Phosphorylation of Both Serine Residues in Cardiac Troponin I Is Required to Decrease the Ca^{2+} Affinity of Cardiac Troponin C*

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The phosphorylation of cardiac muscle troponin I (CTnI) at two adjacent N-terminal serine residues by cAMP-dependent protein kinase (PKA) has been implicated in the inotropic response of the heart to β-agonists. Phosphorylation of these residues has been shown to reduce the Ca^{2+} affinity of the single Ca^{2+}-specific regulatory site of cardiac troponin C (CTnC) and to increase the rate of Ca^{2+} dissociation from this site (Robertson, S. P., Johnson, J. D., Holroyde, M. J., Kranias, E. G., Potter, J. D., and Solaro, R. J. (1982) J. Biol. Chem. 257, 260–263). Recent studies (Zhang, R., Zhao, J., and Potter, J. D. (1995) Circ. Res. 76, 1028–1035) have correlated this increase in Ca^{2+} dissociation with a reduced Ca^{2+}-sensitivity of force development and a faster rate of cardiac muscle relaxation in a PKA phosphorylated skinned cardiac muscle preparation. To further determine the role of the two PKA phosphorylation sites in myocardial CTnI (serines 22 and 23), serine 22 or 23, or both were mutated to alanine. The wild type and the mutated CTnIs were expressed in Escherichia coli and purified. Using these mutants, it was found that serine 23 was phosphorylated more rapidly than serine 22 and that both serines are required to be phosphorylated in order to observe the characteristic reduction in the Ca^{2+}-sensitivity of force development seen in a skinned cardiac muscle preparation. The latter result confirms that PKA phosphorylation of CTnI, and not other proteins, is responsible for this change in Ca^{2+}-sensitivity. The results also suggest that one of the serines (23) may be constitutively phosphorylated and that serine 22 may be functionally more important.

Several lines of evidence have led to a general understanding of how β-agonist stimulation leads to positive inotropic and chronotropic effects by phosphorylation of cellular substrates through cAMP-dependent protein kinase (PKA)1 (1, 2). It is generally agreed that phosphorylation of sarcoplasmic Ca^{2+} channels (3) and phospholamban (4, 5), a sarcoplasmic reticular protein regulating the Ca^{2+} pump, are responsible for the changes seen in the intracellular Ca^{2+} transient. The resulting increases in intracellular Ca^{2+} and the rate of Ca^{2+} resequencing contribute to an increase in cardiac muscle contractility and to faster rates of force development and relaxation. PKA can also phosphorylate contractile machinery proteins, such as C-protein and cardiac troponin I (CTnI) (6–9). Many laboratories, including ours, have focused on the mechanism by which phosphorylation of CTnI may be involved in the inotropic effect brought about by β-agonist stimulation.

Troponin I (TnI), a subunit of the troponin complex, inhibits the actomyosin ATPase activity when muscle is in the resting state. Binding of Ca^{2+} to the low affinity site(s) of troponin C (TnC) releases the TnI inhibition on actomyosin ATPase through protein-protein interactions among the troponin complex, tropomyosin and actin, and leads to muscle contraction (10). In comparison with skeletal TnI, CTnI has an additional 32–33 amino acids at its N terminus, and this segment also contains two adjacent serine residues at positions 22 and 23 or 23 and 24, depending on the species (11–13). By converting the phosphoserine into the stable S-ethylcysteine derivative on bovine CTnI, Swiderek et al. (14, 15) demonstrated that these two serines were able to be fully phosphorylated by PKA. Biochemical studies have shown that CTnI can be phosphorylated to the level of 2 mol of phosphate/mol of protein (16), consistent with the sequence prediction. Considerable effort has been made with either perfused hearts (17, 18) or isolated proteins (14–16) to determine the mechanism by which CTnI phosphorylation may affect cardiac muscle contraction. The decreased Ca^{2+} sensitivity upon phosphorylation of CTnI has been observed in isolated cardiac myofibrils (8, 19), in hyper-permeable cardiac fibers (20), and in skinned cardiac muscle preparations and myocytes (21, 22). Moreover, it has been shown that CTnI phosphorylation increases the rate of Ca^{2+} dissociation from a reconstituted troponin complex (23), suggesting the possibility of the importance of this phosphorylation in modulating the rate of relaxation of cardiac muscle. Some reports, however, have shown that CTnI remains phosphorylated even after removal of β-agonists and the return of muscle contraction to prestimulated basal levels (4, 19). These results have suggested that phosphorylation of CTnI may not be as important as the phosphorylation of other proteins, such as phospholamban (4). In a recent study we have clearly shown that CTnI phosphorylation is directly correlated with the faster muscle relaxation seen after PKA phosphorylation and is probably due to the decreased Ca^{2+} affinity of CTnC and the consequent faster dissociation of Ca^{2+} (22). Our calculations also showed that although PKA phosphorylation of phospholamban is the predominant effecter in the inotropic response, the rate of relaxation is significantly influenced by CTnI phosphorylation.

After many years of study there are still numerous questions regarding the mechanism of action of CTnI phosphorylation and of the physiological significance of this process. In the present study we have focussed on the role of the phosphorylation of the two CTnI serines in this process. In order to study the functional and physiological significance of phosphorylation-
tion of these two serine residues, we have used our cloned mouse CTnI cDNA to create three mutants of CTnI in which either serine 22 or 23, or both, are mutated into alanine (9). Our results demonstrate that both serine residues in CTnI are able to be phosphorylated by PKA in vitro but that the rates of phosphorylation for these two serine residues are different. Using a reconstituted skinned cardiac muscle preparation (CSM), we have studied the effect of phosphorylation of each serine residue on the Ca^{2+} dependence of force development. We show that phosphorylation of both serine residues by PKA is required for the decreased Ca^{2+}-sensitivity of force development. Since the rate of phosphorylation is different, it is possible that the rate of dephosphorylation is different and may account for the slower dephosphorylation seen in intact systems (4) and suggests that one of the serines may be more or less constitutively phosphorylated with the other serine being functionally more important.

**MATERIALS AND METHODS**

In Situ Mutagenesis—Mouse CTnI cDNA was obtained by screening a mouse cardiac cDNA library (Clontech) using an oligonucleotide synthesized according to the published rat CTnI sequence (13, 24). In situ mutagenesis of CTnI was performed using a T7-Gen™ in vitro mutagenesis kit (U. S. Biochemical Corp.) according to the protocol provided by the manufacturer. An oligonucleotide (5'-ATCC AGC GAT GGT CTC CAG -3') was used to create an Ncol restriction site 5' to the translation start site, so that the Ncol and BamHI fragment containing the whole transcriptional cDNA sequence could be subcloned into the pET expression vector (Novagen). The following three oligonucleotides were used to create three mutants: 1) 5'-TTG GCA GGC GCG CGT CG-3' for CTnI, mutant, in which Ser-22 was changed to Ala-22; 2) 5'-TAG TTG GCA GGC CGT CGG-3' for CTnI, mutant, in which Ser-22 was changed to Ala-22; 2) 5'-TAG TTG GCA GGC CGT CGG-3' for CTnI, mutant, in which Ser-22 was changed to Ala-22; 2) 5'-TAG TTG GCA GGC CGT CGG-3' for CTnI, mutant, in which Ser-22 was changed to Ala-22; and 3) 5'-TAG TTG GCA GGC CGT CGG-3' for CTnI, mutant, in which Ser-23 was changed to Ala-23.

Affinity Chromatography—Bovine CTnC was isolated as reported previously (25) and was used to prepare a CTnC affinity column. In brief, the protein (100 mg) was dissolved in coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl) and then mixed with 5 g of CNBr-activated Sepharose 4B (Sigma) equilibrated with 1 M HCl. After 2 h of incubation at room temperature, the gel was transferred to a blocking solution (0.2 M glycine, pH 8.0) and incubated for 2 h at room temperature. The gel was then washed with coupling buffer and then a solution containing 0.1 M sodium acetate, pH 4.0, and 0.5 M NaCl, and finally by coupling buffer again. The Sepharose was packed into a column and equilibrated with desired starting buffer.

Expression and Purification of Wild Type CTnI and the CTnI Mutants—Mutations from wild type CTnI into the CTnI mutants described above were excised from double-stranded M13mp18 DNA using the restriction enzymes Ncol and BamHI and subcloned into the expression vectors pET11d or pET3d (Novagen). The proteins were expressed in Escherichia coli BL21(DE3) (Novagen) using the protocol provided by the manufacturer. The expression was checked by 15% SDS-PAGE and Western blotting using a monoclonal anti-rabbit skeletal TnI antibody (made in Dr. Potter's laboratory). The culture for bacterial expression was collected and centrifuged at 7,000 rpm (J-A.10, Beckman). The bacterial pellet was dissolved in a solution containing 6 M urea, 10 M sodium citrate, pH 7.0, 1 M DTT, 2 M EDTA and sonicated (Sonicator, Heat Systems, Inc., Farmingdale, NY) twice at setting 8 for 2 min at 4°C. After the sonication, the pH of this solution was adjusted to 5.0, and the mixture was centrifuged again at 18,000 rpm (J-A.20, Beckman) at 4°C for 30 min. The supernatant, containing the expressed TnI, was loaded onto a CM-52 ion-exchange column equilibrated with the same buffer used to dissolve the bacterial pellet, except that the pH was 5.0. The TnI elution was monitored with a linear KCl gradient of 0–0.4 M in the equilibration buffer (made in Dr. Potter's laboratory). The fractions containing pure TnI (indicated by SDS-PAGE and Western blotting) were pooled together and dialyzed against 1 M NaCl, 50 mM Tris, pH 7.0, 2 mM CaCl₂, 1 mM DTT and loaded onto a TnC affinity column equilibrated with the same buffer. Pure TnI was eluted with a double gradient of urea (0–6 M) and EDTA (0–3 mM) in a solution containing 50 mM Tris, pH 7.0, 1 M NaCl, and 1 mM DTT. The resulting yield was 2–4 mg of pure CTnI/75 ml culture.

Time Course of Phosphorylation of Wild Type CTnI and the CTnI Mutants—The protocol for TnI phosphorylation was modified based on the protocol kindly provided by Dr. Evangelia Kranias, University of Cincinnati. Briefly, wild type CTnI or its mutants (1 μg of each) were phosphorylated in a solution containing 100 mM NaCl, 50 mM MgCl₂, 50 mM sodium phosphate, pH 6.8, 10 μM ATP, [γ-32P]ATP (specific activity, 250–600 cpm/pmol) and the catalytic subunit of PKA (Sigma) 70 units/ml at 30°C. For measuring maximal phosphorylation incorporation into the wild type or mutated TnI's, phosphorylation was carried out for 2 h and terminated by addition of bovine brain synaptosomal CA for precipitation followed by trichloroacetic acid to 10% to precipitate the proteins. The precipitated proteins were washed again with a 10% trichloroacetic acid solution. The precipitated proteins were mixed with 20 ml of Cytosint (ICN) and subjected to scintillation counting (LS 1801, Beckman). The phosphorylation incorporation was calculated according to a parallel experiment to determine the time course of phosphorylation of TnI, the phosphorylation reactions were terminated after 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, or 60 min with the addition of 1 volume of SDS sample buffer (2.0% SDS, 5 mM Tris-glycine, pH 6.8, 2% β-mercaptoethanol, 20% sucrose, 0.05% bromphenol blue) to each sample. Portions of these phosphorylation samples were electrophoresed on SDS-PAGE. The gels were dried and exposed to x-ray film, or the phosphorylated CTnI was cut from the gel, mixed with 20 ml of CytoSint (ICN) and subjected to scintillation counting. The relative phosphate incorporation for the reactions stopped at different reaction times were calculated by comparing the radioactivity of these gel slices with a gel slice containing a fully phosphorylated Tnl whose phosphorylation incorporation had been determined simultaneously by trichloroacetic acid precipitation as mentioned above.

TnCTnI Complex Formation—A complex of TnC and Tnl was formed by combining TnC and Tnl in a molar ratio of 1:1.2 in a solution containing 6 M urea, 20 mM MOPS, pH 7.0, 0.5 mM CaCl₂, 1 mM KCl, and 1 mM DTT. After dialyzing the protein mixtures against this solution for 2 h, the sample was then successively dialyzed against 1, 0.7, 0.4, 0.2, and 0.05 M KCl in a solution containing 20 mM MOPS, pH 7.0, 0.5 mM MgCl₂, and 1 mM DTT. Any excess TnI that precipitated was removed by centrifugation. The complex formation was confirmed by running the supernatant samples on SDS-PAGE.

Preparation of the Skinned Cardiac and Skeletal Muscle—Cardiac muscle was isolated from isolated left ventricle of porcine hearts and chemically skinned by incubation with 1% Triton X-100 in the pCa 8.0 relaxing solution (see below) at 4°C for 1 h. The skinned cardiac muscle (CSM) was then incubated in the same solution plus 50% glycerol for 24 h at ~20°C and stored in 50% glycerol in the pCa 8.0 solution without Triton X-100 at ~20°C. For experiments, CSM was disintegrated into small fiber bundles (0.5 cm long and 0.1–0.15 cm in diameter).

Cardiac muscle was isolated from rabbit skeletal muscle and immersed in 30 min in pCa 8.0 relaxation solution at 4°C. Then the muscle bundles were excised into small bundles (2–4 mm in diameter, ~8–10 cm in length) and tied with wooden sticks. The bundles were incubated in pCa 8.0 solution containing 50% glycerol for overnight at 4°C. The bundles were charged to a fresh pCa 8.0 solution containing 50% glycerol and then incubated at ~20°C until two contractures were observed.

Determination of the Ca^{2+} Dependence of Force Development—The CSM or skeletal fibers were mounted using stainless steel clips to a force transducer (26) and immersed in a relaxation solution (pCa 8.0) containing 10–4 M [Ca^{2+}], 5 mM [Mg^{2+}], 7 mM EGTA, 20 mM imidazole, 5 mM [Mg^{2+}-ATP], 20 mM creatine phosphate, and 15 units/ml creatine phosphokinase, pH 7.0, ionic strength ~150 mM. The contraction solution (pCa 4.0) had the same composition as the pCa 8.0 solution except the Ca^{2+} concentration was 10–4 M and was used to measure the initial force. To determine the Ca^{2+} dependence of force development, the contracture of CSM was tested in solutions containing intermediate concentrations of Ca^{2+}.

Phosphorylation of the Skinned Cardiac or Skeletal Muscle—The CSM or skeletal fibers was phosphorylated with PKA in a freshly prepared solution containing 50 mM phosphate, 50 mM MgCl₂, 50 mM NaCl, 10 mM Mg^{2+}-ATP, and 0.5 unit/ml of the catalytic subunit of PKA, for 1 h at room temperature. The CSM was then washed with the pCa 8.0 relaxing solution. To monitor the level of phosphorylation of CSM, equal aliquots of phosphorylation samples were loaded onto each lane of a SDS-PAGE gel. The phosphorylation samples were solubilized in SDS sample buffer and analyzed by SDS-PAGE (15%) and autoradiography (22). The Ca^{2+} dependence of the same CSM or skeletal fibers was measured before and after phosphorylation.

CTnI Extraction and Reconstitution of the CSM—The CSM was first tested for its initial force in the pCa 4.0 solution. Extraction of the endogenous CTnI was performed by incubation of the CSM in a 10 mM orthovanadate solution, pH 6.7, for 10 min (27, 28). The vanadate was subsequently removed by washing with the pCa 8.0 relaxing solution. The extent of CnI extraction was estimated from the resultant Ca^{2+}.
independently, force seen in the pCa 8.0 relaxing solution. After the Ca\textsuperscript{2+}-independent contraction had reached the maximum, the CSM was incubated with 10 μM CtnC and urea (0–6 M) in a double gradient to the TnC affinity column. An improvement was made by applying an EDTA (0–3 and near the end of the described gradient, essentially the same as used in purifying skeletal TnI (29). The purification of CTnI was reported previously.

Mutants—The expression of wild type CTnI and its mutants was carried out in BL21(DE3) host cells in which the expression was estimated from the amount of Ca\textsuperscript{2+}-dependent force regained after incubation with the CtnC-TnC complex. Force measurements were initiated when the reconstituted force reached more than 50% of the original force. The Ca\textsuperscript{2+} dependence of force development was determined and compared before and after phosphorylation of the CSM (22). Phosphorylation of the reconstituted CSM was performed under the same conditions as described for the nonextracted CSM. γ\textsuperscript{32P}ATP was included in the phosphorylation solution to monitor the levels of phosphorylation of wild type CTnI and its mutants after they were reconstituted into the CtnI-depleted CSM.

RESULTS

Expression and Purification of Wild Type CTnI and CTnI Mutants—The expression of wild type CTnI and its mutants was carried out in BL21(DE3) host cells in which the expression was controlled by the expression of the T7 RNA polymerase promoter. The purification of CTnI was reported previously and essentially the same as that used in purifying skeletal Tnl (29). An improvement was made by applying an EDTA (0–3 mM) and urea (0–6 M) double gradient to the TnC affinity column to remove other protein contaminants prior to eluting CTnI from the CtnC affinity column. Since the affinity of CTnI for CtnC is quite high, the bound CTnI can only be eluted from the CtnC affinity column in the presence of 6 M urea, 2 mM EDTA, and 1 M NaCl, or near the end of the described gradient, with other contaminating proteins eluted during the first half of the gradient. Fig. 1A shows the SDS-PAGE patterns of the bacterial lysate, and Fig. 1B shows the purified wild type CTnI and the indicated mutants. The levels of expression of CTnI (2–4 mg/liter of culture) were reconstituted into the CSM (22). Phosphorylation of the reconstituted CSM was performed as described under “Materials and Methods.”

Rate of PKA Phosphorylation of Wild Type and Mutated CTnI—The different levels of CTnI phosphorylation detected in cardiac tissues (16) suggest the possible co-existence of two monophosphate and/or biphosphate forms of CTnI. In this experiment we used wild type CTnI and its mutants to study the time course of CTnI phosphorylation of each of the two serine residues (22 and 23), and thus we could carefully control the experimental variables and we could compare our data with other phosphorylation studies using either CTnI isolated from cardiac muscle or synthetic CTnI peptides (14–16). Phosphorylation reactions were stopped at different reaction times. Portions of these samples were electrophoresed on 15% SDS-PAGE, and the dried gels were exposed to x-ray films. A: lanes 1–8, phosphorylation of wild type CTnI; B: lanes 1–8, phosphorylation of CTnI S22A; C: lanes 1–8, phosphorylation of CTnI S23A. Lanes 1–8 in the figures represent the different times of phosphorylation. They are 30 s, 1 min, 2 min, 5 min, 10 min, 30 min, and 60 min, respectively. D, time course of phosphate incorporation into wild type and mutant CTnIs by PKA. The phosphorylation of wild type and mutant CTnIs was calculated as described under “Materials and Methods” and was plotted as an average of three to four experiments (X = mean ± S.E.).

Fig. 2. The time courses of phosphorylation of wild type CTnI and its mutants. Phosphorylation of wild type CTnI and its mutants was performed as described under “Materials and Methods.” A–C, autoradiograms showing the phosphorylation of wild type and mutant CTnIs. 1 μg of either wild type CTnI or mutants were phosphorylated by PKA under conditions described under “Materials and Methods.” The phosphorylation reactions were stopped with the SDS sample buffer added to the reaction mixtures at different reaction times. Portions of these samples were electrophoresed on 15% SDS-PAGE, and the dried gels were exposed to x-ray films. A: lanes 1–8, phosphorylation of wild type CTnI; B: lanes 1–8, phosphorylation of CTnI S22A; C: lanes 1–8, phosphorylation of CTnI S23A. Lanes 1–8 in the figures represent the different times of phosphorylation. They are 30 s, 1 min, 2 min, 5 min, 10 min, 30 min, and 60 min, respectively. D, time course of phosphate incorporation into wild type and mutant CTnIs by PKA. The phosphorylation of wild type and mutant CTnIs was calculated as described under “Materials and Methods” and was plotted as an average of three to four experiments (X = mean ± S.E.).
orthovanadate solution was used to extract endogenous CTnI. CTnI was only half-phosphorylated. Wild type CTnI, was determined again. This was plotted and fit using the Hill equation. We found that phosphorylation of CSM did not change the maximal force at full phosphorylation of CSM by PKA is CTnI. Under our experimental conditions, there was no additional phosphorylation after 60 min of incubation with PKA, indicating that the proteins were fully phosphorylated.

Effect of PKA on the Ca\(^{2+}\) Sensitivity of Muscle Contraction—To determine the effect of PKA on the cardiac skinned muscle, the CSM was phosphorylated as described under “Materials and Methods,” solubilized in SDS sample buffer, and analyzed on SDS-PAGE as shown in Fig. 3. The autoradiogram shown in Fig. 3 indicates that the primary site of phosphorylation of CSM by PKA is CTnI. Under our experimental conditions the amount of C-protein phosphorylation varied from lightly to barely phosphorylated, while the CSM phosphorylation was always high, and this result is consistent with a report by Venema and Kuo (7). Except for CTnI and C-terminal protein, there were no other proteins phosphorylated by PKA under our experimental conditions. The effect of PKA on the Ca\(^{2+}\) sensitivity of muscle contraction was examined and demonstrated in Fig. 3. The Ca\(^{2+}\) dependence of force development of phosphorylated and dephosphorylated CSM was plotted and fit using the Hill equation. We found that phosphorylation of CSM did not change the maximal force at pCa 4.0; however, the pCa\(_{50}\) decreased from 5.51 ± 0.02 to 5.25 ± 0.04 (Table II), demonstrating a decreased Ca\(^{2+}\) sensitivity of force development of the CSM after phosphorylation. Moreover, incubation of CSM in the phosphorylation solution without PKA for up to 2 h did not change the Ca\(^{2+}\) dependence of muscle contraction as compared to unphosphorylated CSM (Fig. 3). Therefore, the decrease in Ca\(^{2+}\) sensitivity of the force development of CSM could only be brought about by PKA treatment.

CTnI Extraction and Reconstitution—Based on the CTnI extraction method reported by Strauss et al. (27), a 10 mM orthovanadate solution was used to extract endogenous CTnI from the CSM. The modification we made here was to use the CTnI-CTnC complex for the reconstitution, since both endogenous proteins, CTnI and CTnC, are extracted from CSM during the vanadate treatment, and previous results have shown that reconstitution is much greater using this protocol (28). As shown in Fig. 4A, the force developed by the CSM after 10-min incubation with 10 mM vanadate, gradually became independent of Ca\(^{2+}\) after incubation in the pCa 8.0 solution for about 30 min. This occurs when the vanadate is washed out by the pCa 8.0 solution, and the CSM contracts even in the absence of Ca\(^{2+}\). After three washes with the pCa 8.0 solution, there was a 10 mM orthovanadate solution described under “Materials and Methods” for 10 min. After washout of the vanadate with the pCa 8.0 solution, the CSM lost its Ca\(^{2+}\) dependence due to the loss of CTnI and CTnC. When maximal force was obtained, the muscle was incubated with 10 \(\mu\)M CTnI-CTnC complex in pCa 8.0 solution. The force restoration was tested after a 3-h incubation in CTnI-CTnC complex. The scales of different time blocks during the experiment are indicated in the figures. The total time of time block II in A and B is 10 min. A, the CTnI-CTnC complex was used for reconstitution; B, control experiment. No CTnI-CTnC was used for the muscle reconstitution. C, Coomassie Blue-stained SDS-PAGE of solubilized CSM which has been extracted by vanadate treatment and reconstituted with CTnI-CTnC complex. Lane 1, bovine CTnI; lane 2, nonextracted CSM; lane 3, extracted CSM; lane 4, same as lane 3, but the load as twice as much; lane 5, reconstituted CSM.
Phosphorylation of Both Serine Residues in CTnI

The effect of PKA on Ca\(^{2+}\) dependence of muscle contraction (pCa\(_{50}\)) of nonextracted CSM and reconstituted CSM

|                        | Before phosphorylation | After phosphorylation | Change pCa\(_{50}\) (n) |
|------------------------|------------------------|------------------------|-------------------------|
| Unextracted skeletal fibers | 5.49 ± 0.02            | 5.49 ± 0.03            | NS                      |
| Unextracted Cardiac muscle | 5.51 ± 0.01            | 5.25 ± 0.01            | 0.26 1.58               |
| Reconstituted CSM with wild type CTnI (n = 7) | 5.72 ± 0.04            | 5.54 ± 0.04            | 0.18 0.93               |
| Reconstituted CSM with CTnI Ser22A (n = 5)  | 5.78 ± 0.08            | 5.78 ± 0.07            | NS                      |
| Reconstituted CSM with CTnI Ser23A (n = 6)  | 5.79 ± 0.03            | 5.76 ± 0.03            | NS                      |
| Reconstituted CSM with CTnI Ser22A, Ser23A (n = 5) | 5.68 ± 0.06            | 5.65 ± 0.05            | NS 0.96                 |

*The midpoint (pCa\(_{50}\)) was determined by the Hill equation: percent change of maximal force = [Ca\(^{2+}\)]/[Ca\(^{2+}\)+pK\(_i\)], where n is the Hill coefficient number. p < 0.05 versus pCa\(_{50}\) value before phosphorylation analyzed by the paired Student’s t test. NS stands for no significance.

The extent of reconstitution is consistent with previously reported results (27, 28). The purpose of this experiment is to show the effect of incubating the extracted fiber in the absence of the CTnI. As expected, wild type CTnI was phosphorylated at the greatest extent. The levels of phosphorylation of CSM Ser22A, and CTnI Ser23A were essentially the same in the reconstituted CSM, but less than that of wild type CTnI, whereas CTnI Ser22A, Ser23A was not phosphorylated at all. These data are consistent with the results shown in Fig. 2. The maximal Ca\(^{2+}\)-dependent force of the reconstituted CSM in pCa4.0 after phosphorylation remained the same as that of before phosphorylation, suggesting that there was no effect of the phosphorylation conditions on the dissociation of CTnI or CTnC from the reconstituted muscle preparation.

Effect of PKA on the Ca\(^{2+}\) dependence of Force Development in CSM Reconstituted with Either Wild Type or Mutated CTnIs—As shown in Fig. 3 and Table II, phosphorylation of CSM resulted in a decrease of pCa\(_{50}\) by -0.26 pCa units. This change appears to be specifically brought about by CTnI phosphorylation, since the Ca\(^{2+}\)-dependence of force development in skeletal muscle (whose TnI does not have the phosphorylation site for PKA) did not change after the skeletal fibers were phosphorylated by PKA (Table II). In addition, no change in the Ca\(^{2+}\)-dependence of muscle contraction was observed when CSM was incubated in the presence of PKA and PKA inhibitor (22). The Ca\(^{2+}\)-dependence of force development of the extracted CSM, subsequently reconstituted with either wild type or mutated CTnIs, was measured before and after phosphorylation. The CSM extraction and reconstitution with the CTnI-CTnC complex, described in detail under “Materials and Methods,” was the same as in Fig. 4, A and B. There was no difference in the recovery of Ca\(^{2+}\)-dependent force between wild type CSM and its mutants, suggesting that reconstitution of the wild type and mutant CTnIs was the same. Fig. 5 shows the phosphorylation of wild type CTnI and its mutants when reconstituted back into the CSM-depleted CSM. As expected, wild type CTnI was phosphorylated to the greatest extent. The levels of phosphorylation of CSM Ser22A, and CTnI Ser23A were essentially the same in the reconstituted CSM, but less than that of wild type CTnI, whereas CTnI Ser22A, Ser23A was not phosphorylated at all. These data are consistent with the results shown in Fig. 2. The maximal Ca\(^{2+}\)-dependent force of the reconstituted CSM in pCa4.0 after phosphorylation remained the same as that of before phosphorylation, suggesting that there was no effect of the phosphorylation conditions on the dissociation of CTnI or CTnC from the reconstituted muscle preparation.

Table I

|                        | TM | CTSnI | LC1 | LC2 |
|------------------------|----|-------|-----|-----|
| Before extraction      | 1  | 0.47 ± 0.06 | 1.02 ± 0.06 | 0.97 ± 0.17 |
| (n = 5)                |    |       |     |     |
| After extraction       | 1  | ND*   | 1.17 ± 0.14 | 0.95 ± 0.10 |
| (n = 4)                |    |       |     |     |
| After reconstitution   | 1  | 1.05 ± 0.67 | 1.52 ± 0.52 | 1.28 ± 0.61 |
| (n = 3)                |    |       |     |     |

*ND = not detectable.

The relative amount of protein before and after extraction by vanadate and following reconstitution. The amount of tropomyosin is arbitrarily designated as 1. The amount of TnI, LC1, and LC2 was compared to that of tropomyosin. These data are expressed as “mean ± S.E.”

The amount of tropomyosin is arbitrarily designated as 1. The amount of TnI, LC1, and LC2 was compared to that of tropomyosin. These data are expressed as “mean ± S.E.”
sumably caused by cross-bridge attachment due to incomplete CTnI-CTnC reconstitution. Therefore, the attached cross-bridges may be responsible for the increased Ca\(^{2+}\) sensitivity seen in the reconstituted CSM (26).

**DISCUSSION**

As we have previously reported (9), mouse CTnI can be expressed in a bacterial expression system. The purification method presented here has proven to be an efficient way to obtain large quantities of CTnI (30). However, the final yield of CTnI purified from the pET expression system is not as high as for skeletal TnI (29). This probably results from the codon usages for the first 32 amino acids in the mouse CTnI cDNA sequence, since the expression of a deletion mutant of CTnI devoid of the first 32 amino acids was much higher than that of the intact CTnI (30).

PKA phosphorylation of CTnI causes a decrease in the Ca\(^{2+}\) dependence of force development or of the myofibrillar ATPase of cardiac muscle and has been observed by many investigators (8, 9, 20, 21, 36). We have recently found that CTnI phosphorylation increases the rate of Ca\(^{2+}\) dissociation from CTnC, thus contributing to the observed faster relaxation (22, 23). CTnI contains two adjacent serine residues immediately adjacent to three arginine residues, i.e. R-R-R-S-S, which meets the minimal sequence requirement (R-R-X-S/T) for PKA phosphorylation of both serines (31). Various studies on CTnI phosphorylation have suggested that CTnI can be phosphorylated up to 2 mol of phosphate/mol of protein (15, 16, 32), which is consistent with the amino acid sequence of the CTnIs. Our phosphorylation studies have confirmed the prediction that these two serine residues are able to be phosphorylated by PKA. Moreover, we have shown that these two serines are the only phosphorylation sites in CTnI, since the mutant, CTnI\(_{S22A/S23A}\), was not phosphorylated by PKA. In agreement with the study by Mittmann et al. (32), we found that the rate constants for the phosphorylation of the two serines are different, with Ser-22 being phosphorylated at a slower rate than Ser-23. However, in contrast to the peptide studies (32), the difference between the rates of phosphorylation of the two serines was not 13-fold but more like 2-fold. The reason of the observed discrepancy is probably due to the different conformation of the two substrates used for phosphorylation. In contrast to our studies on whole CTnI, the peptide used by Mittmann et al. (11) was only ten amino acids in length. The other interesting finding is that the phosphorylation of the wild type CTnI was equal to the sum of the two mutant CTnI phosphorylation. This implies that the phosphorylation of Ser-22 and Ser-23 are independent of each other.

We have demonstrated that only the phosphorylated wild type CTnI was effective in decreasing the Ca\(^{2+}\) sensitivity of force development. The three other mutants, when substituted into CSM, showed no effect of PKA on the Ca\(^{2+}\) dependence of force development. The in vitro experiments (Figs. 2 and 5) have demonstrated that both CTnI\(_{S22A}\) and CTnI\(_{S23A}\) can be fully phosphorylated. Our results suggest then that both serines need to be phosphorylated to lower the Ca\(^{2+}\) sensitivity of muscle contraction. The monophosphate forms of CTnI do
not appear to be effective in decreasing the Ca\textsuperscript{2+} sensitivity of muscle contraction.

It is of course possible that, due to the limitation of the methodology used, we were unable to observe small effects when only Ser-22 or Ser-23 are phosphorylated. The limitation of using the vanadate extraction and reconstitution method is that it is difficult to restore the Ca\textsuperscript{2+}-dependent force close to the initial force before extraction. This is in part due to the fall off in force that occurs during the incubation of the extracted fiber with the CTnI-CTnC complex (Fig. 4, A and B). It may also explain why the change in the Ca\textsuperscript{2+} dependence of force development in CSM reconstituted with wild type CTnI brought about by PKA is only \(-0.18\) pCa units versus a change of \(-0.26\) pCa observed in nonextracted CSM (Table I). Although we cannot rule out the possibility that these intrinsic methodological weaknesses might contribute to our failure to see any effect of the single serine phosphorylation on the Ca\textsuperscript{2+} dependence, our data are consistent with other many experimental results. In any event, our results clearly show that PKA phosphorylation of CTnI, and not some other protein(s), is responsible for the PKA induced change in Ca\textsuperscript{2+} sensitivity.

It has been reported that the majority of the endogenous phosphate in CTnI isolated from cardiac tissue is contained in the second serine (14). It is also possible to isolate the monophosphate form of native CTnI, in which only the second serine is phosphorylated, from cardiac tissue (33). Isolation of the monophosphate form of CTnI in which only the first serine is phosphorylated has not been reported, although it appears to exist (33). Therefore, even in the resting state when \(\beta\)-agonist levels are low, CTnI is probably partially phosphorylated, mostly at the second serine. These data suggest that not only are the kinetics of phosphorylation of these two serine residues different, but more importantly, phosphorylation of serine 23 alone probably does not seem to affect cardiac muscle contractility. Second, CTnI isolated from cardiac muscle always contains different levels of endogenous phosphate, from 0.5 to 1.5 mol of phosphate/mole of protein (16). These differences, as pointed out by Swiderek (14), are probably due to the different methods used to measure the absolute content of phosphate. The interesting point here is that upon \(\beta\)-agonist stimulation, the net increase in the phosphate of CTnI is \(-1\) mol of phosphate/mole of protein (16). Concerning the fact that Ser-23 is phosphorylated even in the resting state, it is reasonable to assume that the net increase of phosphate after \(\beta\)-agonist stimulation is mostly added on the first serine or Ser-22 in the mouse. Thus Ser-23 appears to be constitutively phosphorylated, with the phosphate of Ser-22 being functionally more important. This also suggests that the rate of dephosphorylation of these two serines is probably different and may account for some of the observations in in vivo studies (4, 19). Third, using \(^{31P}\) NMR spectroscopy, Jaquet et al., (33) found that a new \(^{31P}\) NMR signal appeared only when both Ser-23 and Ser-24 of bovine CTnI (equivalent to Ser-22 and Ser-24 in mouse CTnI) were phosphorylated in a complex formed with TnT and TnC. Their result suggests that these two phosphorylated serine residues produce a specific interaction within the troponin complex, possibly causing Ca\textsuperscript{2+} to dissociate faster from CTCn. The two monophospho-forms of CTnI did not show any changes in this specific subunit interaction.

All of these results suggest that partially phosphorylated CTnI has little or no effect on the Ca\textsuperscript{2+} dependence of force development and therefore on cardiac contractility. Only the doubly phosphorylated form of CTnI appears to contribute to these processes. In combining these results with our phosphorylation data on wild type CTnI and its mutants, we hypothesize that this type of ordered phosphorylation may indeed occur in native CTnI with the major change in phosphorylation occurring on serine 22.

These results raise the question as to what the physiological significance of the existence of the two adjacent serine residues in CTnI is and how the phosphorylation of these two serines affects Ca\textsuperscript{2+} binding to CTnC. At this point, we do not have a satisfactory answer. The existing sequence data shows that the existence of the two adjacent serine residues on CTnI is shared by avian, and many other mammalian species, implying the possible physiological necessity of the existence of these two serine residues. The effect of CTnI phosphorylation on the Ca\textsuperscript{2+} affinity of TnC is achieved most likely through a change in the interaction between CTnI and CTnC, which probably requires phosphorylation of both serine residues. Sheng et al. (29) have demonstrated that the N-terminal domain of skeletal TnI interacts with TnC in a Ca\textsuperscript{2+}-Mg\textsuperscript{2+} site-dependent manner, and this interaction serves to maintain the structure of troponin complex. Recent NMR studies on the spatial relationships within the CTnI-CTnC complex demonstrated that CTnI and CTnC also form an antiparallel arrangement similar to skeletal TnI (34, 35). Since the two adjacent serines are close to the TnC binding domain in the N-terminal portion CTnI, the phosphate introduced by PKA possibly influences the interaction between the N terminus of CTnI and the C terminus of CTnC. This structural change may also affect the Ca\textsuperscript{2+}-dependent interaction between the N-terminal region of CTnC and CTnI. The monophospho form of CTnI may play only a transitory role. Further studies on the interaction between CTnI and CTnC using molecular modeling and their structural determination will help to define these interactions.

In summary, our current view of the mechanism by which CTnI phosphorylation controls the increased rate of muscle relaxation is that: 1) in the resting state, CTnI is partially phosphorylated, mostly at the second serine; 2) when PKA is activated by the \(\beta\)-agonist pathway, PKA further phosphorylates TnI, mostly at the first serine; 3) the Ca\textsuperscript{2+} sensitivity of muscle contraction decreases when both serines on CTnI are phosphorylated by PKA, allowing Ca\textsuperscript{2+} to dissociate faster from the single Ca\textsuperscript{2+}-specific regulatory site of CTnC; 4) after removal of \(\beta\)-agonists, CTnI is dephosphorylated, primarily at the first serine.

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Phosphorylation of Both Serine Residues in Cardiac Troponin I Is Required to Decrease the Ca$^{2+}$ Affinity of Cardiac Troponin C

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