**In Vivo and in Vitro Analysis of Cardiac Troponin I Phosphorylation**

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Adrenergic stimulation induces positive changes in cardiac contractility and relaxation. Cardiac troponin I is phosphorylated at different sites by protein kinase A and protein kinase C, but the effects of these post-translational modifications on the rate and extent of contractility and relaxation during β-adrenergic stimulation in the intact animal remain obscure. To investigate the effect(s) of complete and chronic cTnI phosphorylation on cardiac function, we generated transgenic animals in which the five possible phosphorylation sites were replaced with aspartic acid, mimicking a constant state of complete phosphorylation (cTnI-AllP). We hypothesized that chronic and complete phosphorylation of cTnI might result in increased morbidity or mortality, but complete replacement with the transgenic protein was benign with no detectable pathology. To differentiate the effects of the different phosphorylation sites, we generated another mouse model, cTnI-PP, in which only the protein kinase A phosphorylation sites (Ser23/Ser24) were mutated to aspartic acid. In contrast to the cTnI-AllP, the cTnI-PP mice showed enhanced diastolic function under basal conditions. The cTnI-PP animals also showed augmented relaxation and contraction at higher heart rates compared with the nontransgenic controls. Nuclear magnetic resonance amide proton/nitrogen analysis of cardiac troponin C showed that, in the presence of cTnI-AllP and cTnI-PP, the N terminus exhibits a more closed conformation, respectively. The data show that protein kinase C phosphorylation of cTnI plays a dominant role in depressing contractility and exerts an antithetic role on the ability of protein kinase A to increase relaxation.

Recent studies have demonstrated that changes in the phosphorylation states of key cardiac regulatory proteins can have dramatic effects on normal cardiac function (1, 2). Phosphorylation of cardiac troponin I (cTnI), a thin filament regulatory protein, may be particularly important in modulating cardiac function. cTnl, together with cardiac troponin T (cTnT) and cardiac troponin C (cTnC), form the troponin complex. The protein binds to both cTnC and actin and is a critical component in activating contraction as it serves as the Ca²⁺-sensing apparatus. Within the cardiac isoform’s amino-terminal extension, serines are present at residues 23 and 24 (Ser23/Ser24), which serve as substrates for protein kinase A (PKA), which is activated in response to β-adrenergic stimulation of the heart (3). Several investigations report that PKA-mediated phosphorylation of cTnI results in a reduction in myofilament Ca²⁺ sensitivity (4), an increase in cross-bridge cycling (5), and increased binding of cTnI to the thin filament. Cardiac TnI is also a substrate for protein kinase C (PKC) phosphorylation at Ser14/Ser15 and Thr144 (position 143 in the human protein) (6). However, the substrate specificity of these sites is not absolute, since PKC can phosphorylate the PKA sites (7–9). Whereas PKA-mediated phosphorylation is thought to mainly affect the overall Ca²⁺ sensitivity of force development, PKC phosphorylation of TnI inhibits the actin-myosin interaction by decreasing maximum tension, Mg²⁺-ATPase activity, Ca²⁺ sensitivity, and thin filament sliding speed (10). These effects are important determinants in the enhanced relaxation that is critical to the response of the heart to adrenergic stimulation (11, 12).

Normally, there is little or no adrenergic drive in the human left ventricle (13). Whereas increased adrenergic drive is beneficial in initially supporting normal cardiac function, chronic β-adrenergic stimulation can exacerbate an underlying pathology. However, it is unclear whether constant stimulation at the myofibrillar level through the β-adrenergic pathway, as manifested by chronic phosphorylation of the myofibrillar protein targets, causes the deleterious effects. The present study was aimed at determining in vivo whether PKA and/or PKC phosphorylation of TnI were determining factors in mediating contraction or relaxation at base line or during cardiovascular stimulation. In a first iteration, rather than trying to segregate the effects of PKA and PKC phosphorylation, we chose to convert both the PKA and PKC sites in cTnI to aspartic acid to mimic a constant state of complete phosphorylation (cTnI-AllP), since PKC can cross-phosphorylate the PKA sites (8). To investigate the long term effects of TnI charge modification via phosphorylation, we mimicked PKA and PKC cTnI phosphorylation by replacing the endogenous cardiac protein with transmutated to aspartic acid; cTnI-PP, two phosphorylation sites (Ser23/Ser24) mutated to aspartic acid; WT, wild type; PKA, protein kinase A; PKC, protein kinase C; NTG, nontransgenic; TG, transgenic; bpm, beats/min; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LV, left ventricular.
genetically encoded cTnl in which the five substrate residues, Ser32/Ser24 (PKA) and Ser41/Ser45/Thr144 (PKC), were replaced by aspartic acid, whose charge mimics the endogenous residues’ phosphorylated state. To segregate the functional role of PKC-mediated phosphorylation from PKA effects, we also generated a transgenic (TG) mouse in which only the PKA sites (Ser23/Ser24) were mutated to aspartic acid (cTnl-PP). A comparison of their contractile functions and responses to β-adrenergic stimulation allowed us to determine that PKC phosphorylation can antagonize the effects of PKA-induced cTnl phosphorylation in the whole animal and that the primary in vivo function of PKC-mediated cTnl phosphorylation is to decrease contractility at base line and increase relaxation during β-adrenergic stimulation.

EXPERIMENTAL PROCEDURES

Transgene Construction—The wild type (WT) cDNA for mouse cTnl was obtained by reverse transcription-PCR using total RNA isolated from the mouse cardiac ventricle. The cDNA (cTnl-WT) was subcloned into the pET23a vector as described (14). The phosphorylation sites (Ser23/Ser24 by PKA; Ser32/Ser36 and Thr144 by PKC) were mutated to aspartic acid to mimic a constant state of phosphorylation using standard PCR-based methods (cTnl-AlIP). In parallel, the PKA phosphorylation sites of cTnl (Ser23/Ser24) were mutated to aspartic acid to produce the cTnl-PP construct. The cTnl-AlIP and cTnl-PP cDNAs were subcloned into the mouse α-myosin heavy chain promoter (Fig. 1), and the construct was purified from the plasmid backbone after preparation for isoelectric focusing by dilution of 50–75 μg of total RNA isolated from 2–4 mice of mixed gender, multiple sections were carried (18). Using 2–4 mice of mixed gender, multiple sections were analyzed using HT Analyzer version 3.0 (Genomic Solutions) or transcription-specific oligonucleotides (14).

Protein Analyses—Enriched myofibrillar proteins were isolated using F60 buffer (60 mM KCl, 30 mM imidazole, 7.2 mM MgCl2, pH 7.0) with protease/phosphatase inhibitors (Mixture I and II; Sigma) as described (16). Mice were sacrificed, and aliquots of cTnl and human growth hormone probes using transcript-specific oligonucleotides (14).

In Vivo Catheterization and Hemodynamic Studies—Assessment of left ventricular (LV) function in the intact animal was performed using a PowerLab system (AD Instruments, Colorado Springs, CO). Hearts were isolated from five animals of each group. The 100-μl reaction was incubated at room temperature for 5 min and then quenched with 40 μl of 15% trichloroacetic acid. Precipitated proteins were pelleted at 4°C, 80% of the supernatant (containing P1) was transferred to microtiter plates, and 160 μl of FeSO4-aminomonoxybdate solution to react for color development. The absorbance was read, after which the production of inorganic phosphate was determined colorimetrically at 625 nm using a kinetic microplate reader (Molecular Devices). Ca2+-stimulated Mg2+-ATPase activity was measured by subtracting the activity at pH 6.0 from the activity at pH 8.0.

In Vivo PKA and PKC Phosphorylation—Full-length cDNA encoding mouse cTnl-WT and cTnl-AllP were generated by PCR and subcloned into the pET23a vector (Novagen). The phosphorylation assays contained 100 μg of freshly isolated total skinned myofibrils (19). Myofibrils were incubated 120 min at 37°C in 100 μl of 20 mM Tris-HCl, 150 mM NaCl, 20 mM MgCl2, 10 μM ATP, and 100 μM CaCl2. Myofibrils were washed three times in F60 and either solubilized in rehydration/sample buffer (Bio-Rad) for two-dimensional gel electrophoresis or suspended in F60 buffer for the Mg2+-ATPase assay.
FIG. 1. Mouse cardiac TnI-AllP and TnI-PP constructs and characterization of TG expression. A, schematic representation of the mouse cTnI gene (eight exons encoding 211 amino acids). The five phosphorylation sites (PKA/PKC; cTnI-AllP) or two phosphorylation sites (PKA; cTnI-PP) mutated to aspartic acid are indicated. Upstream is the α-myosin heavy chain promoter, and downstream is the human growth hormone (hGH) poly(A) signal. The cardiac specific extension, cTnC- and cTnT-binding domains, and inhibitory regions are shown. B, myofibrillar proteins from three TG lines generated with the cTnI-AllP construct are shown to illustrate how the degree of replacement differed between the various lines. The cTnI-AllP replaced endogenous cTnI partially (lane 13) or completely (lanes 19 and 112) in the TG hearts. cTnI-AllP migrates more slowly in SDS-PAGE (lanes 19 and 112) or completely replaced endogenous cTnI partially (lane 13) b y cTnI-PP, and the degree of replacement can be clearly seen. Complete replacement was confirmed by MALDI-TOF C, Western analyses showed that TG expression did not alter total cTnI protein content. The cTnI-AllP myofibers were compared with cTnI-PP (lane 62) by SDS-PAGE (D) and Western blot (E). Again, total cTnI protein content was unaffected in the TG lane, and the degree of replacement was determined by MALDI-TOF. The molecular weight markers migrate anomalously in the Criterion gel system (Bio-Rad). BD, binding domain.

RESULTS
Expression and Incorporation of cTnI-AllP and cTnI-PP—Cardiac TnI can serve as a substrate for both PKA and PKC (6, 7, 9, 26, 27). However, most of the data bearing on the functional outcome(s) of phosphorylation, with rare exceptions (28, 29), have been obtained in vitro or in isolated systems, with substitution of a noncardiac protein or incomplete replacement (12). To investigate the role of cTnI phosphorylation in cardiac function, we generated two sets of mice in which either the cardiac specific extension, cTnC- and cTnT-binding domains, and inhibitory regions are shown. By guest on July 26, 2018http://www.jbc.org/Downloaded from

unique 32-amino acid sequence at the N terminus, in which two serines at residues 23 and 24 are substrates for PKA. Whereas Ser23/Ser24 and Thr144 are phosphorylated by PKC (Fig. 1A), it is well documented that PKC can cross-phosphorylate the PKA sites as well (6–8). Therefore, to rigorously explore the long term effects of chronic β-adrenergic stimulation, the five sites that can be phosphorylated by PKA and/or PKC were mutated to aspartic acid to mimic the fully charged state of cTnI (Fig. 1A). This transgene was termed cTnI-AllP. To compare and contrast the in vivo effects of PKA-mediated phosphorylation in the absence of PKC-induced modification of cTnI, we generated another construct (cTnI-PP) in which only the PKA phosphorylation sites of cTnI (Ser23/Ser24) were mutated to aspartic acid (Fig. 1A). Each cDNA was then linked to the mouse α-myosin heavy chain promoter and used to generate TG mice. Lines in which replacement was essentially complete were generated with both constructs (Fig. 1, B–E), with replacement confirmed either by SDS-PAGE and Western blots (lane 112; cTnI-AllP) or MALDI-TOF2 (lanes 112 and 62; cTnI-AllP and cTnI-PP, respectively). As described for other contractile proteins, increases observed at the RNA level are not translated into absolute increases of protein (20). Rather, levels of the endogenous protein with the transgenically encoded species. The degree of cTnI-AllP replacement was confirmed by Western blot analyses using a cTnI antibody that recognizes both the endogenous and TG species (Fig. 1C) with line 112 (cTnI-AllP). For cTnI-PP, which could not be resolved from endogenous cTnI on SDS-PAGE (Fig. 1D), MALDI-TOF2 showed greater

\( * \) S. M. Helmke, S. Sakthivel, M. W. Duncan, and J. Robbins, unpublished data.
than 95% replacement. Lines 112 and 62 were chosen for detailed analyses on the basis of essentially complete replacement of endogenous cTnI with the TG species.

Both the cTnI-AllP and cTnI-PP proteins were incorporated normally into the sarcomere (Fig. 2A). Heart/body weight ratios did not differ between NTG (0.0053 ± 0.0008, n = 36) and cTnI-AllP (0.0057 ± 0.0007, n = 28, p = 0.07) or NTG (0.00525 ± 0.0005, n = 18) and cTnI-PP (0.0054 ± 0.0008, n = 15, p = 0.41) littermates. The transcriptional patterns of molecular markers for hypertrophy such as β-myosin, atrial natriuretic factor, and α-skeletal actin were identical for cTnI-AllP, cTnI-PP, and NTG mice. To rule out the possibility that alterations in the overall stoichiometry of the cTnI complex could be responsible for any phenotype observed, comparisons with these lines also included a TG line expressing high levels of cTnI-PP, and NTG mice. To rule out the possibility that the preparation was dephosphorylated or treated with X-100 (skin), the NTG cTnI was completely dephosphorylated and migrated as if it was completely phosphorylated, irrespective of whether the preparation was dephosphorylated or treated with

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Fig. 2. Incorporation of TG cTnI proteins into the sarcomere and histopathological analysis. A, confocal microscopy confirmed that cTnI-AllP and cTnI-PP incorporates normally into the sarcomere (magnification, × 60). The samples were compared both to NTG sarcomeres and material derived from a TG line expressing high levels of cTnI-WT (14). B, longitudinal sections derived from NTG, cTnI-PP, and cTnI-AllP ventricles stained with hematoxylin-eosin display regularly aligned myofibrils (× 20). C, corresponding sections stained with Masson trichrome (× 20) demonstrate the absence of pathology in the two TG lines.

Fig. 3. Evaluation of phosphoproteins by two-dimensional gel electrophoresis. A, total myofibrillar proteins (75 μg) from cTnI-AllP, cTnI-PP, and NTG ventricles were separated in a narrow pH range, 4–7, by two-dimensional gel electrophoresis (see “Experimental Procedures”). This pH range resolves the phosphorylatable contractile proteins cTnT, ELC1v, and RLC2v. Similar gels with different resolving ranges showed that TG expression of either cTnI-PP or cTnI-AllP resulted in no discernable alterations in either the amount or phosphorylation status of the contractile proteins. B, low resolution separation (pH range 7–10) of the charged (phosphorylated) in situ cTnI species from NTG, cTnI-AllP, and cTnI-PP hearts. Under basal conditions, multiple phosphorylation states of cTnI can be observed, with the dephosphorylated protein (P0) predominant. Intermediate phosphorylated (P1) states are also present in small amounts as well as traces of the completely phosphorylated protein (AllP). The cTnI-AllP and cTnI-PP migrate relatively homogeneously, with pI values of 9.08 and 9.41, respectively. High doses of dobutamine (Dob) at 3 ng/g body weight resulted in the complete phosphorylation of the NTG cTnI and the cTnI-PP as well. In contrast, the cTnI-AllP showed no changes upon dobutamine infusion, as would be expected for the already completely charged species. C, migration of NTG cTnI, cTnI-AllP, and cTnI-PP after PKA or PKC treatment. After skimming the muscle with triton X-100 (skin), the NTG cTnI was completely dephosphorylated and migrated at a pI of 9.57. In contrast, detergent treatment did not affect the pI values of either cTnI-AllP or cTnI-PP. PKA or PKC treatment for 2 h resulted in the expected shifts for NTG cTnI, whereas cTnI-AllP was again unaffected. cTnI-PP showed no effects from PKA treatment but was further phosphorylated by PKC. All samples were derived from 15-week mice, and the assays were repeated 4–5 times per sample.
PKA and/or PKC (Fig. 3C). Similarly, cTnI isolated from the cTnI-PP line migrated homogeneously at a position identical to cTnI derived from PKA-treated NTG myofibrillar protein (Fig. 3C). PKA treatment did not affect cTnI-PP mobility, confirming the complete replacement of endogenous cTnI with TG protein. However, upon PKC treatment, cTnI-PP migrated at a pI similar to that exhibited by cTnI-AllP, confirming that the TG protein was a competent substrate for further post-translational modification. The data show that the TG proteins mimic the changes induced by PKC and/or PKA phosphorylation, that the endogenous protein can be phosphorylated completely in vivo, and that the amount and phosphorylation states of the other contractile proteins are relatively unaffected by TG protein substitution.

**Cardiac TnI-AllP Substitution, Maximal Mg\(^{2+}\)-ATPase Activity, and Ca\(^{2+}\) Sensitivity**—Whereas phosphorylation of cTnI by PKA is known to decrease myofibrillar Ca\(^{2+}\) sensitivity and accelerate relaxation (5, 10), the data concerning the role of PKC are contradictory, possibly because of the many different in vivo and in vitro systems used and also because of the potential for cross-phosphorylation of what are normally thought of as PKA sites by PKC (7–9). Using in vitro approaches, phosphorylation of cTnI at Ser\(^{23}/\)Ser\(^{24}\) by PKA was defined as the basis for the decrease in myofilament sensitivity to Ca\(^{2+}\) that follows \(\beta\)-adrenergic stimulation (30). Similarly, PKC phosphorylation of Ser\(^{43}/\)Ser\(^{45}\) and Thr\(^{144}\) resulted in decreases in maximum actomyosin Mg\(^{2+}\)-ATPase activity and velocity. The sites’ functionality could be distinguished, with Ser\(^{43}/\)Ser\(^{45}\) being determinants for sliding speed and Thr\(^{144}\) modulating Ca\(^{2+}\) sensitivity (10). To further understand the consequences of substitution by the phosphorylation mimetics, Ca\(^{2+}\)-stimulated Mg\(^{2+}\)-ATPase activity was determined (Fig. 4), and the data are summarized in Table I. As expected (6, 10), in the skinned fiber system, both PKC and PKA treatment of NTG fibers led to a fall in Ca\(^{2+}\) sensitivity, with PKC resulting in a small but statistically significant decrease in maximal Mg\(^{2+}\)-ATPase activity (Fig. 4A). The cTnI-AllP fibers exhibited behavior similar to that observed for NTG fibers that were treated with PKC (Fig. 4A), showing decreased Ca\(^{2+}\) sensitivity and maximal Mg\(^{2+}\)-ATPase activity (Fig. 4B). Treatment of the cTnI-AllP fibers with either PKA or PKC had no effect. When similar measurements were made on fibers derived from cTnI-PP hearts, the data were similar to those obtained with the PKA-treated NTG fibers, and PKC treatment led to the expected decrease in maximal Mg\(^{2+}\)-ATPase activity (Fig. 4C). Taken together, the data show that complete substitution of endogenous cTnI with cTnI-AllP accurately mimics the effects of PKC phosphorylation, whereas cTnI-PP reflects PKA-mediated modification. Comparison of the data sets confirms that PKC-mediated phosphorylation of cTnI plays a primary role in reducing maximal Mg\(^{2+}\)-ATPase activity, whereas the role of PKA phosphorylation is to mediate decreased Ca\(^{2+}\) sensitivity. The Hill coefficients were not significantly different between the cTnI-AllP, cTnI-PP, and NTG mice (Table I). The unchanged Hill coefficients indicate that cTnI-AllP and cTnI-PP do not affect the cooperativity of the troponin complex and myofibrillar proteins.

**In Vivo Measurement of Cardiac Function**—Although the TG animals appeared overtly healthy in the unstressed state and showed no signs of morbidity or increased mortality, we reasoned that whole organ function would be affected because of the possible effects on motor mechanics and kinetics. To test this hypothesis, we measured ventricular contraction and relaxation at base line and during \(\beta\)-agonist stimulation in the intact closed chest model. There were no significant differences in the basal heart rates, mean arterial pressures, LV end diastolic pressures, and LV systolic pressures between the NTG and cTnI-AllP groups. In contrast, an increase in the basal heart rate (NTG 359 \(\pm\) 22 vs. cTnI-PP 414 \(\pm\) 20 (\(p < 0.02\)) and basal systolic pressure were found in cTnI-PP mice, consistent with a mild increase in \(dP/dt_{\max}\). The in vivo hemodynamic data are summarized in Table II. As might be expected from the decreased Mg\(^{2+}\)-ATPase activity and data gathered from isolated systems (10), substitution of endogenous cTnI with cTnI-AllP resulted in a 40% decrease in LV \(dP/dt_{\max}\) and \(dP/dt_{\min}\) in the TG mice compared with the controls, whereas the cTnI-PP mice were unaffected (Fig. 5, A and B). For the cTnI-PP animals, again the data are consistent with results obtained from isolated systems in which PKA-mediated phosphorylation of cTnI results in enhanced relaxation kinetics (5); contractility is not significantly affected either at base line
or during β-adrenergic stimulation (Fig. 5, A and B), but relaxation at base line is significantly enhanced (Fig. 5B). Interestingly, in the absence of β-adrenergic stimulation, the cTnI-AllP mice, despite containing the same modifications as the cTnI-PP mice, show unaltered relaxation, indicating that PKC phosphorylation can negate the effects of PKA on cTnI.

β-Adrenergic stimulation via increasing dobutamine infusion led to the expected increases in contractility in the NTG, cTnI-AllP, and cTnI-PP mice with values increasing in parallel (Fig. 5, A and B). In contrast, the relaxation kinetics were more complex. Whereas the cTnI-PP mice paralleled the behavior observed in the NTG cohort, with significantly increased relaxation throughout, the cTnI-AllP animals exhibited a complex pattern in response to dobutamine stimulation, with dramatic increases in relaxation as the concentration of the β-agonist increased. The net effect is to unmask the effects of PKA stimulation on cTnI and essentially ablate the antihypertrophic action of PKC on the effect of PKA on cTnI. Although we assumed that this response was mediated by alterations in the other contractile proteins, the relative phosphorylation states of the ryoside receptor, phospholamban, the myosin light chains and voltage-dependent Ca2+-channel did not differ significantly between the cohorts. The main effect of cTnI phosphorylation in vivo is to increase relaxation both at base line (PKA sites) and during β-adrenergic stimulation, and this response may be modulated by the anticipatic effects of the PKA and PKC sites.

Recently, Takimoto et al.,(28) reported that, in a TG mouse similar to cTnI-PP, there was a marked increase in frequency-dependent contractility and relaxation with a rate-dependent enhancement of systolic and diastolic function in vivo. In light of the anticipatic relationships between the PKC and PKA sites observed above, we compared the cTnI-AllP and cTnI-PP cohorts at different heart rates (Fig. 6). At 400 bpm, contraction was decreased by 45% in the cTnI-AllP mice; no differences in relaxation at either 400 or 650 bpm could be detected compared with the NTG mice. Consistent with the data of Takimoto et al.,(28), the cTnI-PP animals showed augmented relaxation and contraction at the higher heart rate when compared with the NTG controls, but this PKA effect is clearly ablated when the PKC sites are also modified.

### Table I

| TABLE I | Ca2+ sensitivity and maximum Mg2+-ATPase activities |
|---------|--------------------------------------------------|
| Vmax (nmol of Pi/min/mg of protein) | EC50 for Ca2+ (mM) | Hill coefficient |
| **NTG** | **Untreated** | **PKA** | **PKC** | **cTnI-AllP** | **Untreated** | **PKA** | **PKC** | **cTnI-PP** | **Untreated** | **PKA** | **PKC** |
| 184.9 ± 5.0 | 5.75 ± 0.04 | 2.1 ± 0.2 |
| 184.8 ± 7.1 | 5.52 ± 0.04a | 1.6 ± 0.2 |
| 150.5 ± 8.2 | 5.53 ± 0.02 | 2.1 ± 0.7 |
| 155.7 ± 15.6 | 5.52 ± 0.14a | 1.5 ± 0.3 |
| 137.6 ± 11.4 | 5.58 ± 0.05 | 1.9 ± 0.3 |
| 143 ± 8.0 | 5.54 ± 0.09 | 2.0 ± 0.6 |
| 191.0 ± 13.3 | 5.55 ± 0.04 | 1.8 ± 0.3 |
| 188.6 ± 12.1 | 5.55 ± 0.1 | 2.0 ± 0.5 |
| 151.5 ± 12.2 | 5.59 ± 0.05 | 1.9 ± 0.2 |

### Table II

| TABLE II | Hemodynamic and physiological measurements |
|----------|------------------------------------------|
| **Basal** | **Dobutamine (32 ng/g/min)** |
| Heart Rate (bpm) | **NTG (n = 12)** | **cTnI-AllP (n = 6)** | **cTnI-PP (n = 6)** | **NTG (n = 12)** | **cTnI-AllP (n = 6)** | **cTnI-PP (n = 6)** |
| MAP (mm Hg/s) | 359 ± 22 | 398 ± 11 | 414 ± 20 | 524 ± 35 | 548 ± 14 | 536 ± 14 |
| Systolic pressure (mm Hg/s) | 72.5 ± 3 | 74.8 ± 4.7 | 86 ± 7 | 72.6 ± 4.6 | 72.1 ± 3.6 | 89.3 ± 7.2 |
| Diastolic pressure (mm Hg/s) | 5.5 ± 1.3 | 6.8 ± 1.6 | 3.2 ± 1.5 | 94.7 ± 6 | 90.7 ± 6.5 | 121 ± 8.05 |
| LVP (mm Hg/s) | 100 ± 3.2 | 102 ± 9.2 | 110.6 ± 11 | 2.5 ± 0.8 | 3.2 ± 1.1 | 3.6 ± 0.4 |
| dP/dtmax (mm Hg/s) | 9097 ± 660 | 4670 ± 589 | 9698 ± 1302 | 1158 ± 3.9 | 1132 ± 4.8 | 1266 ± 6.7 |
| dP/dtmin (mm Hg/s) | 7740 ± 260 | 4553 ± 427 | 8290 ± 465 | 19,009 ± 1441 | 16,584 ± 1122 | 23,598 ± 1286 |
| **Deobutamine (32 ng/g/min)** | 16,360 ± 724 | 12,681 ± 364 | 16,666 ± 216 | 6 | 6 |

* p < 0.05 (compared with untreated NTG).
* p < 0.05 (compared with untreated cTnI-PP).

To evaluate LV function, telemetric measurements of heart rate (bpm), mean arterial pressure (MAP), systolic and diastolic pressures, left ventricular pressure (LVP), the maximum rate of heart contraction (dP/dtmax) and relaxation (dP/dtmin), and the rate of LV pressure increase determined at an LV pressure of 40 mm Hg (dP/dtmin) were measured in NTG, cTnI-AllP, and cTnI-PP littermates as described under “Experimental Procedures.” Data are presented as mean ± S.E.
the N-domain of cTnC (Fig. 7F) (23–25). The largest chemical shift perturbations were observed at Glu^{32}, Gly^{34}, and Ser^{35}, located in inactive site I. Smaller chemical shift perturbations were also observed in Ca^{2+}/H_11001-binding site II (Fig. 7F). The magnitude and direction of the phosphorylation-induced shifts are consistent with loss of the interactions between the cardiac specific amino terminus of cTnI and the regulatory domain of cTnC and a shift in N-domain equilibria toward a more closed regulatory domain conformation (23).

In contrast, amide chemical shifts were observed in both the N- and C-domains of cTnC upon complex formation with cTnI-AllP, containing five negative charges mimicking both PKA and PKC phosphorylation (Fig. 7, E and F). In addition to the previously observed N-domain chemical shift perturbations,
chemical shift perturbations were also observed in C-domain residues mapping to the F/G linker (near the beginning of helix G) of cTnC (Fig. 7E). The introduction of negative charge at Ser43/Ser45 of cTnI, mimicking PKC phosphorylation, induces amide chemical shift perturbations in the F/G loop and helix G of cTnC (46). Thus, chemical shift perturbations mapping to the C-domain of cTnC in the cTnC-cTnI-AllP complex probably result from replacement of Ser43/Ser45 with aspartic acid (Fig. 7E).

Conformational exchange between open/active and closed/inactive regulatory domain conformations, exhibited by multiple amide 1H-15N correlations for regulatory domain residues, was observed in cTnC bound to cTnI-WT (24) and is observed in the cTnI-PP and cTnI-AllP complexes as well (Fig. 7, A and B). Both the magnitude and direction of chemical shifts in resonances belonging to N-domain residues correlate with the opening of the regulatory domain (27). Glycine 34 in the N-domain of cTnC is easily identified in 1H-15N correlation spectra of cTnC in binary cTnC-cTnI complexes and exhibits one of the larger amide proton/nitrogen chemical shifts upon binding cTnI-PP and cTnI-AllP (Fig. 7, C–E). A downfield shift in the amide proton resonance of Gly34 can be correlated with a more closed regulatory domain conformation (24)–(26). In the cTnI-AllP complex, significant chemical shift differences were also observed in residues corresponding to Ca2+-binding site II (Fig. 7E).

Similar to Gly34, located in defunct site I, chemical shift changes in site II could be correlated with a more closed regulatory domain conformation (Figs. 7E and 8). The binding of both cTnI-PP and cTnI-AllP to cTnC induces a downfield amide proton shift in Gly34 (Figs. 7, C and D, and 8). However, the magnitude of the chemical shift change is greatest in the cTnI-AllP complex. Thus, amide proton chemical shifts of regulatory domain residues in the cTnC-cTnI-AllP complex are more consistent with those observed in a closed conformation, where the N-domain hydrophobic cleft is inaccessible to the regulatory region of cTnI, suggesting that the population of closed/inactive regulatory domain conformations increased.

Since incorporation of a negative charge at Ser43/Ser45 induces chemical shift perturbations isolated to the C-domain, the addition of the PKC phosphomimetic at Thr144 to cTnI-PP, containing PKA phosphomimetics at Ser23/Ser24, magnified chemical shift changes in and around Ca2+-binding site II in the N-domain of cTnC (Fig. 7, E and F). Amide proton chemical shifts of regulatory domain residues in the cTnC-cTnI-AllP complex are more consistent with those observed in a closed conformation, where the N-domain hydrophobic cleft is inaccessible to the regulatory region of cTnI, suggesting that the population of closed/inactive regulatory domain conformations increased. Thus, it appears that PKC phosphorylation at Thr144 can enhance the structural consequences of PKA phosphorylation of the cTnI cardiac specific amino terminus by further altering regulatory domain conformational equilibria toward a more closed state, thereby altering the Ca2+ binding behavior of the complex. This is consistent with the reduction in Ca2+ sensitivity that is observed upon PKC phosphorylation of cTnI at Thr144 (10).

DISCUSSION

Constitutive Expression of the Phosphorylation Mimetic Is Beneficial—The present study was undertaken in an effort to understand the long term effects of total TnI phosphorylation on cardiac structure and function and define whether the detrimental effects of long term adrenergic stimulation could be attributed to TnI phosphorylation. Our hypothesis, that the hyperphosphorylation of cTnI at both the PKA and PKC sites would lead to functional pathology, appears to be incorrect, since the mice in which aspartic acid substitution at the relevant residues was used to create a complete and chronic phosphorylation mimetic for cTnI showed no ill effects under normal, unstimulated conditions. We were unable to detect any cardiac pathology or remodeling; the mice tolerated TG replacement well and have shown no signs of increased morbidity, mortality, or cardiac hypertrophy. In contrast to our data, expression of nonphosphorylatable cTnI, in which the five phosphorylation sites were mutated to alanine, showed dilated cardiac hypertrophy in TG mice (34), suggesting that the inability of cTnI to be phosphorylated can result in cardiac pathology.

PKA- Versus PKC-mediated Phosphorylation of cTnI—Chronically, it was unclear what the effects of PKA phosphorylation of cTnI would be in the whole heart. β-Adrenergic stimulation clearly results in increased contractility from the increased [Ca2+] transient, and thus decreased myofibrillar Ca2+ sensitivity might antagonize this effect. The net effect of cTnI PKA phosphorylation in the intact heart, despite a reduction in the Ca2+ sensitivity, is an increased relaxation rate, which would be of obvious benefit in matching relaxation to increased contractility. Just as clearly, PKA phosphorylation appears to have no effect on contractility, consistent with the lack of an effect on maximum Mg2+-ATPase activity.

Elegant in vitro experiments have highlighted the differences resulting from phosphorylation of Ser23/Ser24 via PKA or Ser43/Ser45 and Thr144 via PKC, with PKC-mediated phosphorylation leading to decreased maximal Mg2+-ATPase activity and/or maximum sliding speed in myofibrillar or reconstituted preparations (6, 9, 10, 26, 35). Rather than dealing with the PKC sites in isolation, we elected to study the differences between the two classes by comparing data sets obtained with...
the cTnI-PP mice versus the cTnI-AllP animals, in which both
the PKC and PKA sites were altered. We based the rationale
for this approach on the data showing that PKC could cross-
phosphorylate PKA sites under certain conditions (7–9), and
therefore, it was unlikely in vivo that only the PKC sites
would be modified in the absence of phosphorylated Ser23/Ser24 as
well. Additionally, we wished to determine which effect would
predominate in vivo at the whole organ level if both site classes
were modified. As expected, in NTG myofilaments, PKC mod-
ification effectively decreased maximum Mg²⁺-ATPase activity
compared with untreated myofilaments. There has been some
certainty regarding the effect of PKC-mediated phosphoryl-
ation on myofilament Ca²⁺ sensitivity, with the preponderance
of recent data arguing that PKC-mediated cTnI phosphoryla-
tion results in decreased Ca²⁺ sensitivity (10, 36, 37). Treat-
ment of the cTnI-PP fibers, which already exhibited decreased
Ca²⁺ sensitivity, with PKC did result in an additional slight
decline in Ca²⁺ sensitivity (Fig. 5C and Table I). However, the
major effect of PKC phosphorylation in vivo appears to be
decreased contractility (Fig. 6, A and B), whereas the action of
PKA on cTnI results in accelerated relaxation kinetics (Fig.
6C). These data are consistent with the current theory that
PKC-mediated phosphorylation of the myofilaments results in reduced cross-bridge binding to the thin filament and a consequent reduction in the maximum actomyosin Mg\textsuperscript{2+}-ATPase rate (12) as a result of the affinity of myosin for the thin filament (6). Phosphorylation of cTnI plays a role in both the acceleration of relaxation needed for the cardiac response to β-adrenergic stimulation and the decreased cardiac function that is seen during chronic β-adrenergic stimulation, although, clearly, there are other contributing factors to that pathology, since, despite the reduction in contractility observed in the cTnI-AllP hearts, the unstressed animals survive and thrive with no sign of heart failure over their lifetimes.

**PKC Phosphorylation of cTnI Causes Reduced Contraction and Enhanced Relaxation upon β-Adrenergic Stimulation**—The effects of PKC- and PKA-mediated phosphorylation of cTnI on the heart are complex, which is not surprising, considering the different roles the unique sites can play (5, 10, 38). Recently, the effects of PKC-mediated phosphorylation have been examined in vivo using TG overexpression (28). The data showed only mild increases in systolic and diastolic function that increased at higher heart rates, suggesting that modulation of cardiac function by cTnI PKA-mediated phosphorylation played a role in the force frequency response. However, there are numerous data suggesting that the cross-bridge cycle can play a major role in determining the intrinsic rate of relaxation (39, 40). Faster relaxation kinetics are observed from PKA-mediated phosphorylation of cTnI primarily enhances cardiac relaxation, and PKC phosphorylation depresses cardiac contraction while masking the augmentation of relaxation via PKA. These data clearly suggest that PKC phosphorylation of cTnI plays a dominant role in depressing contractility, presumably by altering the cross-bridge cycling kinetics (10) and also can exert an antithetic role on the ability of PKA to increase relaxation through the phosphorylation of Ser\textsuperscript{43}/Ser\textsuperscript{45} and Thr\textsuperscript{144} sites.

**Structural Studies of cTnI-AllP and cTnI-PP—High-resolution NMR has been useful in understanding the interactions within the troponin complex as well as conformational changes induced by phosphorylation of cTnI** (3, 23, 24, 32, 33). Previously,
conformational changes induced by Ser²³/Ser²⁴ → aspartic acid on cTnC showed that PKA “phosphorylation” of cTnI reduces association of the cardiac specific N-lobe of cTnI with the N-lobe of cTnC (23, 24). Phosphorylation or introduction of negative charge at Ser²³/Ser²⁴ in the cardiac specific amino terminus shifts the open-closed conformational equilibria in the N-domain of cTnC toward the closed state, which may, in part, explain the molecular basis for a decrease in the Ca²⁺ affinity of site II in cTnC. Chemical shift mapping and relaxation studies have previously identified the region around inactive site I in the N-lobe of cTnC as the primary interaction site with the cardiac specific cTnC. Chemical shift mapping and relaxation studies have previously identified the region around inactive site I in the N-lobe of cTnC containing the Ca²⁺/Mg²⁺-dependent protein-protein interaction site (46). These studies suggest that the physiological consequences of PKC phosphorylation of cTnI are transmitted through the Ca²⁺/Mg²⁺-dependent interaction site in the C-lobe of cTnC to the other thin filament proteins.

In the present study, NMR chemical shift perturbation mapping showed that binding of cTnI-ALP induced amide proton/nitrogen chemical shift changes in cTnC residues mapping to the inactive site I and Ca²⁺-binding site II in the N-lobe and the F/G loop and helix G in the C-lobe of cTnC (Fig. 8). Based upon both the magnitude and direction of chemical shift perturbations of residues within the N terminus of cTnC, cTnI-ALP appears to induce a more closed or inactive regulatory domain conformation compared with cTnI-WT, with cTnI-PP being intermediate between the two (Fig. 8). A less accessible, hydrophobic cTnI binding cleft in that domain might lead to faster Ca²⁺ transients and reduced Ca²⁺ sensitivity. It appears that introduction of a negative charge at Thr¹⁴⁴, mimicking PKC phosphorylation, potentiates the effects of PKA phosphorylation at Ser²³/Ser²⁴ (23–25). Thr¹⁴⁴ lies within the inhibitory region of cTnI and is capable of inhibiting actomyosin ATPase activity. This is adjacent to the regulatory region, residues ~150–159 forming an amphiphilic α-helix, which interacts with the N-domain hydrophobic cleft (43). The inhibitory region was not defined in the core troponin structure, possibly because of the inherent flexibility within this region (43). In the presence of bound Ca²⁺ at site II, the cTnI regulatory region stabilizes the open-active N-domain conformation of cTnC by binding in the hydrophobic cleft. In the absence of Ca²⁺, the regulatory region interacts with other components of the thin filament. Thus, the cTnI regulatory region functions as a molecular switch by shifting N-domain conformational equilibria toward the open-active state in the presence of Ca²⁺. Introduction of a negative charge at Thr¹⁴⁴ could alter flexibility within the inhibitory region, possibly through electrostatic interactions between the phosphate group and neighboring basic amino acid residues, thereby altering interactions between the cTnI regulatory region and the N-domain of cTnC. In any case, PKC phosphorylation at Thr¹⁴⁴ of cTnI destabilizes the open conformation of the N-domain of cTnC (Figs. 7 and 8). The synergistic effect of the PKC phosphorylation mimic at Thr¹⁴⁴ on chemical shift perturbations induced by the PKA phosphorylation mimic suggests that PKC-induced Thr¹⁴⁴ phosphorylation in the inhibitory region of cTnI modulates the effects of PKA phosphorylation at the cardiac specific amino terminus of cTnI. Thus, phosphorylation of Ser²³/Ser²⁴ destabilizes the open conformation of the N-domain by weakening interactions between the cardiac specific amino terminus of cTnI and the region around inactive site I in cTnC, whereas phosphorylation of Thr¹⁴⁴ weakens interactions with the regulatory region of cTnI and the region around Ca²⁺ binding site II.

Phosphorylation of cTnI can clearly have major effects on both contractility and relaxation. Of some interest is the observation that chronic β-adrenergic stimulation is a factor in heart failure and that up-regulation of PKC isoforms occurs in decompensated failure and is associated with increased levels of cTnI phosphorylation, which translate into decreases in thin filament sliding velocity (44, 45). Despite the cardiac morbidity associated with chronic adrenergic stimulation and elevated PKC levels, it is apparent that, whereas PKC phosphorylation of cTnI decreases Ca²⁺ responsiveness, maximal Mg²⁺-ATPase activity, and contractility, it cannot, by itself, lead to compromised cardiac function or remodeling, at least in the TG mouse under baseline conditions. Our findings support the precise modulation of cTnI phosphorylation as playing an important role in vivo in modulating cardiac function, but clearly the many other targets of PKA and PKC phosphorylation in the heart must play a determining role in pathologic development, remodeling, and decompensation leading to failure. Our data do not speak to the potential of altered cardiac function as a result of cTnI modification as playing a secondary role in these processes. However, crossing these mice into the many cardiac disease models that are now available as a result of precise genetic manipulation may indicate that selective inhibition of cTnI phosphorylation could provide a novel therapeutic strategy to improve Ca²⁺ responsiveness and increase contractile function.

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