Effects of Protein Kinase A Phosphorylation on Signaling between Cardiac Troponin I and the N-Terminal Domain of Cardiac Troponin C†

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Received April 30, 1997; Revised Manuscript Received July 22, 1997⊗

ABSTRACT: During β-adrenergic stimulation of the heart, there is a decrease in myofilament Ca^{2+} sensitivity mediated by the protein kinase A-(PKA-) induced phosphorylation of troponin I (cTnI). Phosphorylation, which occurs at Ser 23 and Ser 24 in an amino-terminal extension unique to cTnI, decreases the Ca^{2+} affinity of the amino-terminal regulatory site of cardiac troponin C (cTnC). In view of the antiparallel organization of the cTnI−cTnC complex [Krudy, G. A., Kleerekoper, Q., Guo, X., Howarth, J. W., Solaro, R. J., and Rosevear, P. R. (1994) *J. Biol. Chem. 269*, 23731−23735], it is not clear how the phosphorylation signal at one end of the complex affects the Ca^{2+} binding site at the other end. To address this question, we probed the interaction between cTnI and cTnC fragments, $cTnC_{1-89}$ and $cTnC_{90-162}$ (recombinant peptides corresponding to the N- and C-domains of cTnC). cTnI-Cys 5 mutant (S5C/C81I/C98S) and $cTnC_{1-89}$ were fluorescently labeled with IAANS. When cTnI was phosphorylated, the affinity of Ca^{2+} for the cTnI−$cTnC_{1-89}$ complex decreased significantly as indicated by a shift in the pCa_{50} value from 6.65 to 5.25. Upon phosphorylation, the affinity of cTnI for $cTnC_{1-89}$ decreased by 3.8-fold in the absence of Ca^{2+} and 1.7-fold in the presence of Ca^{2+}. In contrast to the case with full-length cTnC, neither $cTnC_{1-89}$ nor $cTnC_{90-162}$ induced significant structural changes in cTnI-Cys 5 as determined from intersite distance measurements between Cys 5 and Trp 192. Moreover, neither fragment of cTnC could significantly restore Ca^{2+} regulation of force generation, when exchanged into fiber bundles from which cTnC had been extracted. Our findings indicate that the transduction of PKA-induced phosphorylation signal from cTnI to the regulatory site of cTnC involves a global change in cTnI structure.

In vertebrate striated muscle, activation of thin filaments from an off to on state is controlled by binding of Ca^{2+} to a regulatory domain of troponin C (TnC).[1] TnC together with TnI, TnT, and Tm forms a regulatory complex controlling contraction by regulating the interactions between myosin cross-bridges and actin (*1*). Ca^{2+}-induced conformational changes are believed to promote strong binding between TnC and the inhibitory protein, TnI, thus weakening an actin−TnI association. These changes, coupled with the movement of Tm on the thin filament and changes in actin structure, switch thin filaments to an on state that facilitates ATP-driven cross-bridge cycling. Although cycling cross-bridges themselves are important for full activation of thin filaments (*2*), it is certain that TnC−Ca^{2+}-induced changes in the state of thin filaments are essential for the triggering of cardiac muscle contraction (*3*).

Evidence derived largely from studies of fast skeletal proteins indicated that transduction of the Ca^{2+} binding signal involves interesting aspects of the dumbbell shape of the TnC molecule. The dumbbell shape, which was first revealed in the crystal structure of fast skeletal TnC (fsTnC), demonstrated two globular N- and C- terminal domains connected by a central helix, whose exposed side chains showed no interaction with either of the globular domains (*4*). In fsTnC the N-domain contains two low-affinity Ca^{2+} binding sites (sites I and II) and the C-domain contains two high-affinity Ca^{2+} binding sites (sites III and IV), which also bind Mg^{2+}. Metal binding to the C-domain is required for the stability of the TnC−thin filament complex, whereas Ca^{2+} binding to the N-domain is essential for the activation of myofilaments (*5*). Upon binding of Ca^{2+}, an extensive patch of hydrophobic amino acids in the regulatory N-domain is exposed from a partially buried state (*6*). This newly exposed hydrophobic patch is believed to form a binding interface for the target protein, TnI. However, it is generally understood that both fast skeletal and cardiac TnI−TnC are arranged in an antiparallel manner (*7*, *8*) and that the binding involves extensive contacts between the two molecules (*9*). The central region of TnC, which has been shown to bind to both TnI and TnT, is considered to be important in

† This work was supported in part by National Institutes of Health Grants HL 49934 (to R.J.S.) and HL 52508 (to H.C.C.) and by postdoctoral fellowships from the American Heart Association of Metropolitan Chicago (to M.C.) and from the Muscular Dystrophy Association (to W.-J.D.).

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: fs, fast skeletal; c, cardiac; TnC, troponin C; TnI, troponin I; TnT, troponin T; Tm, tropomyosin; Tn, troponin complex; hcTnC, recombinant human cardiac TnC; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FRET, fluorescence resonance energy transfer; PMSF, phenylmethanesulfonyl fluoride; IAANS, 2-[(4-iodoacetamido)-anilino]naphthalene-6-sulfonic acid; PKA, protein kinase A; PCR, polymerase chain reaction.

S0006-2960(97)01012-X CCC: $14.00

regulating the transmission of information generated by the binding of Ca^{2+} (*10, 11*). However, the exact role of the central helix remains poorly understood.

The general features of cardiac TnI−TnC interaction appear to be similar to those in fast skeletal muscle. Yet, important differences between primary structures of both cTnC and cTnI and their counterparts in fast skeletal muscle suggest functional specializations. For example, cTnC has only one Ca^{2+} binding site (site II) in the regulatory domain that is responsible for the activation of cardiac myofilaments (*3, 12*). Moreover, cTnI has an N-terminal extension that contains unique sites (Ser 23 and Ser 24) of phosphorylation by PKA. Phosphorylation of these sites has been shown to occur *in situ* (*13, 14*) and to increase the rate of dissociation of Ca^{2+} from cTnC (*15*).

In view of the known antiparallel organization of cTnI-cTnC (*8*), we asked the following: (1) How do phosphorylation-induced conformational changes in the N-terminal extension of cTnI affect Ca^{2+} binding properties of the N-domain of cTnC? (2) Can isolated N- and C-domains of cTnC induce structural changes in cTnI similar to that of intact cTnC? (3) Can N- and C-domains of cTnC function independently? To address these questions, we studied the interaction of N- and C-domains ($cTnC_{1-89}$ and $cTnC_{90-162}$, respectively) of cTnC with cTnI under various conditions. We also tested their ability to function in cTnC-extracted cardiac fiber bundles. Our results demonstrate that the phosphorylation-induced conformational changes near the N-terminal end of cTnI can influence properties of the N-domain of cTnC independently of the C-domain. Our study also demonstrates that fragments of cTnC fail to restore full Ca^{2+}-dependent regulation of force in skinned cardiac fiber bundles.

EXPERIMENTAL PROCEDURES

Preparation of cTnI and cTnC Mutants. A mouse cTnI mutant containing a single cysteine at position 5 was isolated as described before (*16*). A pET3d plasmid construct (phcTnC) containing the complete coding sequence for hcTnC was obtained as previously described (*17*). Recombinant cTnC fragments $cTnC_{1-89}$ and $cTnC_{90-162}$ were generated as described below. For the amplification of the DNA fragment corresponding to $cTnC_{1-89}$ from phcTnC, two oligonucleotides were used as primers. The forward primer (primer 1) was designed to incorporate codons for amino acids 1−7 of hcTnC. This was flanked on the 5′ side by nucleotides of pET3d plasmid including the *Nco*I restriction enzyme site.

primer 1

GAGATATACCATGGATGACATCTACAAGGCT-GCG

The reverse primer (primer 2) corresponded to the noncoding strand of the hcTnC gene that included codons for amino acids 85−90 of hcTnC gene. This was flanked on the 5′ side by a stop codon and the *Bam*HI restriction enzyme site.

primer 2

GCGTGTCTGGATCCCTAGCTGTCGTCCTTCAT-GCAC

The DNA fragment corresponding to the $cTnC_{90-162}$ of cTnC was amplified from phcTnC by using following two oligonucleotides. The forward primer (primer 3) was designed to incorporate codons for amino acids 91−97 of hcTnC. This was flanked on the 5′ side by nucleotides of the pET3d plasmid including the *Nco*I restriction enzyme site.

primer 3

GAGATATACCATGGGGAAATCTGAGGAGGAG-CTG

The reverse primer (primer 4) corresponded to the noncoding strand of hcTnC gene that included codons for amino acids 156−161 of the hcTnC gene. This sequence was flanked on the 5′ side by a stop codon and the *Bam*HI restriction enzyme site.

primer 4

GCGTGTCTGGATCCCTACTCCACACCCTTCAT-GAAC

PCR products were digested with *Nco*I−*Bam*HI restriction enzymes and ligated into the pET3d expression vector (Novagen). Plasmid DNA with proper inserts were sequenced to ascertain the authenticity of the sequence.

Purification of Proteins. Bovine cTnI was purified as described by Potter (*18*). The cTnI-Cys 5 mutant was expressed in bacteria and purified as previously described (*19*). Expression and purification of full-length cTnC was as described by Pan and Johnson (*17*). For the purification of recombinant peptides $cTnC_{1-89}$ and $cTnC_{90-162}$, pET3d plasmid DNA constructs containing the coding sequences for $cTnC_{1-89}$ and $cTnC_{90-162}$ were transformed into BL21-(DE3)pLysS cells (Novagen). Transformed cells were inoculated into LB medium containing 200 μg/mL ampicillin. Typically 4 L of inoculated cultures were grown at 37 °C until the optical density reached 0.8−1.0 (600 nm). Protein expression was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM, and cells were harvested after 4 h. The cell pellet was resuspended in DEAE-Sepharose column buffer containing 50 mM Tris (pH 8.0), 0.1 M KCl, 6 M urea, 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT (50 mL/L of culture). After resuspension, the pellet was sonicated and centrifuged to remove cell debris. For both $cTnC_{1-89}$ and $cTnC_{90-162}$, supernatant fraction containing the protein was initially fractionated on an anion-exchange column (DEAE-Sepharose, Pharmacia). Protein was eluted with a gradient of 0.1−0.5 M KCl in column buffer. Partially purified $cTnC_{90-162}$ was further purified on a phenyl-Sepharose (Pharmacia) column equilibrated with a buffer containing 50 mM Tris (pH 7.5), 1 M NaCl, 0.1 mM $CaCl_2$, and 1 mM DTT. $cTnC_{90-162}$ was eluted from the phenyl-Sepharose column using a buffer containing 50 mM Tris (pH 7.5), 1 M NaCl, 5 mM EDTA, and 1 mM DTT. Partially purified $cTnC_{1-89}$ was further chromatographed on a DEAE-Sephadex (A-25, Pharmacia) column in a buffer containing 50 mM Tris (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT. $cTnC_{1-89}$ was eluted from the column using a gradient of 0.1−0.55 M NaCl in column buffer. After purification, both $cTnC_{1-89}$ and $cTnC_{90-162}$ were dialyzed extensively against water containing 10 mM β-mercaptoethanol and lyophilized. The authenticity of the purified protein was analyzed by SDS−15% polyacrylamide gel electrophoresis (*20*). Protein concentrations were determined by the procedure of Lowry et al. (*21*).

Phosphorylation of cTnI. The catalytic subunit of protein kinase from bovine heart was used to phosphorylate cTnI. cTnI (1 mg/mL) was incubated with the kinase (125 units/mg of cTnI) in 50 mM KH_2PO_4 (pH 7.0), 10 mM $MgCl_2$, 0.5 mM EGTA, and 0.5 mM DTT at 30 °C. The reaction was initiated by the addition of ATP to a final concentration of 1 mM. After 20 min, protein was dialyzed extensively against the buffer at 4 °C. Previously, it has been shown that phosphorylation by this protocol yields nearly 90% phosphorylation of PKA sites in cTnI (*15*).

Fluorescence Studies. The single Cys in cTnI-Cys 5 mutant was labeled with IAANS as described before (*16*). Cys 35 and Cys 84 in $cTnC_{1-89}$ were labeled with IAANS following the protocol described for cTnC labeling (*19*). For measurement of titration of $cTnC_{1-89\ IAANS}$ with Ca^{2+}, all proteins were dialyzed against a buffer containing 30 mM MOPS (pH 7.0), 0.1 M KCl, 3 mM $MgCl_2$ 1 mM EGTA, and 0.5 mM DTT. Steady-state fluorescence measurements were carried out on a Perkin-Elmer LS-5B fluorescence spectrometer using a 0.5 mL cuvette. Excitation and emission slits were set at 5 nm bandwidth. All fluorescence measurements were made at room temperature. Data from the binding isotherms for the interaction of $cTnC_{1-89}$ with cTnI were fitted to a simple 1:1 binding equation to derive the apparent binding constant (K) as described by Liao et al. (*22*).

Determination of Intersite Distances Using Fluorescence Measurements. Steady-state fluorescence measurements were carried out at 20 °C on an SLM 8000C spectrofluorometer as previously described (*23*). The band-pass of both the excitation and emission monochromators was set at 3 nm, and the measurements were made in the ratio mode. Emission spectra were corrected for variations of the detector system with wavelengths. Fluorescence intensity decay of tryptophan in the protein was measured at 20 °C on a PRA single photon counting system (Model 3000) with a rhodamine 6G dye laser synchronously pumped by a mode-locked argon ion laser (Model 171, Spectra-Physics). The output from the dye laser was frequency-doubled to 295 nm by an angle-tuned KDP crystal (Model 390, Spectra-Physics). The emission wavelengths were selected with a 4-nm band-pass monochromator (Instrument SA, Inc.) or a Ditric three-cavity 334 nm interference filter. The procedures previously described (*16*) were used for measurements and analysis of the intensity decays.

The distance between the donor site Trp 192 and the acceptor site Cys 5 (labeled with IAANS) of cTnI-Cys 5 mutant was determined from FRET measurements between the donor and the acceptor. The distribution of the distance was recovered by using a comparative analysis of the donor fluorescence intensity decay and the decay of the donor−acceptor pair. The theory and procedures for measurements of FRET to determine distance distributions have been previously described (*16, 24, 25*).

Force Measurements. Left ventricular papillary muscles were excised from the hearts of male Sprague-Dawley rats and dissected into fiber bundles approximately 100−150 μm in width and 3−4 mm in length. The bundles were placed in a high relaxing (HR) solution containing 20 mM MOPS (pH 7.0), 10 mM EGTA, 1 mM free Mg^{2+}, 5 mM $MgATP^{2-}$, 12 mM creatine phosphate, 10 IU/mL creatine kinase (bovine heart, Sigma), and 0.5 mM DTT. The amount of $CaCl_2$ required to make the pCa 4.5 solution from HR and the amount of KCl required to adjust the ionic strength of all solutions to 150 mM were calculated using a computer program (*26*). All solutions included a cocktail of protease inhibitors as described before (*27*). After the fiber bundle was mounted between a micromanipulator and a force transducer, skinning was performed in HR containing 1% Triton X-100 for 30 min at room temperature. The sarcomere length was adjusted to 2.2 μm. Initial maximum isometric tension of the fiber was measured in pCa 4.5 solution. Fibers were then relaxed in HR solution. cTnC was extracted from these fibers by incubating them in a buffer containing 40 mM Tris (pH 7.8), 5 mM CDTA, and 0.5 mM DTT for 55−60 min (*28*). After extraction, the fibers maintained approximately 20% residual force. Reconstitution was performed by incubating cTnC-extracted fibers with 2−3 mg/mL intact cTnC in HR or 1−2 mg/mL $cTnC_{1-89}$ and $cTnC_{90-162}$ in pCa 4.5 solution. All measurements were made at room temperature.

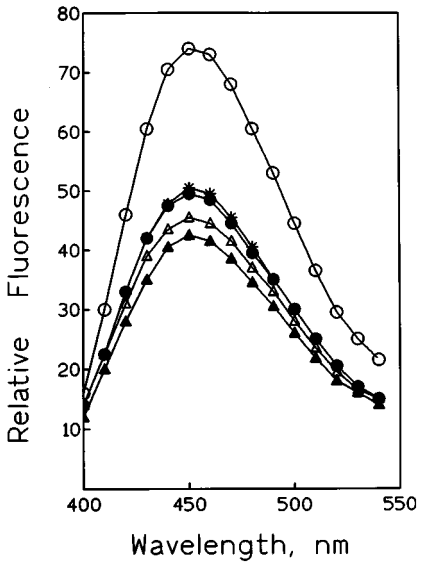

FIGURE 1: Effects of cTnC and its fragments on fluorescence emission spectra of cTnI-Cys 5_{IAANS}. (○) cTnI-Cys 5_{IAANS}; (●) cTnI-Cys 5_{IAANS} + $cTnC_{90-162}$; (*) cTnI-Cys 5_{IAANS} + cTnC; (△) cTnI-Cys 5_{IAANS} + $cTnC_{90-162}$ + 2 mM $CaCl_2$; (▲) cTnI-Cys 5_{IAANS} + cTnC + 2 mM $CaCl_2$. Buffer conditions were 30 mM MOPS, pH 7.0, 0.3 M KCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM DTT; 23 °C; cTnI-Cys 5_{IAANS} concentration was 2 μM. When present, concentrations of cTnC and $cTnC_{90-162}$ were 2 μM each. Excitation was at 330 nm.

RESULTS

Effect of $cTnC_{1-89}$ and $cTnC_{90-162}$ on the Spectral Properties of $cTnI_{IAANS}$. We used a fluorescently labeled cTnI-Cys 5 mutant containing a single cysteine at position 5 to study the interaction between cTnI and fragments of cTnC. Our previous measurements (*16, 23*) showed that fluorescence properties of IAANS attached to Cys 5 in the cTnI-Cys 5 mutant were quite sensitive to the phosphorylation-induced state of cTnI. Figure 1 shows the emission spectra of IAANS-labeled cTnI-Cys 5. Addition of cTnC or $cTnC_{90-162}$ caused a significant decrease in fluorescence intensity of cTnI-Cys 5_{IAANS} in both the presence and absence of Ca^{2+}. Under these conditions $cTnC_{1-89}$ did not induce any changes in emission spectra of cTnI-Cys 5_{IAANS} (data not shown). These results are consistent with the notion that the N-terminal region of cTnI binds to the C-terminal region of cTnC (*8*).

Effect of cTnI Phosphorylation on the Binding of Ca^{2+} to $cTnC_{1-89\ IAANS}$. Changes in the fluorescence intensities of

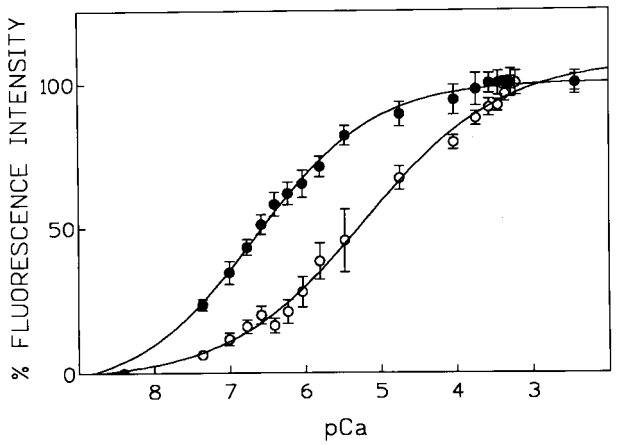

FIGURE 2: Effect of cTnI phosphorylation on the Ca^{2+}-dependent changes in the fluorescence of $cTnC_{1-89}$ IAANS. (●) $cTnC_{1-89}$ IAANS + unphosphorylated cTnI; (○) $cTnC_{1-89}$ IAANS + phosphorylated cTnI. Buffer conditions were 30 mM MOPS, pH 7.0, 0.1 M KCl, 1 mM EGTA, 3 mM $MgCl_2$ and 0.5 mM DTT; 23 °C; $cTnC_{1-89\ IAANS}$ concentration was 0.5 μM. Concentrations of unphosphorylated and phosphorylated cTnI were 2.5 μM each. Excitation was at 330 nm and the emission was monitored at 455 nm. The error bars represent the standard deviation of at least five experiments.

the environmentally sensitive probe IAANS labeled at Cys 35 and Cys 84 of $cTnC_{1-89}$ were monitored to probe the binding of Ca^{2+} to the cTnI−$cTnC_{1-89\ IAANS}$ complex. Figure 2 shows the effect of PKA-dependent phosphorylation of cTnI on the binding of Ca^{2+} to $cTnC_{1-89\ IAANS}$. When $cTnC_{1-89\ IAANS}$ was titrated with Ca^{2+} in the presence of phosphorylated cTnI, there was a significant decrease in the affinity of Ca^{2+} for $cTnC_{1-89\ IAANS}$ as indicated by a rightward shift in pCa_{50} of 1.4 pCa units (from a pCa_{50} value of 6.65 to 5.25).

Effect of cTnI Phosphorylation on the Binding of cTnI to $cTnC_{1-89\ IAANS}$. The decrease in affinity of intact cTnC for Ca^{2+}, associated with cTnI phosphorylation, has also been shown to be accompanied by a 2−3-fold decrease in the binding constant of cTnI to cTnC (*16, 22*). In the present study, we tested the effect of cTnI phosphorylation on the binding of cTnI to $cTnC_{1-89}$. This binding was probed by titrating IAANS-labeled $cTnC_{1-89}$ with the phosphorylated and unphosphorylated cTnI. Figure 3 shows the effect of Ca^{2+} on the binding of $cTnC_{1-89\ IAANS}$ to cTnI under various conditions. The apparent binding constant (*K*) derived from the fitted curves shown in Figure 3 indicate that Ca^{2+}-free and Ca^{2+}-bound $cTnC_{1-89\ IAANS}$ differed slightly in their affinities for unphosphorylated cTnI ($2.76 \times 10^6\ M^{-1}$ vs $3.48 \times 10^6\ M^{-1}$). In the absence of Ca^{2+}, PKA-induced phosphorylation of cTnI decreased the affinity of $cTnC_{1-89\ IAANS}$ for cTnI by almost 4-fold, from $2.76 \times 10^6\ M^{-1}$ to $0.73 \times 10^6\ M^{-1}$. The phosphorylation-induced decrease in the affinity of $cTnC_{1-89\ IAANS}$ for cTnI was also seen in the presence of Ca^{2+}, but the magnitude was smaller as indicated by a change in the binding constant from $3.48 \times 10^6\ M^{-1}$ to $2.05 \times 10^6\ M^{-1}$.

Effects of cTnC Fragments on the Intersite Distance Distribution between Trp 192 and Cys 5 of cTnI. The distribution of intersite distances between Trp-192 and Cys 5 of the cTnI-Cys 5 mutant was calculated as described before (*16*). Our previous observations showed that intact cTnC induced a distance increase between Cys 5 and Trp 192 of nonphosphorylated cTnI in the cTnI−cTnC complex as indicated by an increase in the distance from 44.4 to 48.3

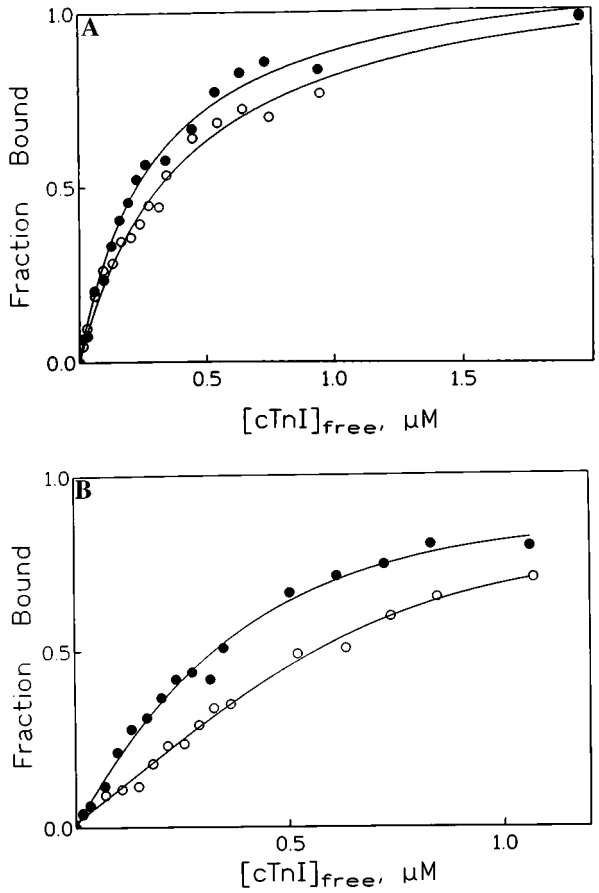

FIGURE 3: Effect of phosphorylation on the binding of cTnI to $cTnC_{1-89\ IAANS}$. The binding was monitored by change in fluorescence of $cTnC_{1-89\ IAANS}$ when titrated with either unphosphorylated or phosphorylated cTnI. Titrations were performed in 30 mM MOPS, pH 7.0, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM DTT; 23 °C; The concentration of $cTnC_{1-89\ IAANS}$ was 0.1 μM. Excitation was at 330 nm and emission was monitored at 455 nm. (A) Titration of $cTnC_{1-89\ IAANS}$ with the unphosphorylated cTnI. (○) 1 mM EGTA; (●) 2 mM $CaCl_2$. (B) Titration of $cTnC_{1-89\ IAANS}$ with the phosphorylated cTnI; (○) 1 mM EGTA; (●) 2 mM $CaCl_2$.

Å (*16*) As summarized in Table 1, fragments of cTnC were not effective in inducing this distance increase, indicating a potentially important role for the central helix of cTnC in the regulatory function of cTnI. In the case of phosphorylated cTnI, it is not clear why either the intact cTnC (*16*) or the fragments failed to induce any significant changes in distance between Cys 5 and Trp 192 of cTnI.

Biological Properties of cTnC Fragments. In order to probe the functional properties of cTnC fragments, we tested the ability of $cTnC_{1-89}$ and $cTnC_{90-162}$ to restore Ca^{2+} regulation of force in cTnC-extracted skinned rat cardiac muscle fibers. Endogenous cTnC was extracted from these fibers by bathing in a buffer containing 5 mM CDTA. Figure 4 shows the amount of force generated by various species of cTnC when reconstituted into cTnC-extracted fibers. For example, when compared to preparations reconstituted with intact cTnC, fiber bundles reconstituted with either $cTnC_{1-89}$ or $cTnC_{90-162}$ could not generate significant force above that of the residual force. When force was measured at pCa 4.5, intact cTnC was able to restore 84% of the initial force, whereas $cTnC_{1-89}$ and $cTnC_{90-162}$ could only restore 32% and 22% of the initial force, respectively. When the two were tested together ($cTnC_{1-89}$ and $cTnC_{90-162}$ present in equimolar amounts), the amount of force restored was found to be smaller than that observed for $cTnC_{1-89}$ alone, indicating that $cTnC_{90-162}$ has an inhibitory effect on force

Table 1: Intersite Distance Distribution between Trp 192 and Cys 5 of cTnI-Cys 5_{IAANS} When Complexed with cTnC or Its Fragments[a]

sample	R_0 (Å)	r (Å)	hw (Å)	χ^2_R
cTnI	24.1	45.3	7.0	1.3
cTnI + $cTnC_{1-89}$	24.2	45.7	9.7	1.0
cTnI + $cTnC_{1-89}$ + Ca^{2+}	23.7	47.2	9.4	0.98
cTnI + $cTnC_{90-162}$	24.0	44.8	7.3	0.92
cTnI + $cTnC_{90-162}$ + Ca^{2+}	24.1	45.1	8.0	1.2
cTnI + $cTnC_{1-89}$ + $cTnC_{90-162}$	23.9	45.9	8.0	1.0
cTnI + $cTnC_{1-89}$ + $cTnC_{90-162}$ + Ca^{2+}	24.2	48.1	9.0	1.1
cTnI[b]	24.3	44.4	9.5	1.07
cTnI + cTnC[b]	24.6	48.3	8.1	1.0
cTnI + cTnC + Ca^{2+} [b]	24.3	51.5	11.1	1.1
P-cTnI	23.6	35.8	2.8	1.2
P-cTnI + $cTnC_{1-89}$	23.8	38.6	2.9	0.97
P-cTnI + $cTnC_{1-89}$ + Ca^{2+}	23.1	39.1	2.1	1.1
P-cTnI + $cTnC_{90-162}$	23.5	35.9	2.8	0.99
P-cTnI + $cTnC_{90-162}$ + Ca^{2+}	23.4	38.2	3.8	1.1
P-cTnI + $cTnC_{1-89}$ + $cTnC_{90-162}$	23.2	36.6	3.1	1.1
P-cTnI + $cTnC_{1-89}$ + $cTnC_{90-162}$ + Ca^{2+}	23.3	39.2	2.9	1.1
P-cTnI[b]	24.8	35.8	3.7	1.1
P-cTnI + cTnC[b]	25.5	36.6	6.0	1.0
P-cTnI + cTnC + Ca^{2+} [b]	24.6	39.3	6.6	1.0

[a] R_0 is the Forster distance, r is the mean distance, and hw is the half-width of the distribution. All other conditions for measurements and calculations are as described previously (*16*). [b] Data from previous observations (*16*) are included for comparison.

generation. This was confirmed by the observation that the amount of force restored by the intact cTnC was considerably reduced when $cTnC_{90-162}$ was present (data not shown). These observations are similar to those of Francois et al. (*29*), who showed that fragments from the C-terminal region of cTnC had a blocking effect on the binding of cTnC to the thin filament.

DISCUSSION

Our main objective in this study was to investigate the mechanism by which phosphorylation of Ser 23 and Ser 24 influences the interaction of cTnI with cTnC. It is possible that this information is transmitted to the N-domain of cTnC through the central helix of cTnC or changes in cTnI structure itself. To probe the molecular mechanism involved in such signal transduction, we constructed $cTnC_{1-89}$ and $cTnC_{90-162}$ corresponding to the N- and C-domains of cTnC following an approach used with fsTnC by Li et al. (*30*). They showed that isolated N- and C-domains of fsTnC showed little differences in structural properties to those in the intact fsTnC (*30*). Using isolated domains of cTnC in binding experiments, we have made a significant finding that phosphorylation-induced conformational changes at the N-terminal region of cTnI are able to modulate Ca^{2+} binding properties of $cTnC_{1-89}$ independently of $cTnC_{90-162}$. This most likely occurs through a global change in cTnI structure rather then through what would appear to be a pathway involving direct interaction between the N-terminal extension of cTnI and the N-domain of cTnC.

Our hypothesis that a global change in cTnI structure is associated with the phosphorylation-induced change in cTnC Ca^{2+} binding is supported by previous experiments demonstrating structural changes in cTnI upon phosphorylation of Ser 23 and Ser 24. On the basis of measurements of quantum yield and acrylamide quenching of Trp 192 in cTnI, Liao et al. (*31*) reported that Trp 192 undergoes a conformational change when cTnI is phosphorylated by PKA. We extended this observation in subsequent experiments by performing measurements using an engineered monocysteine derivative

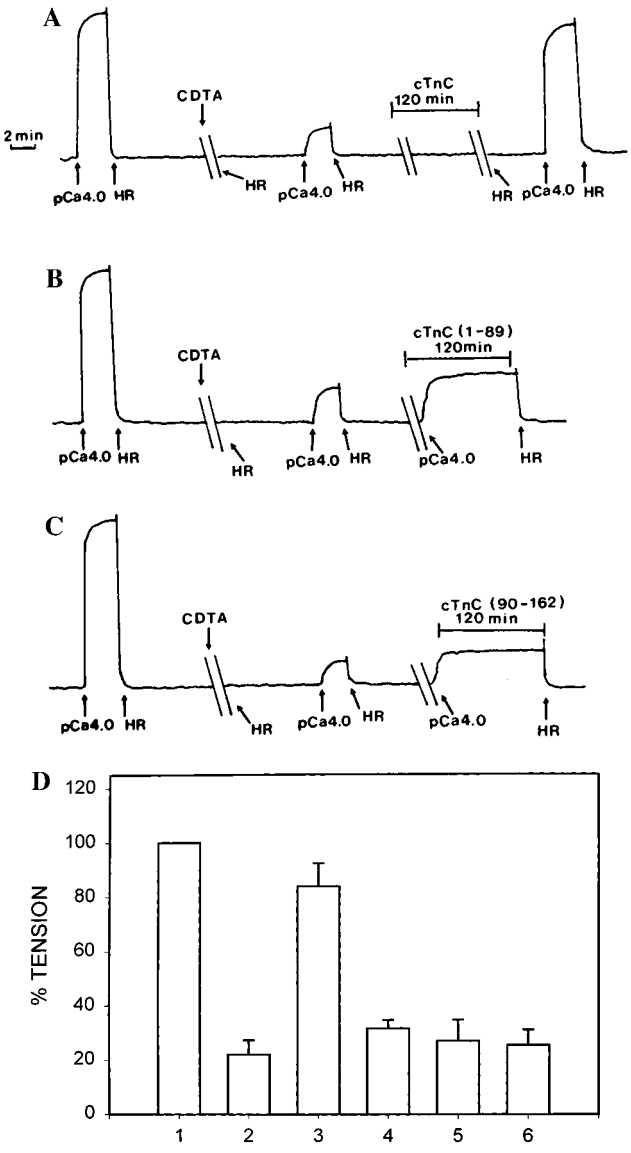

FIGURE 4: Force restoring capacities of cTnC and its fragments in cTnC-extracted skinned rat cardiac fibers. After the initial force was measured in pCa 4.5 solution, endogenous cTnC was extracted by bathing the fiber bundles in buffer containing 5 mM CDTA at pH 7.8. Reconstitution of **(A)** cTnC, **(B)** $cTnC_{1-89}$, and **(C)** $cTnC_{90-162}$ were carried out for 120 min in either HR or pCa 4.0 solution as described in the figure. In panel **D**, the amounts of force restored by cTnC, $cTnC_{1-89}$ and $cTnC_{90-162}$ are expressed as percent of force generated by the unextracted fiber in pCa 4.5. (1) Unextracted fiber, (2) cTnC-extracted fiber, (3) cTnC reconstituted fiber, (4) $cTnC_{1-89}$ reconstituted fiber, (5) $cTnC_{90-162}$ reconstituted fiber, and (6) $cTnC_{1-89}$ + $cTnC_{90-162}$ reconstituted fiber.

of cTnI labeled at Cys 5 with IAANS (*23*). Quenching of the steady-state fluorescence of the probe attached to Cys-5 of cTnI-Cys 5 mutant provided evidence that PKA phosphorylation is associated with a more compact hydrodynamic shape. A possible conformational change giving rise to the more compact shape is a phosphorylation-induced folding of the N-terminal extension associated with phosphorylation of Ser 23 and Ser 24. We tested this hypothesis using FRET measurements between Cys 5 and Trp 192 of cTnI-Cys 5 mutant (*16*). Results of these experiments demonstrated that there was a decrease in intersite distance from 44.4 Å in the unphosphorylated cTnI to 35.8 Å when cTnI was phosphorylated by PKA. A similar decrease in intersite distance occurred in the cTnI−cTnC complex. Because these measurements were made with intact cTnI and cTnC, it was not possible to correlate changes in Ca^{2+} binding properties of

the N-domain of cTnC to the involvement of either the central helix or changes in cTnI structure. However, in view of results of the experiments reported here, we now think that the phosphorylation-induced conformational changes near the N-terminal region of cTnI are transmitted mainly by a global change affecting the other end of cTnI, thereby modulating its interaction with the N-domain of cTnC.

The finding that phosphorylation signaling is manifested over a long distance in a protein molecule is not a new one. X-ray crystallographic analysis of glycogen phosphorylase has shown that the conformational changes associated with the phosphorylation of a single Ser at position 14 is transmitted to the catalytic site 66 Å from the phosphorylation site through a series of changes in the protein structure (*32*). These include folding of the N-terminal segment (residues 10−20) into a compact helical structure. Transition to this structure alters intra- and intersubunit contacts of the N-terminal segment associated with a 120° swing in their position. Here the dianionic nature of the phosphate group at position 14 in glycogen phosphorylase appears to be important in this folding process by forming ionic pair bonds with Arg 69 from the same subunit and Arg 43 from another subunit. Our present findings with cTnI do not permit such a direct correlation between phosphorylation and changes in cTnI structure. Even so, there is clear evidence indicating that phosphorylation of cTnI induces significant global changes in cTnI structure (*16, 33*), which can be correlated to changes in its activity. These data taken together with the results presented here provide important pieces of information in the objective to further define the detailed mechanism by which PKA phosphorylation affects myofilament activity and regulation.

An interaction between the N-terminal region of cTnI and the C-domain of cTnC was suggested by previous observations. In NMR measurements, ^{13}C-methionine was incorporated into bacterially expressed cTnC and used as a spectral probe of the interaction between cTnC and a cTnI mutant missing the N-terminal extension (cTnI/NH_2). When ^{13}C-cTnC was complexed with cTnI/NH_2, changes in the magnetic environment of the ^{1}H−^{13}C correlations for the Met methyl groups of cTnC showed major spectral shifts in the C-domain of cTnC. These data provided the first clear evidence of an antiparallel configuration of the cTnI−cTnC binary complex (*8*). Our results support the idea that the cTnI−cTnC interaction is antiparallel. We showed in the presence of either Mg^{2+} or Mg^{2+} and Ca^{2+} that both $cTnC_{90-162}$ and intact cTnC induced similar changes in the emission spectra of cTnI-Cys 5_{IAANS}, indicating that the $cTnC_{90-162}$ binding region is either near or around the PKA phosphorylation site of cTnI. The observation that $cTnC_{1-89}$ did not induce any significant changes in the spectral properties of cTnI-Cys 5_{IAANS} is a strong indication that there is no direct contact between the N-terminal extension of cTnI and the regulatory domain of cTnC.

Our results indicate that the Ca^{2+} affinity of the $cTnC_{1-89}$−cTnI complex decreases by 1.4 pCa units when cTnI is phosphorylated. Yet previous reports indicated that the Ca^{2+} affinity of cTnC was decreased by 0.2−0.3 pCa unit when cTnI was phosphorylated (*15, 16*). The underlying cause for this difference probably involves the specific conformation of the intact cTnC, which may restrict such major changes in the activity of the cTnC−cTnI complex. This is clearly shown by our observation that fragments of cTnC either alone or when present together failed to induce any significant changes in the intersite distance between Trp 192 and Cys 5 of cTnI-Cys 5. Thus, these fragments of cTnC do not function independently. Further support for this conclusion comes from results of our experiments on force measurements in reconstituted skinned fiber bundles.

When exchanged into fiber bundles, neither $cTnC_{1-89}$ nor $cTnC_{90-162}$ was effective in restoring Ca^{2+} regulation of force. Thus, our observations confirm and extend the findings of Dotson and Putkey (*34*), who showed that cTnC fragments 1−97 and 81−161 were biologically ineffective in regulating the myofibrillar ATPase activity. These findings clearly indicate that the central helix of cTnC serves the important role of determining a precise disposition of the domains that is functional in the regulatory process. This may also account for the ineffectiveness to induce a significant change in intersite distance between Cys 5 and Trp 192 of cTnI when complexed with the isolated domains of cTnC. Consistent with this observation is the recent finding that the structural integrity of the native central helix is an important determinant of the ability of TnC to function normally (*35*). Inasmuch as there is no direct domain−domain interaction (*4, 36, 37*), it appears that the central helix serves to regulate an organization of the two domains that is optimal for target binding. However, despite various biochemical and biophysical investigations on the central helix mutants of fsTnC (*38*−*41*), structure−function correlations of fsTnC activity to the central helix is not clearly understood. On the other hand, the importance of central helix flexibility has been well-established in calmodulin (CaM), which shares a common structure with TnC as visualized by X-ray crystallography (*42*). For example, in CaM the central helix acts as a flexible tether regulating the disposition of the two domains as various target enzymes are recognized (*43, 44*). Using isotope- and spin-labeled cTnC, Kleerekoper et al. (*45*) showed that there is central helix flexibility in cTnC. Binding of cTnI to cTnC decreased the interdomain flexibility, thus maintaining cTnC in an extended conformation. Our results suggest that this aspect of the signaling process is lost in the reconstituted fiber bundles containing isolated N- and C-domains of cTnC. In view of the experimental evidence for fsTnT−fsTnI interaction (*46*) and a direct regulatory role for fsTnT *(47*), it is possible that the influence of cTnC fragments on the Ca^{2+} regulation may be lost due to altered interactions between cTnT and cTnC fragments and/or through an indirect effect involving cTnI−cTnT interaction.

In conclusion, our results indicate that the desensitization of Ca^{2+} binding to the regulatory domain of cTnC can occur even in the absence of $cTnC_{90-162}$ or the central helix of cTnC, suggesting that phosphorylation-induced changes are transmitted to the regulatory site of cTnC through a global change in cTnI structure. It is well-known that the Ca^{2+} signaling process is modulated by the covalent modification of proteins (*48*). For example, there is evidence that the increased rate of relaxation associated with β-adrenergic stimulation of the heart during exercise is related in part to an increase in the off rate for Ca^{2+} exchange with cTnC induced by the phosphorylation of cTnI (*48, 49*). Figure 5 shows schematically the interrelations between Ca^{2+} binding, which promotes the cTnI−$cTnC_{1-89}$ interaction, and PKA-dependent phosphorylation of cTnI, which inhibits the cTnI−$cTnC_{1-89}$ interaction. This reciprocal effect of phosphorylation on the cTnI−cTnC interaction diminishes the binding of Ca^{2+} to $cTnC_{1-89}$, which has been shown to occur by an

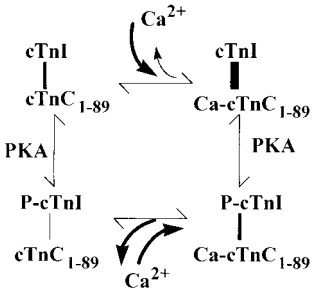

FIGURE 5: Schematic diagram showing the effects of Ca^{2+} and phosphorylation on the interaction between cTnI and $cTnC_{1-89}$. In this scheme, Ca^{2+} promotes and phosphorylation inhibits the cTnI−$cTnC_{1-89}$ interaction as indicated by bold lines. Weakening of the cTnI−$cTnC_{1-89}$ interaction by the PKA-induced phosphorylation of cTnI is shown to result in an enhanced off rate (*15*) for Ca^{2+}-exchange with $cTnC_{1-89}$ as indicated by the bold arrow. The phosphorylated cTnI is shown as P-cTnI.

increase in the off rate for Ca^{2+} binding to the regulatory site of cTnC (*15*). Understanding of the detailed mechanism by which cTnI phosphorylation alters cTnC Ca^{2+} binding is important in the context of both cardiac physiology and pathophysiology. Moreover, this mechanism is altered in human heart failure. It has been demonstrated that the myofilament response to Ca^{2+} is increased in heart failure by a mechanism involving a depression in cTnI phosphorylation (*50*). In a related line of investigation, it has been demonstrated that pharmacologically induced changes in the cTnC−cTnI interaction may be a useful approach in rational design of drugs for heart failure (*51*).

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BI9710129