Phosphorylation or Glutamic Acid Substitution at Protein Kinase C Sites on Cardiac Troponin I Differentially Depress Myofilament Tension and Shortening Velocity*

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There is evidence that multi-site phosphorylation of cardiac troponin I (cTnI) by protein kinase C is important in both long- and short-term regulation of cardiac function. To determine the specific functional effects of these phosphorylation sites (Ser-43, Ser-45, and Thr-144), we measured tension and sliding speed of thin filaments in reconstituted preparations in which endogenous cTnI was replaced with cTnI phosphorylated by protein kinase C-ε or mutated to cTnI-S43E/S45E/S414E, cTnI-S43E/S45E, or cTnI-T144E. We used detergent-skinned mouse cardiac fiber bundles to measure changes in Ca\(^{2+}\)-dependence of force. Compared with controls, fibers reconstituted with phosphorylated cTnI, cTnI-S43E/S45E/T144E, or cTnI-S43E/S45E were desensitized to Ca\(^{2+}\), and maximum tension was as much as 27% lower, whereas fibers reconstituted with cTnI-T144E showed no change. In the in vitro motility assay actin filaments regulated by troponin complexes containing phosphorylated cTnI or cTnI-S43E/S45E/T144E showed both a decrease in Ca\(^{2+}\) sensitivity and maximum sliding speed compared with controls, whereas filaments regulated by cTnI-S43E/S45E showed only decreased maximum sliding speed and filaments regulated by cTnI-T144E demonstrated only desensitization to Ca\(^{2+}\).

Our results demonstrate novel site specificity of effects of PKC phosphorylation on cTnI function and emphasize the complexity of modulation of the actin-myosin interaction by specific changes in the thin filament.

Specific modifications of charge in thin filament proteins are known to have significant effects on myofilament regulation and heart function. Single point mutations linked to familial hypertrophic cardiomyopathies involve charged amino acids on a number of thin filament proteins that induce alterations in myofilament regulation by Ca\(^{2+}\), as well as alterations in modulation of Ca\(^{2+}\) regulation by pH and sarcomere length (1, 2). Moreover, these charge modifications of the thin filament are well correlated with altered myocardial dynamics that may be significant in the evolution of hypertrophy and sudden death (3). An important question is whether charge changes introduced into the thin filament proteins by protein phosphorylation may also be causal in cardiac dysfunction (4). In experiments reported here we introduced charge changes into specific protein kinase C (PKC) sites of cardiac troponin I (cTnI) by either protein phosphorylation or mutation of these sites to glutamic acid. We tested the effect of these changes on Ca\(^{2+}\) regulation of steady-state tension generated by the myofilaments as well as the speed of thin filament sliding on myosin heads in the in vitro motility assay.

cTnI is a key regulatory and inhibitory protein of the thin filament, which together with the tropomyosin binding unit of troponin (Tn), cardiac troponin T (cTnT), the Ca\(^{2+}\) binding unit, cardiac troponin C (cTnC), and tropomyosin (Tm) confers Ca\(^{2+}\) sensitivity to the actin-myosin reaction. The phosphorylation of cTnI at specific sites (Ser-23 and Ser-24) that are substrates for PKC appears to be of special significance with regard to modulation of myofilament function and control of cardiac dynamics (5–8). Our hypothesis has been that covalent modification of cTnI not only plays a role in the homeostasis of cardiac function, but also in the decline of cardiac function that occurs in the transition from compensated hypertrophy to heart failure. Ample evidence indicates that phosphorylation of cTnI at Ser-23 and Ser-24 results in a reduction in myofilament Ca\(^{2+}\) sensitivity (9) and an increase in cross-bridge cycling (6, 10). These effects of protein kinase A-mediated phosphorylation of cTnI are important elements in the enhanced relaxation that is critical to the response of the heart to adrenergic stimulation (6, 11–13). The role of the PKC sites on cTnI in the regulation of myofilament response to Ca\(^{2+}\) and regulation of cross-bridge cycling is less clear. An understanding of the role of these sites is particularly important in view of evidence from studies on failing human hearts (14, 15) and on hearts from transgenic mouse models (16, 17). These studies indicate that increased activity and expression of PKC may lead to excessive and/or persistent phosphorylation of cTnI.

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1 The abbreviations used are: PKC, protein kinase C; Tn, troponin; cTn, cardiac troponin; Tn1-P, PKC phosphorylated Tn; Tm, tropomyosin; AB, low salt assay buffer; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; TPCK, N-tosyl-L-lysine chloromethyl ketone; pCa, –log(Ca\(^{2+}\)); ANOVA, analysis of variance; WT, wild-type; TG, transgenic; NTG, non-transgenic.
by PKC with an associated depression in tension-generating capability. Myofilaments from hearts of transgenic mice expressing the mutant cTnI-S43A/S45A (substitution of alanine residues, A, for serine residues, S) demonstrate a blunting of the effect of PKC-mediated phosphorylation to depress maximum tension (17). Moreover, these transgenic mouse hearts contract and relax much faster than the controls and demonstrate significant alterations in the Ca\textsuperscript{2+}-pressure relationship of isolated, perfused hearts (18).

Exactly how phosphorylation of the PKC sites on cTnI affects the crossbridge function in the myofilament lattice remains unknown. Studies with transgenic hearts are revealing but difficult to interpret. Apart from the existence of multiple sites for PKC-mediated phosphorylation, substitution of alanine for serine in the transgene is not benign. This substitution itself causes a depression in both maximum tension and maximum Ca\textsuperscript{2+}-stimulated actomyosin MgATPase activity in fully reconstructed preparations (7). In addition, modification of the PKC sites on cTnI influences the ability of PKC to phosphorylate cTnT (19). The reverse is true as well (17).

We report here new evidence on the role of the specific PKC sites of cTnI in regulation of the actin-myosin interaction. We phosphorylated cTnI directly by PKC-\(\varepsilon\) (TnI-P), one of the predominant isoforms of PKC found in the adult cardiac myocyte (20), and incorporated either the unphosphorylated or the phosphorylated cTnI into thin filaments. To determine the specific role of the phosphorylation induced change in charge, we also generated the following mutant forms of cTnI: cTnI-S43E/S45E/T144E, cTnI-S43E/S45E, and cTnI-T144E in which specific role of the phosphorylation induced change in charge, phosphorylated cTnI into thin filaments. To determine the extent of endogenous troponin removed. Next, the cTnI-TnC-treated fiber was treated with 90 min with a ThC reconstitution solution, prepared as previously described (24). The exchange of endogenous cTnI for phosphorylated cTnI was accomplished by first forming a 10 M MOPS (pH 7.0), 2 mM DTT, 0.01% NaN\textsubscript{3}, 12 mM creatine phosphate, 10 IU/ml creatine kinase, and 1 mM DTT. The fiber bundle was then placed in high relaxing solution containing 20 mM MOPS (pH 7.0), 35 mM KCl, 10 mM EGTA, 9.96 mM CaCl\textsubscript{2}, 5.39 mM ATP, 6.47 mM MgCl\textsubscript{2}, 12 mM creatine phosphate, 10 IU/ml creatine kinase, and 1 mM DTT. The fiber bundle was then placed in high relaxing solution containing 20 mM MOPS (pH 7.0), 35 mM KCl, 10 mM EGTA, 250 mM KCl, 5 mM EGTA, 5 mM MgCl\textsubscript{2}, and 0.1 mM DTT at 4°C. The next day, insoluble protein was removed by centrifugation at 5,000 rpm for 30 min. After centrifugation, protease inhibitors were added to the supernatant fraction containing the cTnT-cTnI complex.

Preparation of Detergent-skinned Fiber Bundles and Treatment with cTnT-C—Left ventricular papillary muscle fiber bundles were dissected from male CD-1 mice (age 3 months) and detergent-skinned overnight as previously described (25). The detergent-skinned fiber bundle was mounted between a force transducer and a micro-manipulator, and the sarcomere length was adjusted to 2.5\,\mu\text{m}. Initial maximum isometric force was measured in activating solution (pCa 4.5) containing 20 mM MOPS (pH 7.0), 33.8 mM KCl, 10 mM EGTA, 9.96 mM CaCl\textsubscript{2}, 5.39 mM ATP, 6.47 mM MgCl\textsubscript{2}, 12 mM creatine phosphate, 10 IU/ml creatine kinase, and 1 mM DTT. The fiber bundle was then placed in high relaxing solution containing 20 mM MOPS (pH 7.0), 35 mM KCl, 10 mM EGTA, 0.25 mM CaCl\textsubscript{2}, 1 mM free Mg\textsuperscript{2+}, 5 mM MgATP\textsuperscript{2+}, 1 mM EGTA, and 0.2 mM phenylmethylsulfonyl fluoride. Force was measured while the fibers were bathed in sequentially increasing Ca\textsuperscript{2+} concentrations ranging from pCa 8 to 4.5. pCa values were calculated as previously described using binding constants reported by Godt and Lindley (26). The Tn exchange was carried out by treating the fiber bundle with 6 M urea to inactivate the PKC and with 2 ml of extraction solution containing cTnT-cTnI, prepared as previously described (25), then 10 min with 2 ml of extraction buffer (without cTnT-cTnI) and 10 min with 2 ml of high relaxing. Maximum Ca\textsuperscript{2+}-activated force was then measured in pCa 4.5 solution to determine the extent of endogenous troponin removed. Next, the cTnT-cTnI-treated fiber was treated with 90 min with a ThC reconstitution solution, prepared as previously described (24). The exchange of endogenous cTnI for phosphorylated cTnI was accomplished by injection of 40\,\mu\text{g} of 1 mg/ml bovine serum albumin (Sigma), which incubated for 1 min. The slide chamber was then washed with two 40\,\mu\text{l} aliquots of low salt assay (AB) buffer (20 mM Tris-HCl (pH 7.4), 25 mM KCl, 2 mM MgCl\textsubscript{2}, 2 mM EGTA (with varying ratios of CaEGTA to K\textsubscript{2}EGTA), and 10 mM DTT. The chamber was incubated in this solution for 5 min, and the solution replaced by two 40-\mu\text{l} aliquots of AB buffer. This was followed by injection of 40\,\mu\text{g} of a 50-\mu\text{M} ionic strength reconstitution solution containing regulatory proteins (Tn and Tm, ranging in concentrations from 0.1–0.4\,\mu\text{M}). The reconstitution solution consisted of 25 mM MOPS (pH 7.4), 25 mM KCl, 2 mM MgCl\textsubscript{2}, 2 mM EGTA (with varying ratios of CaEGTA to K\textsubscript{2}EGTA), and 10 mM DTT. The chamber was incubated in this solution for 5 min, and the solution replaced by two 40-\mu\text{l} washes of motility solution, which was a reconstitution solution to which 1 mM Na\textsubscript{2}ATP and a glucose/oxidase/catalase mixture had been added to slow photobleaching (3 mg/ml glucose, 100 \mu\text{g/ml glucose oxidase}, 10 \mu\text{g/ml catalase}).

**Polyacrylamide Gel Electrophoresis and Western Blot Analysis—**Cardiac myofilament proteins from skinned fiber bundles were analyzed on 12.5% SDS-polyacrylamide gels as well as 8% alkaline urea polyacrylamide gels as previously described (25). The exchange of endogenous Tn with recombinant Tn components was demonstrated by Western blot analysis using a primary antibody (polyclonal goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Sigma)).

**Statistical Analysis—**Data from the pCa-force measurements in skinned fibers were normalized and fitted to the Hill equation by using a nonlinear least-square regression procedure to obtain the pCa\textsubscript{50} (log concentration of activating agent required for half-maximal force) and the Hill coefficient (n). Statistical differences were analyzed by an unpaired t test and ANOVA with the criteria for significance set at p < 0.05. Data are expressed as means ± S.E. The in vitro motility assay data were acquired and analyzed as previously described in detail (22).
Typically the speed at a given pCa is reported as a mean for ~150–300 filaments. Data are reported as means ± S.E. Plots of the mean filament sliding speed versus pCa were fitted to the Hill equation. Fits to the Hill equation were made from a regression using all of the observed speeds so that a fit was generated from over 1200 filaments.

RESULTS

Tension Measurements in Skinned Fiber Bundles—Our strategy to analyze the functional role of charge modification at the PKC sites on cTnI involved exchange of native Tn components in detergent extracted (skinned) fiber bundles with Tn containing phosphorylated or mutant forms of cTnI. We used a procedure illustrated in a chart recording depicted in Fig. 1A, in which the tension of the skinned fiber bundle was first measured over a range of pCa values, ranging from pCa 8 to 4.5. Removal of the native Tn complex is accomplished by incubating the fiber in a cTnT-cTnI extraction solution. B, Coomassie-stained SDS-PAGE analysis of myofilament proteins from skinned fiber bundles with endogenous Tn complex (lane 1), after removal of endogenous Tn complex by extraction with recombinant cTnT-cTnI complex (lane 2), and after subsequent reconstitution of Tn complex with cTnC treatment (lane 3). A pure cTnC standard is shown in lane 4. C, Western blot analysis of cTnT presence (using anti-cTnT antibody) from skinned fiber bundles with endogenous (e) Tn complex (lane 1), after removal of endogenous Tn complex by extraction with recombinant (r) cTnT-cTnI complex (lane 2), and after subsequent reconstitution of Tn complex with cTnC treatment (lane 3). Pure cTnT standard is shown in lane 4. D, non-SDS alkaline urea PAGE analysis of TnC presence from skinned fiber bundles with endogenous Tn complex (lane 1), after removal of endogenous Tn complex by extraction with cTnT-cTnI complex (lane 2), and after subsequent reconstitution of Tn complex with cTnC treatment (lane 3). Pure cTnC standard is shown in lane 4.
Fig. 2. Force-pCa relationships of skinned fibers in which the endogenous Tn complex was exchanged for Tn containing either cTnI WT or cTnI with charge changes at PKC sites of phosphorylation. A, force-pCa curve for skinned fibers containing cTnI WT (filled squares) or cTnI that had been phosphorylated by PKC (cTnI-P (open circles)). Skinned fibers reconstituted with cTnI-P (pCa50 = 5.38 ± 0.01) were significantly desensitized to Ca2+ compared with skinned fibers containing cTnI WT (pCa50 = 5.55 ± 0.01). cTnI WT, n = 10; cTnI-P, n = 4. B, force-pCa curve for skinned fibers containing cTnI WT (filled squares) or cTnI-S43E/S45E/T144E (open circles). Skinned fibers reconstituted with cTnI-S43E/S45E/T144E (pCa50 = 5.55 ± 0.01) were significantly desensitized to Ca2+ compared with skinned fibers containing cTnI WT (pCa50 = 5.55 ± 0.01), cTnI WT, n = 10; cTnI-S43E/S45E/T144E, n = 11. C, force-pCa curve for skinned fibers containing cTnI WT (filled squares) or cTnI-S43E/S45E (open circles). Skinned fibers reconstituted with cTnI-S43E/S45E (pCa50 = 5.34 ± 0.01) were significantly desensitized to Ca2+ compared with skinned fibers containing TnI WT (pCa50 = 5.55 ± 0.01). cTnI WT, n = 10; cTnI-S43E/S45E, n = 13. D, force-pCa curve for skinned fibers containing cTnI WT (filled squares) or cTnI-T144E (open circles). Skinned fibers reconstituted with cTnI-T144E (pCa50 = 5.55 ± 0.02) were not desensitized to Ca2+ compared with skinned fibers containing TnI WT (pCa50 = 5.55 ± 0.01). cTnI WT, n = 10; cTnI-T144E, n = 14.

complex by extraction with cTnT-cTnI complex results in loss of cTnC from the fiber bundle (lane 2), which accounts for the decrease in tension generated by the fiber bundle at pCa 4.5 (Fig. 1A). After reconstitution of Tn complex with cTnC treatment the cTnC band reappears (lane 3). This incubation of the fiber bundle in cTnC solution restored maximum tension to a value close (−75%) to that of the control, and was maintained during a series of measurements over a range of pCa values. A pure cTnC standard is shown in lane 4.

The pCa-tension curves obtained after the exposure of the fiber bundle to the same series of solutions, which did not contain cTnT-cTnI or cTnC, were essentially the same as those obtained after replacement of the endogenous Tn with exogenous native Tn (data not shown). This result indicated that the fall-off of maximum tension was time-dependent and that the exchange protocol using native Tn components did not result in changes in the myofilament activity due to nonspecific interactions of the cTnT-cTnI complex to other regions of the thin filament. The pCa-tension relation of fiber bundles incubated with exchange solutions without proteins (cTnT-cTnI or cTnC) gave pCa50 values of 5.70 ± 0.01, whereas fiber bundles incubated with exchange solutions containing native Tn components gave pCa50 values of 5.72 ± 0.01.

Fig. 2 compares pCa-tension relations for fiber bundles in which the endogenous cTnI was exchanged with various forms of exogenous cTnI. Data in Fig. 2A show that compared with controls, exchanged with unphosphorylated WT cTnI, which had a pCa50 = 5.55 ± 0.01, fiber bundles containing cTnI exhaustively phosphorylated by PKC (cTnI-P) had a reduced sensitivity to Ca2+ (pCa50 = 5.38 ± 0.01). As summarized in Fig. 3B and Table I, there was also a significant 26% decrease in maximum tension of the myofilaments induced by cTnI phosphorylation at the PKC sites. Replacement of endogenous Tn with Tn complex containing cTnI-S43E/S45E/T144E, in which charged glutamic acid residues replaced the two serines and one threonine that are substrates for PKC (Fig. 2B), resulted in a desensitization of the myofilaments to Ca2+ with a decrease in the pCa50 to 5.31 ± 0.01 and a 26% depression in maximum tension (Fig. 3B and Table I). Thus, the presence of cTnI-S43E/S45E/T144E in place of WT cTnI decreased the pCa50 by 0.24 units and decreased Ca2+ sensitivity to a slightly greater extent than that of cTnI-P, which gave a decrease in the pCa50 of 0.17 pCa units. When unphosphorylated cTnI was replaced with cTnI-S43E (Fig. 2C), the pCa50 decreased by 0.21 units and tension decreased by 27% (Fig. 3B and Table I). Replacement of cTnI with cTnI-S43D/S45D (serine residues replaced by aspartic acid residues), however, induced only a relatively small change in myofilament Ca2+ sensitivity (a decrease in the pCa50 of 0.11 units) and a 20% decrease in maximum tension (data not shown) compared with the effects of replacement of cTnI with cTnI-S43E/S45E. Lastly, when unphosphorylated cTnI was replaced with cTnI-T144E (Fig. 2D), there was neither a decrease in myofilament Ca2+ sensitivity nor a decrease in maximum tension (Fig. 3B and Table I).

In Vitro Motility Assay Measurements—Our studies employing the in vitro motility assay also demonstrated that specific charge changes at the PKC sites on cTnI sites on cTnI depress thin filament sliding over myosin heads and its sensitivity to Ca2+. Compared with control filaments regulated by WT Tn, the speed of sliding of thin filaments regulated by Tn complexes containing cTnI phosphorylated at cTnI-P sites decreased by as much as 60% at various pCa values. Thin filaments containing cTnI-S43E/S45E/T144E demonstrated a decrease in maximum sliding speed (Vmax) of 43% and a decrease in the pCa50 of 1.3 pCa units (Fig. 4A and Table I) compared with controls. Sliding speed of actin filaments regulated by Tn complexes containing cTnI-S43E/S45E/T144E decreased by as much as 85% at various free Ca2+ concentrations; Vmax decreased by 55% and the pCa50 de-
Data presented here provide new evidence on the scope of functional effects of cTnI phosphorylation on myofilament function. Previous data indicated that phosphorylation of the N-terminal protein kinase A sites is able to decrease Ca\textsuperscript{2+} sensitivity with no effect on maximum tension and to increase shortening velocity and cross-bridge cycling rate (6, 11–13). We report here that phosphorylation of Ser-43 and Ser-45 (not Thr-144) dominates regulation of the level of maximum tension. However, phosphorylation of Thr-144, in addition to phosphorylation of Ser-43 and Ser-45, appears to be required for regulation of thin filament sliding speed. This result may not be surprising in that the cTnI domain containing Ser-43 and Ser-45 is located at sites distinct and apparently distant from the domain containing Thr-144. Ser-43 and Ser-45 are located in the near N-terminal region of cTnI that binds to the C-terminus of cTnT and the C-lobe of cTnC, whereas Thr-144 is located in the highly basic inhibitory region. Together with a C-terminal region of cTnI (28), the cTnI inhibitory region is the molecular switch that turns on contraction when this region of cTnI moves from actin-Tm to the N-terminal lobe of cTnC upon Ca\textsuperscript{2+} binding to the regulatory site. It is also apparent from our data that Thr-144, which is a cardiac-specific residue (Pro in ssTnI and fsTnI), may represent a structural specialization in cTnI related to the regulation of cardiac muscle, which lacks the ability to regulate function through the recruitment of motor units.

The depression of maximum tension and Ca\textsuperscript{2+} sensitivity induced by phosphorylation or glutamic acid substitution at Ser-43 and Ser-45 may involve altered interactions of the near...
N-terminal region of cTnI with the C-terminal lobe of cTnC. Data on the crystal structure of a complex containing cTnC, a large proportion of cTnI, and a large peptide comprised of most of the C-terminal region of cTnT demonstrate an interaction between the C-lobe of cTnC and the cTnI region containing Ser-43 and Ser-45 (29). Although the C-lobe of cTnC is generally considered to be important in the structural stability of the thin filament, evidence on the binding sites and functional effects of the Ca$^{2+}$ sensitizing agent EMD 57033 indicates a role for the C-lobe of cTnC and its interaction with the near N terminus of cTnI in tension regulation. EMD 57033, which increases sub-maximal and maximal tension developed by skinned fiber preparations (30), docks in a stereospecific manner to the C-lobe of cTnC (31). Moreover, a cTnI peptide comprised of the near N-terminal region of cTnI (cTnI$_{34-71}$) and containing Ser-43 and Ser-45 was demonstrated to displace EMD 57033 from its binding site (31). Interestingly, EMD 57033 was not displaced by the presence of a cTnI peptide containing the inhibitory region (cTnI$_{128-147}$).

The effect of a charge change at Ser-43 and Ser-45 of cTnI on steady-state tension may involve effects on the affinity of cross-bridges for the thin filament as well as cross-bridge-dependent activation. In earlier studies of ATPase rate of reconstituted preparations, we reported a decrease of the affinity ($K_{app}$) of myosin S-1 for regulated thin filaments containing cTnI phosphorylated at Ser-43 and Ser-45 residues when compared with those containing unphosphorylated residues (7). However, data reported by Morimoto et al. (32) also indicate that phosphorylation-induced modulation of interactions of cTnI with the C-lobe of cTnC may alter cross-bridge-dependent activation of cardiac myofilaments. In these studies, activation of myofilaments with and without cTnC was accomplished at pCa 9 by varying MgATP concentration and thus the number of strongly bound rigor cross-bridges. The ability of rigor cross-bridges to activate the thin filament depended on the presence of cTnC and was also dependent on the isoform population of TnI. Inasmuch as the C-lobe of cTnC forms the major interaction with cTnI at low Ca$^{2+}$, Morimoto et al. (32) concluded that activation of the myofilaments by the strongly bound cross-bridges is dependent on an interaction between the C-lobe of cTnC and cTnI. The activation of the thin filament by myosin is generally thought to involve a movement of Tm induced by strongly bound cross-bridges (33). Yet, the data of Morimoto et al. (32) indicate that the ability of strong cross-bridges to move Tm may also involve cTnI and its interaction with the C-lobe of cTnC. It seems reasonable to speculate that this role of cTnI in cross-bridge-dependent activation may be modified by phosphorylation at Ser-43 and Ser-45.

The mechanism by which phosphorylation at Ser-43 and Ser-45 together with phosphorylation at Thr-144 might alter the unloaded velocity of thin filament sliding is not likely to involve altered binding of cross-bridges to the thin filament. Previous studies (34, 35) have demonstrated that unloaded shortening velocity is independent of the filament overlap and thus cross-bridge availability. Velocity of thin filament sliding is more likely to be modified by the step-size of the cross-bridge.
(i.e. how far the cross-bridge pulls the thin filament in its cycle) or the rate of cross-bridge detachment (which is a function of ADP release and ATP binding to the cross-bridge).

Our results support the hypothesis that alterations in the thin filament proteins affect the reaction of cross-bridges with the thin filament by an allosteric mechanism. The depression in speed of thin filament sliding especially at the saturating levels of Ca^{2+} suggests that the charge change at the PKC sites of cTnI either decreases the step-size of the cross-bridge or decreases the rate of cross-bridge detachment at the end of the power stroke. As argued previously (36) it seems unlikely that phosphorylation of cTnI would alter the step-size. However, there is evidence that phosphorylation of cTnI at Ser-43 and Ser-45 may affect cross-bridge detachment. In studies comparing force and ATPase rate of myofilaments from non-transgenic (NTG) mice and transgenic (TG) mice expressing a mutant cTnI, cTnI-S43A/S45A, Pyle et al. (19) reported that compared with the skinned fiber bundles from NTG mice, skinned fiber bundles from the TG cTnI-S43A/S45A mice demonstrated an increase in tension cost. That is, the ATPase rate at a given level of tension was higher in the TG than the NTG preparations. As expected, levels of phosphorylation at PKC sites were higher in the NTG preparations than the TG preparations. These data indicate that the cross-bridge detachment is indeed affected by PKC-mediated phosphorylation of the myofilaments.

Although the precise physiological significance of signaling through the PKC pathway and phosphorylation of myofilament proteins in the intact heart remains unknown, there is indirect evidence that PKC-mediated phosphorylation of cTnI may have important effects on short-term, beat-to-beat regulation of the heart. PKC-mediated phosphorylation of the myofilaments has been shown to be associated with reduced myofibrillar ATPase rate (7, 37–39) and also with decreased unloaded shortening velocity (40, 41). Studies on transgenic mice missing PKC phosphorylation sites on cTnI do not indicate that these changes in myofilament activity are translated into altered cardiac function (17, 42). Montgomery et al. (17) reported that the depression of maximum tension of papillary muscles from NTG controls by the α-adrenergic agonist, phenylephrine, was substantially blunted in papillary muscles from TG cTnI-S43A/S45A hearts. Montgomery et al. (17) reported no differences in twitch dynamics between NTG and TG preparations before or after treatment with phenylephrine. In contrast, the studies of Pi et al. (42), in which all three PKC sites of cTnI were mutated to alanine, implicated altered cross-bridge cycling kinetics as a possible result of phosphorylation of cTnI. Pi et al. (42) investigated the effect of the PKC agonist endothelin 1 on ventricular myocytes from wild-type mice or mouse lines that expressed non-phosphorylatable cTnI in which protein kinase A and PKC sites of phosphorylation (Ser-23, Ser-24, Ser-43, Ser-45, and Thr-144) were substituted with alanine (cTnI-Ala5) on a cTnI-null background. In response to endothelin 1, twitches were prolonged by 24–41% in WT mice, but only by 5–8% in cTnI-Ala5 mice. The data of Pi et al. (42) indicate that the depression of cross-bridge cycling has the more predominant effect on twitch duration than desensitization of the myofilaments to Ca^{2+}, which we found to be the case in the present investigation. Therefore, PKC-mediated phosphorylation of cTnI could play an important role in prolonging the cardiac twitch, which may be a direct result of altered cross-bridge cycling kinetics. Myofilament protein phosphorylation through the PKC pathway may also be of significance in long-term regulation of heart function in adaptation to hemodynamic stressors such as hypertension and myocardial infarction. Up-regulation of the PKC pathway is well known to promote transcription and growth of heart cells (15, 43, 44). Our hypothesis is that this increased activity of PKC is associated with myofilament phosphorylation and decreased force and shortening. Thus, at a time when the heart has engaged pathways to induce cellular hypertrophy, the power of muscle contraction may be declining and leading to a vicious cycle resulting in decompensation and heart failure. Evidence in support of this idea comes from the data of Bowing et al. (14), who reported an increase in the expression level of PKC-α, -β1, and -β2 in failed heart cells.

More recent data indicate that phosphorylation of cTnI is responsible for decreases in sliding velocity of thin filaments isolated from explanted hearts of patients with end-stage heart failure (45). Moreover, expression of PKC-β2 in the hearts of transgenic mice induces hypertrophy as well as reduced myofilament response to Ca^{2+} (16, 46). It thus seems reasonable to speculate that increases in the activity of PKC-β2, induced by some hemodynamic stress, may initially promote compensatory hypertrophic mechanisms that with sustained stress lead to decompensation and heart failure. This is a particularly important mechanism to understand as such agents that specifically inhibit PKC are being developed (47, 48).

Yet, it is a challenge to understand the exact role of PKC in the myocardium. PKC has multiple and varied effects on cardiac function that could be attributed to the multiple PKC phosphorylation sites on cTnI, as well as on its other substrates, which include cTnI, myosin binding protein C, and myosin light chain-2 (49). Furthermore, PKC can also phosphorylate ion pumps, channels, and exchangers. We think data presented here, which show the specificity of functional effects of phosphorylation of a specific substrate, provide an important tool for understanding the complex integration of PKC-mediated signaling in both short- and long-term regulation of cardiac function.

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