cTnT has a functional effect on myofilament mechanics and energetics. This pseudo-phosphorylation method was recently used in our laboratory to study the effect of PKC phosphorylation on cTnI residues Ser$^{43}$, Ser$^{45}$, and Thr$^{144}$ (8) by selectively mutating them to glutamic acid. The same approach was employed by Hoshijima et al. (32) in constructing a S16E phospholamban (PLN) recombinant adeno-associated vector (rAAV) to imitate the conformational changes induced by PKA phosphorylation. This method proved to be useful in mimicking the effects of PKA phosphorylation of PLN. In a similar fashion, Wick et al. (33) showed that substitution of the Thr$^{316}$ with glutamic acid, mimicking phosphorylation, confers constitutive activity to mouse PDK-1 in cells. There is an increase utilization of glutamate substituted Ser/Thr residues as a useful model for studying the functional consequences of protein phosphorylation (33–37).

Our approach was to selectively mutate the main PKC phosphorylation sites on cTnT (Thr$^{197}$, Ser$^{201}$, Thr$^{206}$, and Thr$^{287}$) to Glu, express and purify the recombinant proteins, reconstitute the cTn complex using wild type (see above) or mutated cTnT with cTnI-wt and cTnC-wt, and then measure simultaneously the effect on myofilament activation and ATP consumption at various [Ca$^{2+}$]. Our experimental rationale was to have different permutations of the mutated residues to Glu resulting in single, double, triple, and quadruple mutants. Fig. 3 schematically depicts the amino acid substitution and the location in the adult mouse cTnT. Table II summarizes the mechanical and energetic profiles of left ventricular fiber bundles harboring the various forms of cTnT as part of the exchanged cTn complex.

The cTn complex containing all four PKC sites mutated to Glu (T3SE) compared with cTn-wt had a severe inhibiting effect (see Fig. 4) on the myofilament tension development (reduced by 62%), actomyosin Mg-ATPase activity (reduced by 51%), Ca$^{2+}$ sensitivity and cooperativity. EC$^{50}$ of tension changed from 1.7 to 22.8 $\mu$M and the Hill coefficient from 4.7 to 2.5. The EC$^{50}$ of actomyosin Mg-ATPase activity changed from 1.5 to 20.9 $\mu$M and the Hill coefficient from 4.8 to 2.0. A similar trend, although not so pronounced, was seen in fiber bundles containing the cTn complexes harboring the triple Glu mutant (T3E), double (T2E), or single T206E. The introduction of the charged Glu only at position 206 in place of Thr induced severe changes on mechanoenergetics of the fiber bundles containing it. Data illustrated in Fig. 4 and summarized in Table II demonstrate a 51% depression in tension development and a 42% decrease in actomyosin Mg-ATPase rate. Myofilament Ca$^{2+}$ sensitivity and cooperativity were also affected. The EC$^{50}$ of tension changed from 1.7 to 18.6 $\mu$M and the Hill coefficient from 4.7 to 2.8; also EC$^{50}$, and Hill coefficient of actomyosin Mg-ATPase activity changed from 1.5 to 15.7 $\mu$M and from 4.8 to 2.4, respectively.

These results indicate that T206E is sufficient for the functional effect seen in the mutants containing this charge modification. To verify this result we generated a set of 3 constructs T2SE, TSE, and T197E in which the Thr$^{206}$ site was left unchanged. Fig. 5 shows the results of experiments in which left ventricular fiber bundles underwent whole cTn exchange with recombinant complexes that were “pseudo-phosphorylated” at residues other than Thr$^{206}$. Compared with cTn-wt exchange T2SE, TSE, T197E demonstrated no significant differences for any parameter (see Table II). T197E demonstrated a slight increase in isometric tension and actomyosin Mg-ATPase rate, displaying features that resemble the native fiber profile even closer than the wild-type exchange. These data thus support our conclusion that Thr$^{206}$ is the functionally critical cTnT PKC phosphorylation residue.

**Effects of Ala Substitution at the cTnT PKC Phosphorylation Sites**—We investigated the effect of Ala substitution at the cTnT PKC sites on myofilament mechanics and energetics. The Ala mutants were originally designed to serve as controls in our exchange experiments and we expected no major changes of mechanoenergetic parameters. However, the results obtained with these mutants were quite intriguing. As shown by the...
The isometric tension and actomyosin Mg-ATPase of native (no exchange) and recombinant cTn-treated (exchanged) fiber bundles were determined at varying Ca\(^{2+}\) concentrations (see "Materials and Methods"). Tension cost is defined as the slope of the relation between actomyosin Mg-ATPase rate and tension. N represents the number of fibers (hearts) used for each group.

### Table II

| Isometric Tension | Actomyosin Mg-ATPase |
|-------------------|----------------------|
| Maximum nM/mm\(^2\) | EC\(_{50}\) µM | Hill Coefficient | Maximum pmol/mm\(^2\) | EC\(_{50}\) µM | Hill Coefficient |
| Native | 42.3 ± 3.3 | 1.6 ± 0.1 | 4.9 ± 0.8 | 369.2 ± 24.3 | 1.4 ± 0.1 | 5.3 ± 1.1 |
| WT | 39.2 ± 1.0 | 1.7 ± 0.1 | 4.7 ± 0.6 | 362.9 ± 15.9 | 1.5 ± 0.1 | 4.8 ± 0.8 |
| T3SE | 14.9 ± 2.5 \(^a\) | 22.8 ± 0.8 \(^a\) | 2.5 ± 0.2 \(^a\) | 181.1 ± 19.8 \(^a\) | 20.9 ± 0.7 \(^a\) | 2.0 ± 0.2 \(^a\) |
| T3E | 26.3 ± 1.6 \(^a\) | 7.5 ± 0.7 \(^a\) | 2.5 ± 0.5 \(^a\) | 227.3 ± 17.8 \(^a\) | 6.4 ± 0.3 \(^a\) | 2.8 ± 0.8 \(^a\) |
| T2E | 24.3 ± 3.9 \(^a\) | 15.7 ± 0.4 \(^a\) | 2.2 ± 0.1 \(^a\) | 230.2 ± 19.0 \(^a\) | 12.0 ± 1.0 \(^a\) | 1.8 ± 0.2 \(^a\) |
| T206E | 19.1 ± 1.3 \(^a\) | 18.6 ± 0.7 \(^a\) | 2.8 ± 0.6 \(^a\) | 210.3 ± 14.4 \(^a\) | 15.7 ± 0.8 \(^a\) | 2.4 ± 0.5 \(^a\) |
| T2SE | 38.2 ± 1.8 | 2.3 ± 0.2 | 4.2 ± 0.2 | 359.8 ± 16.0 | 2.1 ± 0.2 | 3.9 ± 0.1 |
| STE | 36.8 ± 4.1 | 2.3 ± 0.1 | 4.5 ± 0.8 | 350.6 ± 23.6 | 1.6 ± 0.1 | 3.9 ± 0.4 |
| T197E | 42.4 ± 2.0 | 2.5 ± 0.1 | 4.9 ± 0.2 | 392.9 ± 21.1 | 2.2 ± 0.1 | 4.2 ± 0.3 |
| T3SA | 30.6 ± 2.0 \(^a\) | 6.6 ± 0.3 \(^a\) | 3.7 ± 0.7 | 280.3 ± 17.0 | 6.2 ± 0.6 \(^a\) | 3.7 ± 0.5 |
| T3A | 36.8 ± 2.2 | 7.5 ± 0.1 \(^a\) | 4.1 ± 0.3 | 319.1 ± 14.6 | 6.9 ± 0.6 \(^a\) | 3.5 ± 0.3 |
| T206A | 21.1 ± 3.2 \(^a\) | 12.0 ± 0.8 \(^a\) | 2.9 ± 0.1 \(^a\) | 229.9 ± 32.2 \(^a\) | 10.8 ± 0.7 \(^a\) | 2.6 ± 0.1 \(^a\) |
| WT-P | 30.4 ± 1.3 \(^a\) | 5.3 ± 0.2 \(^a\) | 2.8 ± 0.2 \(^a\) | 231.4 ± 24.6 \(^a\) | 6.4 ± 0.3 \(^a\) | 1.5 ± 0.1 \(^a\) |
| T206-P | 29.7 ± 1.4 \(^a\) | 2.7 ± 0.1 \(^a\) | 2.6 ± 0.1 \(^a\) | 260.3 ± 13.6 \(^a\) | 2.7 ± 0.1 \(^a\) | 2.4 ± 0.3 \(^a\) |

\(^a\) p < 0.05 as compared with wild-type exchange.

*Fig. 3. Schematic representation of the cTnT mutants.* Cartoon of cTnT showing the PKC phosphorylation sites (Thr\(^{197}\), Ser\(^{201}\), Thr\(^{206}\) and Thr\(^{287}\)). The cTnT mutants used in the present study are arranged in three groups. The first four mutants have in common the presence of the Thr\(^{206}\) residue mutated to Glu. The second group contains an unaltered Thr\(^{206}\) residue, whereas the third group has Thr\(^{206}\) mutated to Ala.

Data summarized in Table II and Fig. 6, the substitution of Thr\(^{206}\) with Ala, had an effect comparable in severity with T206E (depressed tension and actomyosin Mg-ATPase), and a notable change in Ca\(^{2+}\) sensitivity and cooperativity). We also observed an increase in tension cost, but somewhat smaller than with T206E. T3SA and T3A also contain T206A, but its effect seems to be buffered by the presence of Ala at the other PKC sites. Overall we conclude that Thr\(^{206}\) is positioned in a hypersensitive region of the protein and any changes (such as phosphorylation by PKC) have the potential of depressing myocardial functional properties and energetics.

**Analysis of Glu Substitutions on cTn-Tm Binding**—To test whether cTnT phosphorylation affects the binding between cTnT and Tm we used a modified cosedimentation assay of Hill and Tobacman (31). For these experiments the recombinant cTn complexes were selected from the same batch used in fiber exchange measurements. Fig. 7 shows the binding curves of cTn-wt and mutants T3SE and T2SE to Tm. Our results indicated that there is no significant difference between the binding of cTn-wt and cTn containing the mutants cTnT to Tm. This result fits with previous findings (31) that identified the cTnT fragment spanning amino acids 95–153 (bovine sequence) as responsible for most of the binding affinity of cTnT to Tm. This area of the molecule remained unchanged by our charged mutations.

**Effects of Thr\(^{206}\) and cTnT-wt Phosphorylation by PKC**—The above “pseudo-phosphorylation” study proved to be a useful tool in identification of Thr\(^{206}\) as the functionally critical phosphorylation residue. The next logical step was to phosphorylate Thr\(^{206}\) by PKC and see if we get the same functional effect. However, we found out that the PKC-ε used in the previous study (8) to phosphorylate cTnT performed poorly in phosphorylating cTnT. In order to find which PKC isozyme prefers cTnT and especially Thr206 we compared recombinant PKC-α, -βII, and -ε. Our data demonstrate that PKC-α preferentially phosphorylates Thr\(^{206}\), followed by PKC-βII and -ε. The PKC-α phosphorylated cTnT-wt-P and cTnT-T2SA-P (used for exclusive Thr\(^{206}\) phosphorylation and from now on referred to as cTnT-T206-P), were reconstituted in the cTn and incorporated in fiber bundles. Fig. 8 and Table II show the mechan and energetic profile of these proteins. The phosphorylation of both Thr\(^{206}\) and cTnT-wt induced a significant reduction in maximal tension of 22–24% and maximal ATPase of 28–36%, as well as a significant Ca\(^{2+}\) desensitization and change in cooperativity. It is interesting that both cTnT-T3SE and cTnT-wt-P gave a more pronounced Ca\(^{2+}\) desensitization than the cTnT-T206E and the corresponding cTnT-T206-P, implying that the effect of Thr\(^{206}\) phosphorylation may be enhanced by the phosphorylation of the other sites (1). Here we show that phosphorylation and Glu substitution at Thr\(^{206}\) differentially affect isometric tension and actomyosin Mg-ATPase rate. Fiber bundles containing cTnT-wt-P (but not T3SE) exhibited a significant decrease of tension cost (see Table II) as compared with cTnT-wt, implicating cTnT phosphorylation in regulation of cross-bridge detachment rate.

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\(^a\) M. P. Sumande and R. J. Solaro, manuscript in preparation.
DISCUSSION

Data presented here are the first to report the specific effect of phosphorylation and charge change at the PKC sites on cTnT in the cardiac myofilament lattice generating tension and hydrolyzing ATP. Moreover, our systematic analysis of the functional role of the multiple PKC phosphorylation sites on cTnT identified a localized region that controls maximum tension, ATPase activity and Ca\textsuperscript{2+} desensitization. An important and novel finding of our study was that Thr 206 is a functionally critical cTnT PKC phosphorylation residue. PKC phosphorylation and Glu incorporation at this site, as a mimic of phosphorylation, resulted in a significant reduction of isometric tension and actomyosin Mg-ATPase activity, Ca\textsuperscript{2+} desensitization, change in cooperativity. Glu modification of the other three phosphorylation sites Thr\textsuperscript{197}, Ser\textsuperscript{201}, and Thr\textsuperscript{287} had no significant effect on any of the parameters studied. Interestingly the incorporation of cTnT-wt-P in papillary muscle fiber bundles resulted in a decrease of tension cost that was not observed with T3SE.

The only other investigations of the specific role of cTnT phosphorylation were reported by Noland and Kuo (38, 39), who measured Ca\textsuperscript{2+}-dependent Mg-ATPase activity of a fully reconstituted thin filament preparation reacting with myosin or myosin heads. These studies reported that PKC-dependent phosphorylation of cTnT induced about a 50% depression in maximum actomyosin Ca\textsuperscript{2+}-dependent Mg-ATPase activity. They showed that specific PKC-dependent phosphorylation of cTnI was also able to inhibit the Mg-ATPase activity, but to a lesser extent than cTnT phosphorylation. Moreover, whereas increasing the ratio of myosin to actin was able to greatly overcome the inhibition by the phosphorylated cTnI, the inhibition of Ca\textsuperscript{2+}-dependent Mg-ATPase activity by phosphorylated cTnT remained about the same regardless of the myosin to actin ratio. These data not only indicated a difference between cTnI and cTnT in the mechanism by which phosphorylation affects myofilament activation, but also that the state of cTnT has more significant control over the activation state of the thin filament. Indirect evidence from our previous work (18) also indicated that specific phosphorylation of cTnT may modulate myofilament activity. We showed that pan-activation of endog-
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enous PKC in mouse heart muscle preparations resulted in a phosphorylation of cTnT and cTnI that induced a 30% reduction of maximum tension generated by the skinned fiber bundles. This effect of PKC activation was no longer evident, when cTnT was partially replaced by transgenesis with a fast skeletal isoform that is not phosphorylated by PKC.

The depression of myofilament function by the PKC-dependent phosphorylation of cTnT may be of significance in the decompensation seen at end-stage heart failure and associated with a history of hypertrophic signaling. Animal and human studies strongly support the idea that PKC activation is a key signal in the hypertrophic process and that PKC-dependent phosphorylation of myofilament proteins may be a critical factor in hypertrophy/failure (40, 41). However there are no detailed studies showing precisely how PKC phosphorylation of contractile proteins is linked to alterations in contractile function.

Several lines of evidence indicate that signaling through the PKC pathway, which involves phosphorylation of sites on cTnI and cTnT, is important in regulation of cardiac myofilament function. In the case of cTnI, this role has been demonstrated in a transgenic mouse model (TG-S43A/S45A) expressing a mutant cTnI in which the PKC sites, Ser^{43} and Ser^{45}, were mutated to Ala (18, 42). Compared with wild-type controls the TG-S43A/S45A hearts, demonstrated an enhanced response to intracellular Ca^{2+} with activation of PKC, and an enhanced

FIG. 6. Mechanoenergetic characteristics of cTn-wt versus mutants containing T206A. Steady-state isometric tension (A) and actomyosin Mg-ATPase activity (B) were simultaneously measured in fiber bundles at various [Ca^{2+}]. T3A was indistinguishable from wild type at maximal Ca^{2+}, but showed a significant desensitization at submaximal Ca^{2+} concentrations. Average values ± S.E. are presented in Table II.

FIG. 7. Analysis of Glu substitutions on cTnT-Tm binding. The plot represents the binding curves of cTn-wt, T3SE and T2SE to tropomyosin, as determined from a cosedimentation assay. The binding constants (K_d) of cTnT to Tm were determined in the presence of Ca^{2+} or EGTA. The K_d values were: wt-Ca^{2+} = 0.409 μM, wt-EGTA = 0.759 μM, T2SE- Ca^{2+} = 0.434 μM, T2SE-EGTA = 0.540 μM, T3SE-Ca^{2+} = 0.560 μM, T3SE-EGTA = 0.841 μM. Each data point is an average from duplicate measurements.
induction of contracture with ischemia. These results fit with the hypothesis that cTnI in wild-type hearts was partially phosphorylated at Ser15 and Ser16, and that this phosphorylation inhibits contraction. Previous indirect support for this hypothesis came from the studies of Strang and Moss (43), who reported a significant depression in unloaded shortening velocity of cardiac myocytes skinned after pretreatment with the α-1 adrenergic agonist, phenylephrine. In support of this hypothesis, studies comparing intact and detergent extracted papillary muscle from controls and TG hearts demonstrated a diminished response to activation of the PKC pathway by phenylephrine in papillary muscles from the TG-S43/S45-A. Maximum tension development by skinned fiber bundles from TG-S43/S45A hearts was depressed by activation of the PKC pathway prior to skinnig, but to a significantly lower extent than the controls. Interestingly, determination of levels of protein phosphorylation demonstrated that phosphorylation of cTnI in the TG-S43/S45-A heart preparations was increased relative to controls following activation of the PKC pathway. These studies thus indicated the potential for thin filament PKC-dependent protein phosphorylation to regulate contractility, and indirectly demonstrate the potential for an in vitro effect of cTnT phosphorylation by PKC. Further evidence for this hypothesis must await the generation of heart muscle preparations either by transgenesis or by viral transfer of cDNA that contain variations in the PKC sites of cTnT.

There is evidence that Thr206 phosphorylation or Glu substitution may have short and long range effects on regulation of the thin filament by cTnT. cTnT can be separated into 2 domains: T1 (residues 1–183, adult mouse sequence) and T2 (residues 184–229). T1 is considered as essential in cTnT binding to Tm, whereas T2 is the region of interaction with cTnI-cTnC. Recent studies reported that the T1 fragment, bovine cardiac residues 1–153 (44) and rabbit fast skeletal residues 1–158 (45), inhibits myosin binding to actin, potentially by stabilizing the Tm binding to actin. Maytum et al. (45) showed that T1 causes a much greater inhibitory effect on actomyosin Mg-ATPase activity than the whole cTnT. This suggests that the T2 region of cTnT might have an important role in the thin filament activation by modulating the inhibitory effect of the T1. This fits well with the evidence presented by Potter (46) that cTnT is not just a scaffold molecule anchoring cTnI and cTnC to Tm, but is directly involved in the Ca2+ regulation of actomyosin S1-ATPase activity. Therefore alterations of the T2 half of cTnT, either by phosphorylation or by charge mutations, could prevent the release of inhibitory effect of T1 resulting in the decrease of actomyosin Mg-ATPase activity, isometric tension generation and desensitization to calcium as reported in our study. Malnic et al. (47) demonstrated that a cTnT fragment (1–191) encompassing the T1 plus a small fragment of the T2 region can actually activate actomyosin Mg-ATPase activity rather than inhibit it. Furthermore Oliveira et al. (48) showed that a region of chicken fsTnI (158–191), corresponding to mouse cardiac cTnT region (190–229), has actin binding properties and stimulates ATPase activity. Taken as a whole these results indicate that the cTnT region (190–229) is important in controlling the thin filament activation. This could explain why an alteration of this region by phosphorylation could have such an inhibitory effect on tension development and actomyosin ATPase activity.

It is noteworthy that charge alterations in this region of cTnT (190–229) by the deletion of the charged amino acid Lys210 (ΔLys210) have been shown to cause DCM, characterized by cardiac dilation and reduced systolic function leading to heart failure with high mortality (7). In vitro motility studies showed that ΔLys210 resulted in a decrease in the maximum ATPase activity, sliding filament velocity and an alteration in the Ca2+ activation of the thin filament (49). When the TnT containing the ΔLys210 was exchanged into fiber bundles it caused a significant Ca2+ desensitization of force generation (50). Lys210 is part of the region 190–229 and is also part of the α-helix structure (as Lys213 in mouse sequence) that has Thr206 as its starting (N-cap) residue (51). These results also show the importance of this cTnT region in thin filament regulation and homeostasis.

The decrease in tension cost observed with fiber bundles containing cTnT-wt-P (but not with T3SE) supports the hypothesis that alterations in the thin filament proteins affect the reaction of cross-bridges with the thin filament by an allosteric mechanism (8). This result implicates that cross-bridge detachment rate (which is a function of ADP release and ATP binding to the cross-bridge) could be regulated by cTnT phosphorylation.

Computational analysis of cTnT fragment (184–227) containing the PKC phosphorylation cluster Thr197, Ser201, and Thr206 was performed using the facility at EMBL-Heidelberg (www.embl-heidelberg.de/Services/serrano/Agadir). The prediction algorithm AGADIR (52) is based on the helix/coil transi-
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Fig. 9. Computational analysis of cTnT (184–227) peptide helical content. The drawing depicts the cTnT fragment residues 184–227. The PKC sites are underlined and in bold face. Under each fragment the straight line represents random coil structure, and the tube represents α-helical content. Glu or Ala substitutions at Thr\textsuperscript{206} site result in an extension of the α-helix content.

filament structure and function (55–58), we consider cTnT as a lever in transmitting the Ca\textsuperscript{2+}-induced conformational changes in cTnC-cTnT structures to Tm. We hypothesize that the fullness of the lever is located in the cTnT region (190–229). Simple lever mechanics suggest that the closer the fulcrum is to the load the less effort is needed, and the farther the fulcrum is to the load the greater the effort needed to move it. We propose that PKC phosphorylation of cTnT might induce its effect on the mechanics and energetics of the myofilament through repositioning the "fulcrum" through α-helix (206–225) stabilization and extension.

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