There is evidence for PKC-dependent multisite phosphorylation of cardiac troponin I (cTnI) at Ser-23 and Ser-24 (also PKA sites) in the cardiac-specific N-terminal extension and at Thr-144, a unique residue in the inhibitory region. The functional effect of these phosphorylations in combination is of interest in view of data indicating intramolecular interaction between the N-terminal extension and the inhibitory region of cTnI. To determine the role of PKC-dependent phosphorylation of cTnI on sarcomeric function, we measured contractile regulation at multiple levels of complexity. Ca²⁺ binding to thin filaments reconstituted with either cTnI(wild-type) or pseudo-phosphorylated cTnI(S23D/S24D), cTnI(T144E), and cTnI(S23D/S24D/T144E) was determined. Compared with controls regulated by cTnI(wild-type), thin filaments with cTnI(S23D/S24D) and cTnI(S23D/S24D/T144E) exhibited decreased Ca²⁺ sensitivity. In contrast, there was no significant difference between Ca²⁺ binding to thin filaments with cTnI(wild-type) and with cTnI(T144E). Studies of the pCa-force relations in skinned papillary fibers regulated by these forms of cTnI yielded similar results. However, in both the Ca²⁺ binding measurements and the skinned fiber tension measurements, the presence of cTnI(S23D/S24D/T144E) induced a much lower Hill coefficient than either wild type, S23D/S24D, or T144E. These data highlight the importance of thin filament-based cooperative mechanisms in cardiac regulation, with implications for mechanisms of control of function in normal and pathological hearts.

In experiments reported here, we have investigated functional effects of multisite phosphorylation in cardiac troponin I (cTnI). cTnI is an inhibitory subunit of the cardiac troponin (cTn) complex, which consists also of troponin C (TnC), a Ca²⁺ binding component and troponin T (TnT), a tropomyosin (Tm) binding component. cTnI functions as a key protein in the cardiac muscle contraction-relaxation cycle by relieving or restoring its inhibitory influence that is regulated by Ca²⁺ association to or dissociation from the regulatory Ca²⁺ binding site of TnC (1–4). It is now well recognized that cTnI has multiple sites, which are substrates for a variety of kinases, especially PKA and PKD (Ser-23 and Ser-24), and PKC (Ser-43, Ser-45, and Thr-144). These phosphorylations have been demonstrated to occur in various signaling pathways that control cardiac dynamics and growth (for review, see Ref. 5). There is reasonable agreement that phosphorylation of Ser-23/Ser-24 located in the unique N terminus of cTnI enhances the “off” rate for Ca²⁺ dissociation from cTnC and is important in the abbreviated cycle time associated with β-adrenergic stimulation of the heart. However, the functional significance of the PKC sites remains unclear and controversial.

Previous work has focused on PKC-dependent phosphorylation of cTnI at Ser-43/Ser-45, which decreases maximum Ca²⁺-activated force, ATPase activity, and sliding velocity, and desensitizes myofilaments to [Ca²⁺] by stabilizing the off-state of the thin filaments (6, 7). More recent work, however, has shown PKC primarily cross-phosphorylates the typical PKA sites Ser-23 and Ser-24 (8, 9). We previously found PKCβ phosphorylates Thr-144, as well as PKA sites (Ser-23 and Ser-24), but not other PKC sites, Ser-43 and Ser-45 of cTnI in the Tn complex and in the thin filaments (8). Wang et al. (9) also demonstrated that PKCβ preferably phosphorylates Ser-23, Ser-24, and Thr-144 of cTnI in skinned cardiomyocytes. The difference between previous studies (e.g. 10, 11) and recent results (8, 9) may be attributed to the different PKC preparations or conditions. For example, we used recombinant PKCβ (8), whereas previous studies used pan-PKC (a mixture of PKC isoforms) purified from brain tissue. In addition, we have demonstrated that PKCδ phosphorylates cTnI at Ser-23 and Ser-24 when activated by lipid cofactors, whereas Ser-23, Ser-24, and Thr-144 are phosphorylated when PKCδ is phosphorylated at Tyr-311/Tyr-505 (12). The effects of PKC-mediated cTnI phosphorylation at different sites with regard to integrated heart function remain poorly understood. It is particularly surprising that the functional consequences of PKC-dependent phosphorylation of cTnI at Thr-144, in the critical inhibitory region of cTnI, are not clear. Thr-144 is unique to cardiac TnI (slow and fast skeletal TnI have Pro at the corresponding position). It has been demonstrated that Thr-144 is involved in length-dependent activation of cardiac muscle fibers (13) and strong cross-bridge-dependent activation of actomyosin S1-ATPase activity (14).

The abbreviations used are: cTn, cardiac troponin; TnT, troponin T; TnC, troponin C; Tm, tropomyosin; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; SIM, selected ion monitoring; IAANS, 2-(4’-iodoacetylamidino)-naphthalene-6-sulfonic acid; NEM, N-ethylmaleimide; MOPS, 4-morpholinepropanesulfonic acid.
In addition to functional consequences of Thr-144 phosphorylation alone, recent studies suggest an intramolecular interaction occurs between the N-terminal extension of cTnI, which contains Ser-23 and Ser-24, and the switch region and/or the inhibitory region, which contains Thr-144 (15–18). Therefore, phosphorylation of cTnI at site Thr-144 might influence this intramolecular interaction in cTnI. Hence, the modification of the functional consequences of Ser-23/Ser-24 phosphorylation.

In this study, we investigated the functional effects of Thr-144 phosphorylation and its impact on the functional consequences of Ser-23/Ser-24 phosphorylation at the level of the myofilaments. We introduced aspartic acids at Ser-23, Ser-24, and glutamic acid at Thr-144 of cTnI to mimic the fully phosphorylated state of cTnI (e.g. 7, 19–23). Thin filaments were reconstituted with these pseudo-phosphorylated cTnI species. We determined the functional properties of the reconstituted thin filaments by measuring Ca$^{2+}$-binding affinity and actomyosin S1-ATPase activity. Moreover, we incorporated recombinant cTn complexes into skinned cardiac muscle fibers to investigate how Thr-144 pseudo-phosphorylation affects the pCa-force relations in the myofilament lattice. Our study provides new insights into functional regulation of the myocardium, as well as, potential mechanisms in effect during the pathogenesis of cardiomyopathy and heart failure.

**MATERIALS AND METHODS**

**Protein Purification and Tn Complex Reconstitution**—Actin was isolated from rabbit fast skeletal muscle acetone powder and tropomyosin was prepared from bovine cardiac ether powder as described previously (24). Myosin-S1 was made by chymotryptic digestion of rabbit psoas muscle myosin. Recombinant mouse cTnI (wild-type and mutant) in pET3d, mouse cTnT containing an N-terminal myc tag in pSBET, and human cTnC in pET3d were expressed and purified as described previously (20, 25). It was shown that myc tag at the N terminus of cTnT has no effect on myofilament activity (20). Tn complex was reconstituted with equimolar amounts of purified Tn components, dialyzed sequentially to remove urea and decrease salt, and purified using Resource Q (1 ml; Amersham Biosciences) (25). The subunit composition was confirmed by 12% SDS-PAGE as described previously (26).

**PKC-dependent cTnI Phosphorylation Assay**—PKCβ phosphorylation of the cTn complex was carried out as described previously (8). Following PKCβ treatment, wild-type cTn complex was digested with lysyl endopeptidase. The digests were separated using a ThermoFinnigan Surveyor HPLC systems with a Vydac C18 1.0 mm ID × 250 mm length column. The peptide peaks were measured using a SIM (selected ion monitoring) mode in cooperation with Thermo Electron (Finnigan) LCQ Deca.

**Ca$^{2+}$ Binding Measurements of Reconstituted Thin Filaments**—Ca$^{2+}$ binding to the reconstituted thin filaments was determined by changes in fluorescence emission intensity of IAANS attached to Cys-53 in a single Cys mutant cTnC(C35S/T53C/C84S) (27) by using a model 2000–4 spectrophotometer equipped with two 814 PMT photon-counting detectors (Photon Technology International) and a cell holder containing a thermostat and magnetic stirrer. Previously we showed that skinned trabeculae reconstituted with cTnC(C35S/T53C/C84S) with IAANS developed force with almost the same Ca$^{2+}$-sensitivity and Hill coefficient values as those with cTnC (wild-type). Furthermore, maximum tensions were developed to a similar extent as those with cTnC (wild-type) (27). IAANS-labeled cTnC, myc-tagged cTnT and one of the mutant cTnIs were combined in the presence of urea, and the IAANS-labeled Tn complex was purified as described above. The solution conditions for Ca$^{2+}$-binding measurements were 100 mM NaCl, 5 mM MgCl$_2$, 20 mM MOPS, 2 mM EGTA at pH 7.0 and 25 °C. The free Ca$^{2+}$-concentrations were calculated with WEBMAX program (28). The ratio of actin/tropomyosin/troponin was 7:1:0.8. PKA treatment of the thin filaments was employed in the presence of 1 mM ATP, 10 μM of E-64, and 200 μM of AEBSF for 30 min at 15 °C with 1 unit of PKA/1 μg of cTnI. Almost complete phosphorylation of cTnI was confirmed under these conditions.

**Actomyosin S1-ATPase Activity Assay**—ATPase activity measurements were carried out in 35 mM NaCl, 5 mM MgCl$_2$, 20 mM MOPS, and either 2 mM EGTA or 0.1 mM CaCl$_2$, pH 7.0 at 25 °C. The concentrations of S1, actin, tropomyosin, troponin were 0.2, 5.0, 2.0 μM, respectively, with troponin complexes, consisting of wild-type cTnC, myc-tagged cTnT, and one of the recombinant cTnIs, titrated from 0 to 3.0 μM. Reactions were initiated by addition of 1.0 mM ATP. Every 2 min (up to 12 min), a 10-μl aliquot was removed into 90 μl of 0.2 mM perchloric acid to terminate the reaction. ATPase activity was determined from the time course (typically 6 time points/one time course) of phosphate liberation using the malachite green method (14, 29). In the NEM-S1 ATPase activity measurements, the initial concentrations of S1, actin, tropomyosin, troponin were 0.2, 5.0, 1.0, 1.0 μM with concentrations increased by an amount equal to the NEM-S1 to maintain the same level of free actin/tropomyosin/troponin (6, 14). The ATPase rate of S1 alone was subtracted from all measurements.

**Preparation of Skinned Fibers and Mechanical Measurements**—Animals were treated and housed in accordance with the Animal Care and Use Committee guidelines at the University of Illinois at Chicago Biologic Resources Laboratory. Mouse cardiac skinned muscle fibers were prepared from the left ventricular papillary muscles of male FVB mice (3–5-month old). Endogenous Tn was exchanged for recombinant Tn, comprising wild-type cTnC, myc-tagged cTnT, and one of the recombinant cTnIs, by overnight incubation at 4 °C in 18 μM recombinant cTn containing 200 mM KCl, 5 mM MgCl$_2$, 5 mM EGTA, 1 mM dithiothreitol, 20 mM MOPS at pH 6.5 as previously described (20). The skinned fiber bundles were attached between a force transducer and torque motor and sarcomere length of each muscle was set to 2.2 μm by laser diffraction. Myofilament Ca$^{2+}$-sensitivity of tension was determined by measuring total steady-state tension development over a range of Ca$^{2+}$-concentrations as described previously (20). All the mechanics measurements were performed at 15 °C.

**Statistical Analysis**—To determine the pCa value at half-maximal fluorescence change (pCa$_{50}$) in reconstituted thin filament or force generation in skinned fiber and the Hill coefficient ($n_H$), the fluorescence and force were normalized to maximum. The relationship of fluorescence change or force...
Functional Consequences of Thr-144 Phosphorylation in cTnI

RESULTS

PKC Phosphorylation of cTnI in the Thin Filament—Although Ser-43 and Ser-45 in cTnI have been considered to be the major PKC-dependent phosphorylation sites, recent reports indicate PKC isoforms beta, epsilon, and delta phosphorylate cTnI at sites Ser-23, Ser-24, and Thr-144 (8, 9). To confirm these results and ascertain the sequence of phosphorylation events, we determined the time-dependent changes in PKC-phosphorylation of cTnI by HPLC. Fig. 1A shows the SIM chromatogram of lysyl endopeptidase digests of PKC-treated cTn. Chromatogram of lysyl endopeptidase digest after 0 min (a, b) or 30 min (c, d) PKC-treated cTn are shown. B, relative abundance of phosphopeptides as a function of time after PKC-treatment. Ser-23 and Ser-24 in cTnI peptide 1–36 and Thr-144 in cTnI peptide 141–164 were previously established to be the phosphorylated residues in this assay (8).

Ca\(^{2+}\) Binding to the Thin Filaments—Results of measurements of Ca\(^{2+}\) binding to reconstituted thin filament preparations are shown in Fig. 2 and summarized in Table 1. Compared with thin filaments with cTnI(wild-type), those containing cTnI(S23D/S24D) and cTnI(S23D/S24D/T144E) exhibited decreased Ca\(^{2+}\) sensitivity. In contrast, cTnI(T144E) did not affect the Ca\(^{2+}\) binding properties of the reconstituted thin filaments. Importantly, thin filaments containing cTnI(S23D/S24D/T144E) have a significantly lower steepness of the curve (n_{st}) compared with cTnI(wild-type), cTnI(S23D/S24D), and 14E (Table 1). Thus our data indicate that Thr-144 pseudo-phosphorylation interferes with the cooperative behavior of the thin filaments by a mechanism dependent upon Ser-23/Ser-24 pseudo-phosphorylation.

To test whether the observed effects are due to pseudo-phosphorylation of Ser-23/Ser-24/Thr-144, rather than marginal mutational effects, we measured Ca\(^{2+}\) binding properties of the thin filaments with cTnI(T144A), cTnI(S23A/S24A), or cTnI(T144E) mutant cTnI were reacted with PKA and the rate and extent of phosphorylation determined by SDS-PAGE separation and phosphostaining. The results demonstrated no significant change in terms of time-course of phosphorylation or maximum phosphorylation level (data not shown).

Generation with pCa was fit to a modified Hill equation (25, 30). Statistical significance was determined by ANOVA followed by the Student Newman-Keuls test. Data are presented mean ± S.E. with significance set at p < 0.05.
Summary for the effects of pseudo-phosphorylated or pseudo-non-phosphorylated cTnI and PKA-treated cTnI on Ca\(^{2+}\) binding properties in the reconstituted thin filaments

| cTnI       | \(\text{pC}_{50}\) | \(\Delta \text{pC}_{50}\) | \(n_{H}\) | n  |
|------------|------------------|------------------|---------|----|
| Wild type  | 5.71 ± 0.03      | N/A              | 2.69 ± 0.09 | 8  |
| T144E      | 5.67 ± 0.03      | -0.04 ± 0.04     | 2.54 ± 0.09 | 7  |
| S23D/S24D  | 5.55 ± 0.03\(^b\) | -0.16 ± 0.04     | 2.67 ± 0.13 | 5  |
| S23D/S24D/T144E | 5.51 ± 0.03\(^b\) | -0.20 ± 0.04     | 1.79 ± 0.13\(^*\) | 5  |
| T144A      | 5.72 ± 0.03      | 0.01 ± 0.04      | 2.66 ± 0.16 | 5  |
| S23A/S24A  | 5.64 ± 0.04      | -0.07 ± 0.05     | 2.70 ± 0.21 | 5  |
| S23A/S24A/T144A | 5.62 ± 0.03      | -0.09 ± 0.04     | 2.66 ± 0.13 | 5  |
| Wild type with PKA | 5.42 ± 0.03\(^b\) | -0.29 ± 0.04     | 2.51 ± 0.10 | 6  |
| T144E with PKA | 5.37 ± 0.02\(^b\) | -0.34 ± 0.04     | 2.00 ± 0.07\(^a\) | 6  |

\(^a\) \(\Delta \text{pC}_{50}\) change in \(\text{pC}_{50}\) from wild-type. Data are presented as mean ± S.E.

\(^b\) \(p < 0.05\) vs. wild-type and T144E.

\(^\ast\) \(p < 0.05\) vs. wild-type, T144E and wild-type with PKA (Newman-Keuls Multiple Comparison Test).

We also conducted the Ca\(^{2+}\) binding measurements with thin filaments with PKA-treated cTnI(wild-type) or PKA-treated cTnI(T144E) to verify pseudo-phosphorylation mutations mimic phosphorylation states. As expected, thin filaments with PKA-treated cTnI(T144E) showed a reduced cooperativity compared with those with cTnI(wild-type), cTnI(T144E), or cTnI(S23D/S24D) (Table 1). Thus phosphorylation mutation (Ser to Asp) at PKA site mimicked the actual phosphorylated state of the PKA sites well, consistent with previous studies (e.g. Refs. 22, 23).

**Actomyosin S1-ATPase Activity**—Relationships between the concentration of Tn complex and actomyosin S1-ATPase activity are shown in Fig. 3. Each of the pseudo-phosphorylated cTnls inhibited ATPase activity to a similar extent as cTnI(wild-type) in the presence of Ca\(^{2+}\). In the presence of Ca\(^{2+}\), however, S23D/S24D and S23D/S24D/T144E displayed strong suppressing effects, whereas T144E exhibited a moderate but
statistically significant inhibitory effect on the maximum acto-
imyosin S1-ATPase activity. A decrease in Ca\(^2+\)/H11001
-activated acto-
imyosin S1-ATPase activity with the S23D/S24D mutation is
consistent with a previous report with PKA-treated cTnI (38).
Effects of T144E mutations have not previously been assessed.
To assess the effects of strong cross-bridge-dependent activa-
tion, we measured actomyosin S1-ATPase activity in the pres-
ence of NEM-treated S1 (NEM-S1). NEM-S1 mimics the strong
cross-bridge, binds strongly to actin even in the presence of
ATP, and activates the thin filaments to fully active state (6, 14,
39). Fig. 4 shows the relationships between the concentration of
NEM-S1 and actomyosin S1-ATPase activity in the absence
(Fig. 4A) and the presence (Fig. 4B) of Ca\(^2+\). As the concentra-
tion of NEM-S1 increased, ATPase activity increased. Although
there was a tendency for the pseudo-phosphorylation muta-
tions to suppress ATPase activity in the presence of low [NEM-
S1], this difference was not statistically significant. Thus we
concluded these mutations did not affect strong cross-bridge-
dependent activation of myofilament.

**Effect of Pseudo-phosphorylated cTnI on Force Development
of Skinned Cardiac Fibers**—To determine whether the alter-
ations in cooperative thin filament Ca\(^2+\) binding associate with
the presence of cTnI(S23D/S24DT144E) translated to tension
generation in the myofilament lattice, we measured pCa force
relation in skinned fiber bundles. As compared with fibers
exchanged with cTnI(wild-type), those exchanged with
cTnI(S23D/S24D) or cTnI(S23D/S24D/T144E) exhibited
decreased Ca\(^2+\) sensitivity of tension (Fig. 5 and Table 2).
In contrast, there was no alteration of Ca\(^2+\) sensitivity in fibers
with cTnI(T144E). Interestingly, fibers with cTnI(S23D/S24D/
T144E) showed a much lower Hill coefficient (n\(_H\)) compared
with those containing either cTnI(wild-type), cTnI(S23D/
S24D), or cTnI(T144E) (Fig. 5A, Table 2), consistent with our
findings in the Ca\(^2+\) binding measurements (Fig. 2). We also
measured the rate constant of force redevelopment (k\(_{tr}\)). The
obtained maximal Ca\(^2+\)-activated k\(_{tr}\) values were 12.74 ± 0.65,
11.45 ± 0.52, 11.01 ± 0.74, and 11.93 ± 0.95 s\(^{-1}\) for the fibers
with cTnI(wild-type), cTnI(T144E), cTnI(S23D/S24D), and

![FIGURE 4. ATPase activity in the absence and presence of Ca\(^2+\) with
increasing concentrations of NEM-S1. The conditions were as follows: 35
mM NaCl, 5 mM MgCl\(_2\), 20 mM MOPS, and either 2 mM EGTA or 0.1 mM CaCl\(_2\), pH
7.0 at 25 °C. The concentrations of myosin S1, actin, tropomyosin, and tropo-
in were 0.2, 5, 1, and 1 μM, respectively. Histograms display effect of cTnI on
ATPase activity in the presence of EGTA (A) and in the presence of Ca\(^2+\) (B).
Data are presented as mean ± S.E., n = 4–6.](chart1)

![FIGURE 5. Effects of pseudo-phosphorylated cTnIs on force generation
parameters of skinned fibers. A, force-pCa relationships in skinned cardiac
muscle fibers into which wild-type cTnI (circle), or mutant of pseudo-phos-
phorylated cTnI(T144E) (square), cTnI(S23D/S24D) (triangle), or cTnI(S23D/
S24D/T144E) (diamond) was incorporated. B, effects of the pseudo-phosphor-
ylated cTnIs on the maximal force. The minimum and maximal force levels
were not statistically significantly different between wild type and mutants.
Data are presented as mean ± S.E., n = 4–5.](chart2)

**TABLE 2** Summary for the effects of pseudo-phosphorylated cTnI on Ca\(^2+\)
sensitivity (pCa\(_{50}\)) and Hill coefficient (n\(_H\)) in skinned cardiac muscle
fibers

| cTnI         | pCa\(_{50}\) ± S.E. | ΔpCa\(_{50}\) ± S.E. | n\(_H\) ± S.E. | n  |
|--------------|---------------------|---------------------|----------------|---|
| Wild type    | 5.69 ± 0.04         | 0.34 ± 0.25         | 4              | 4  |
| T144E        | 5.73 ± 0.07         | 0.04 ± 0.04         | 3.70 ± 0.14    | 4  |
| S23D/S24D    | 5.54 ± 0.03         | -0.15 ± 0.06        | 3.46 ± 0.52    | 5  |
| S23D/S24D/T144E | 5.41 ± 0.03       | -0.28 ± 0.06        | 2.36 ± 0.04    | 5  |

ΔpCa\(_{50}\) change in pCa\(_{50}\) from wild-type. Data are presented as mean ± S.E.

\( ^p < 0.05 \) vs. wild-type and T144E.

\( ^c \) p < 0.05 vs. wild-type, T144E and S23D/S24D.
cTnI(S23D/S24D/T144E), respectively. No differences in the maximal rate constant of force redevelopment were determined \((p > 0.05)\). These results suggest the phosphorylation of cTnI at Thr-144 may affect cardiac contractile function by depressing cooperative behavior of myofibrils with Ser-23/Ser-24 phosphorylation.

**DISCUSSION**

Our results provide novel insights into the role of processes at the level of the thin filaments in control of cardiac function. Our data demonstrate for the first time that pseudo-phosphorylation at Thr-144 of cTnI depressed the cooperative behavior of cardiac thin filaments exclusively in the presence of the S23D/S24D mutation of cTnI. Importantly this mechanism translated to the level of intact myofilaments as demonstrated by altered cooperativity of the pCa-isometric tension relationship. Thus our data demonstrate that interactions between the N-terminal extension, of cTnI, which contains Ser-23 and Ser-24, and the inhibitory region, which contains Thr-144, occur and are capable of modulating cardiac muscle contractility.

The relative role of thin and thick filaments related mechanisms in the steep Ca\(^{2+}\) dependence of force in cardiac preparations is under debate (41–44). The activation of cardiac myofilaments by Ca\(^{2+}\) is a highly cooperative process involving protein-protein interactions within thin filament proteins and between thin and thick filaments. Whereas cardiac TnC has only single functional regulatory Ca\(^{2+}\)-binding site in the N-lobe, cardiac thin filaments bind Ca\(^{2+}\) cooperatively (25, 27, 45, 46). This observed cooperativity in the regulatory Ca\(^{2+}\)-binding sites along thin filaments may involve transmission of signals through head-to-end interaction of Tm molecules and/or actin monomers. Another explanation for the steepness of Ca\(^{2+}\)-dependent myofilament activity is through the contribution of strong cross-bridges formation, which enhances Ca\(^{2+}\) binding affinity of thin filaments. Whether phosphorylation of cTnI modulates the relative contribution of these two activation mechanisms remains poorly understood. Data reported here support the idea that thin filaments related cooperative mechanism, independent of force generating cross-bridge reactions, is a significant determinant of the steep relation between Ca\(^{2+}\) and force generation in skinned cardiac muscle fibers. There were striking similar responses to pseudo-phosphorylation occurred at the level of Ca\(^{2+}\) binding to the thin filaments (Fig. 2) and Ca\(^{2+}\)-dependent steady-state tension (Fig. 5), even though these were two separate experiments and were carried out under two different set-ups. Thus it is evident that under the conditions employed modulation of Ca\(^{2+}\) binding properties of the thin filament is translated into the level of the sarcomere and is sufficient to modulate skinned cardiac tension development. This observation is consistent with a recent report by Sun et al. (44), who probed structural transitions in the C-helix and E-helix of cTnC in the skinned trabeculae during active force production. The structural transition, as reported using fluorescence polarization, demonstrated a steep dependence on Ca\(^{2+}\) concentration similar to that observed in force generations. Furthermore, the Ca\(^{2+}\) dependence of the structural transition was the same in the absence of force generating strong cross-bridges. Thus Sun et al. (44) concluded that the physiological cooperative activation of cardiac contractility is intrinsic to the thin filaments. Our data agree with and extend this conclusion to show factors directly affecting cooperativity within the thin filament, such as post-translational modification of cTnI, are translated to the level of the intact myofibrillar lattice, and possible modulation of cardiac contractility.

Results reported above indicate apparent discrepancy in the effects of the cTnI variants on activities in actomyosin ATPase measurements and skinned fiber experiments. Whereas pseudo-phosphorylation of Thr-144 alone showed little effect in the experiments presented in Figs. 2 and 5, it showed a decrease in actomyosin S1-ATPase activity in the presence of Ca\(^{2+}\) (Fig. 3). We also found pseudo-phosphorylation of Ser-23/Ser-24 decreased the ATPase activity in the presence of Ca\(^{2+}\) (Fig. 3), similar to a previous report (31). ATPase activity in the absence of Ca\(^{2+}\) was not changed in either T144E or S23D/S24D. There are several mechanisms by which ATPase rate can be modified, such as \(a\) altered kinetics of the elementary steps of the active state, \(b\) the shift of the thin filament states, and \(c\) a change in the affinity of the thin filament for myosin. Because NEM-S1-dependent activation of ATPase rates with these mutations did not show significant difference from those with cTnI(wild-type) (Fig. 4), it is reasonable to exclude \(a\). If we assume the changes in ATPase activity are due to the shift of the equilibrium of the thin filament states \(b\), our observations suggest that the pseudo-phosphorylation mutations destabilize the active state of thin filaments and shift the equilibrium of the thin filament states to the intermediate and/or inactive states. The resulting equilibrium should slower the \(P_i\) release step, which would be predicted to decrease power generating capacity (47), but not directly affect isometric tension production. Previous studies indicate thin filaments in the off-state bind Ca\(^{2+}\) relatively weakly, whereas the intermediate state bind Ca\(^{2+}\) more tightly than the active state (6, 14). Thus, shifts in the distribution of these states may provide an explanation for the changes observed in Ca\(^{2+}\) binding measurements (Fig. 2). Current experiments cannot rule out the possibility of a change in the affinity of the thin filament for myosin (c). However it was reported that PKA phosphorylation of cTnI does not affect S1 binding to the thin filaments (48), it is likely that the physiologically important myosin-actin association step(s) involves M-ADP-P\(_i\), rather than S1 (myosin) without nucleotide (49–51). Also it was implicated that, from the relationship between thin filament length and the velocity, the apparent binding affinity of myosin and the thin filament was reduced after PKA treatment in the *in vitro* motility assay (52). Thus it seems to be plausible that both S23D/S24D and T144E mutations modify the affinity between myosin and the thin filaments.

It is apparent that further experiments are needed to fully elucidate the molecular mechanisms underlying a discrepancy in the effects of the cTnI variants on myofilament activities. In any cases, in the reconstituted solution system, the functional properties modified by mutant cTnIs may be exaggerated. On the other hand, under the isometric conditions in the myofilament lattice, the local concentration of myosin for actin is high enough and myosin heads may be ready to interact with actin.
Thus a small increase of the population of the intermediate state of the thin filaments and/or slight decrease in affinity between the thin filament and myosin may not be detected. In a working heart, the situations are more dynamic and complicated, such as loading conditions and sarcomere–sarcomere interactions. Thus a small change in the properties of the thin filament may be amplified and have significant effects on function.

Our data also support the idea that intramolecular interactions in cTnI are functionally significant. Howarth et al. (17) determined the solution structure of the bis-phosphorylated form of the N-terminal extension of cTnI. Based on their NMR structure, the structural model derived from the previously published neutron scattering data, and the crystal structure of the core domain of cTnI complex, they proposed the N-terminal extension of cTnI interacts with the switch and the inhibitory regions of cTnI. Consistent with their model, we reported that a benzophenone moiety attached to either Cys-5 or Cys-19 of cTnI cross-linked to the switch region of cTnI in the ternary cTnI complex (15). It is also significant that interactions between the N-terminal extension and the switch region of cTnI were shown to be phosphorylation-dependent (18). According to Howarth et al. (17), the N-terminal acidic region of the extension interacts with the basic residues in the inhibitory region of cTnI, but otherwise there seems to be no direct interaction between the inhibitory region and Ser-23/Ser-24. Thus it is likely that Thr-144 pseudo-phosphorylation allosterically modulates the functional consequence of the Ser-23/Ser-24 pseudo-phosphorylation.

Importantly, PKC and PKA regulate cardiac contractile function through phosphorylation of several myofilament proteins including myosin-binding protein C, myosin regulatory light chain, titin, TnT, and TnI. However, cTnI phosphorylation has been shown to be necessary for clinically relevant modulatory effects (37, 40, 53), emphasizing the importance of understanding its molecular regulation. In addition, thin filament dysfunction was associated with increased PKCβ expression in failing human myocardium and reduced after left ventricular assist device support (54). Thus Ser-23/Ser-24/Thr-144 may be physiologically important phosphorylation sites of cTnI for specific PKC isoforms involved in the response to physiological stimuli or pathological conditions, such as heart failure and oxidative stress (3, 12, 55). Our results provide novel mechanisms for modulation of myofilament activity through PKC-dependent phosphorylation of cTnI.

Acknowledgments—We thank Richard van Breemen for mass spectrometry analysis, Julie Mouannes for providing myosin-S1, and Chad Warren for NEM-S1.

REFERENCES

1. Tobacman, L. S. (1996) Annu. Rev. Physiol. 58, 447–481
2. Gordon, A. M., Homsher, E., and Regnier, M. (2000) Physiol. Rev. 80, 853–924
3. Metzger, J. M., and Westfall, M. V. (2004) Circ. Res. 94, 146–158
4. Kobayashi, T., and Solaro, R. J. (2005) Annu. Rev. Physiol 67, 39–67
5. Solaro, R. J. (2008) J. Biol. Chem. 283, 26829–26833
6. Mathur, M. C., Kobayashi, T., and Chalovich, J. M. (2008) Biophys. J. 94, 542–549
7. Burkart, E. M., Sumandea, M. P., Kobayashi, T., Nili, M., Martin, A. F., Homsher, E., and Solaro, R. J. (2003) J. Biol. Chem. 278, 11265–11272
8. Kobayashi, T., Yang, X., Walker, L. A., Van Breemen, R. B., and Solaro, R. J. (2005) J. Mol. Cell Cardiol. 38, 213–218
9. Wang, H., Grant, J. E., Doede, C. M., Sadayappan, S., Robbins, J., and Walker, J. W. (2006) J. Mol. Cell Cardiol. 41, 823–833
10. Noland, T. A., Jr., Raynor, R. L., and Kuo, J. F. (1989) J. Biol. Chem. 264, 20778–20785
11. Noland, T. A., Jr., Guo, X., Raynor, R. L., Jideama, N. M., Averhart-Fullard, V., Solaro, R. J., and Kuo, J. F. (1995) J. Biol. Chem. 270, 25445–25454
12. Sumandea, M. P., Rybin, V. O., Hinken, A. C., Wang, C., Kobayashi, T., Harleton, E., Sievert, G., Balke, C. W., Feinmark, S. J., Solaro, R. J., and Steinberg, S. F. (2008) J. Biol. Chem. 283, 22680–22689
13. Tachampa, K., Wang, H., Farman, G. P., and de Tombe, P. P. (2007) Circ. Res. 101, 1081–1083
14. Kobayashi, T., Patrick, S. E., and Kobayashi, M. (2009) J. Biol. Chem. 284, 20052–20060
15. Warren, C. M., Kobayashi, T., and Solaro, R. J. (2009) J. Biol. Chem. 284, 14258–14266
16. Sadayappan, S., Finley, N., Howarth, J. W., Osinski, H., Klevitsky, R., Lorenz, J. N., Rosevear, P. R., and Robbins, J. (2008) FASEB J. 22, 1246–1257
17. Howarth, J. W., Meller, J., Solaro, R. J., Trewella, J., and Rosevear, P. R. (2007) J. Mol. Biol. 373, 706–722
18. Baryshnikova, O. K., Li, M. X., and Sykes, B. D. (2008) J. Mol. Biol. 375, 735–751
19. Dohet, C., al-Hillawi, E., Trayer, I. P., and Ruegg, J. C. (1995) FEBs Lett. 377, 131–134
20. Sumandea, M. P., Pyle, W. G., Kobayashi, T., de Tombe, P. P., and Solaro, R. J. (2003) J. Biol. Chem. 278, 35135–35144
21. Finley, N. L., and Rosevear, P. R. (2004) J. Biol. Chem. 279, 54833–54840
22. Takimoto, E., Soergel, D. G., Janssen, P. M., Stull, L. B., Kas, D. A., and Murphy, A. M. (2004) Circ. Res. 94, 496–504
23. Yasuda, S., Coutou, P., Sadayappan, S., Robbins, J., and Metzger, J. M. (2007) Circ. Res. 101, 377–386
24. Barick, H. M., Opgenorth, T. J., von Geldern, T. W., Wu-Wong, J. R., and Solaro, R. J. (1996) J. Biol. Chem. 271, 27039–27043
25. Kobayashi, T., and Solaro, R. J. (2006) J. Biol. Chem. 281, 13471–13477
26. Takeda, S., Kobayashi, T., Taniguchi, H., Hayashi, H., and Maeda, Y. (1997) Eur. J. Biochem. 246, 611–617
27. Davis, J. P., Norman, C., Kobayashi, T., Solaro, R. J., Swartz, D. R., and Tikunova, S. B. (2007) Biophys. J. 92, 3195–3206
28. Patton, C., Thompson, S., and Epel, D. (2004) Cell Calcium 35, 427–431
29. Onishi, H., Maeda, K., Maeda, Y., Inoue, A., and Fujiwara, K. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 704–708
30. Lu, Q. W., Morimoto, S., Harada, K., Du, C. K., Takahashi-Yanaga, F., Miwa, Y., Sasaguri, T., and Ohtsuki, I. (2003) J. Mol. Cell Cardiol. 35, 1421–1427
31. Mittmann, K., Jaquet, K., and Heilmeyer, L. M., Jr. (1992) FEBS Lett. 302, 133–137
32. Keane, N. E., Quirk, P. G., Gao, Y., Patchell, V. B., Perry, S. V., and Levine, B. A. (1997) J. Mol. Biol. 268, 329–337
33. Zhang, R., Zhao, J., and Potter, J. D. (1995) J. Biol. Chem. 270, 30773–30780
34. Zubroukov, V., Ge, Y., Schwartz, J., and Walker, J. W. (2008) Mol. Cell Proteomics 7, 1838–1849
35. Pi, Y., Kemnitz, K. R., Zhang, D., Kranias, E. G., and Walker, J. W. (2002) Circ. Res. 90, 649–656
36. Pi, Y., Zhang, D., Kemnitz, K. R., Wang, D., and Walker, J. W. (2003) J. Physiol. 552, 845–857
37. Westfall, M. V., Lee, A. M., and Robinson, D. A. (2005) J. Biol. Chem. 280, 41324–41331
38. Deng, Y., Schmidtmann, A., Redlich, A., Westerdorf, B., Jaquet, K., and Thieleczek, R. (2001) Biochemistry 40, 14593–14602
39. Nagashima, H., and Asakura, S. (1982) J. Mol. Biol. 155, 409–428
40. Kentish, J. C., McCloskey, D. T., Layland, J., Palmer, S., Leiden, J. M., Martin, A. F., and Solaro, R. J. (2001) *Circ. Res.* **88**, 1059–1065
41. Moss, R. L., Razumova, M., and Fitzsimons, D. P. (2004) *Circ. Res.* **94**, 1290–1300
42. Regnier, M., Martin, H., Barsotti, R. J., Rivera, A. J., Martyn, D. A., and Clemmens, E. (2004) *Biophys. J.* **87**, 1815–1824
43. Hanft, L. M., Korte, F. S., and McDonald, K. S. (2008) *Cardiovasc. Res.* **77**, 627–636
44. Sun, Y., Lou, F., and Irving, M. (2009) *J. Physiol.* **587**, 155–163
45. Tobacman, L. S., and Sawyer, D. (1990) *J. Biol. Chem.* **265**, 931–939
46. Pan, B. S., and Solaro, R. J. (1987) *J. Biol. Chem.* **262**, 7839–7849
47. Hinken, A. C., and McDonald, K. S. (2004) *Am. J. Physiol.* **287**, C500–C507
48. Reiffert, S. U., Jaquet, K., Heilmeyer, L. M., Jr., Ritchie, M. D., and Geeves, M. A. (1996) *FEBS Lett.* **384**, 43–47
49. Dantzig, J. A., Goldman, Y. E., Millar, N. C., Lacktis, J., and Homsher, E. (1992) *J. Physiol.* **451**, 247–278
50. Steffen, W., and Sleep, J. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 12904–12909
51. Sleep, J., Irving, M., and Burton, K. (2005) *J. Physiol.* **563**, 671–687
52. Hünlich, M., Begin, K. J., Gorga, J. A., Fishbaugh, D. E., LeWinter, M. M., and VanBuren, P. (2005) *J. Mol. Cell Cardiol.* **38**, 119–125
53. Phan, T. T., Abozguia, K., Nallur Shiva, G., Mahadevan, G., Ahmed, I., Williams, L., Dwivedi, G., Patel, K., Steendijk, P., Ashrafian, H., Henning, A., and Frenneaux, M. (2009) *J. Am. Coll. Cardiol.* **54**, 402–409
54. Noguchi, T., Hünlich, M., Camp, P. C., Begin, K. J., El-Zaru, M., Patten, R., Leavitt, B. J., Ittleman, F. P., Alpert, N. R., LeWinter, M. M., and VanBuren, P. (2004) *Circulation* **110**, 982–987
55. Molkentin, J. D., and Dorn, G. W., 2nd (2001) *Annu. Rev. Physiol.* **63**, 391–426