Cardiac Troponin I Mutants

PHOSPHORYLATION BY PROTEIN KINASES C AND A AND REGULATION OF Ca2+-STIMULATED MgATPase OF RECONSTITUTED ACTOMYOSIN S-1

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The significance of site-specific phosphorylation of cardiac troponin I (Tni) by protein kinase C and protein kinase A in the regulation of Ca2+-stimulated MgATPase of reconstituted actomyosin S-1 was investigated. The Tni mutants used were T144A, S43A/S45A, and S43A/S45A/T144A (in which the identified protein kinase C phosphorylation sites, Thr-144 and Ser-43/Ser-45, were, respectively, substituted by Ala) and S23A/S24A and N32 (in which the protein kinase A phosphorylation sites Ser-23/Ser-24 were either substituted by Ala or deleted). The mutations caused subtle changes in the kinetics of phosphorylation by protein kinase C, and all mutants were maximally phosphorylated to various extents (1.3-2.7 mol of phosphate/mol of protein). Protein kinase C could cross-phosphorylate protein kinase A sites but the reverse essentially could not occur. Compared to wild-type Tni and T144A, unphosphorylated S43A/S45A, S43A/S45A/T144, S23A/S24A, and N32 caused a decreased Ca2+-sensitivity of Ca2+-stimulated MgATPase of reconstituted actomyosin S-1. Phosphorylation by protein kinase C of wild-type and all mutants except S43A/S45A and S43A/S45A/T144 caused marked reductions in the maximal activity of Ca2+-stimulated MgATPase and apparent affinity of myosin S-1 for reconstituted (regulated) actin. It was further noted that protein kinase C acted in an additive manner with protein kinase A by phosphorylating Ser-23/Ser-24 to bring about a decreased Ca2+-sensitivity of the myofilament.

It is suggested that Ser-43/Ser-45 and Ser-23/Ser-24 in cardiac Tni are important for normal Ca2+-sensitivity of the myofilament, and that phosphorylation of Ser-43/Ser-45 and Ser-23/Ser-24 is primarily involved in the protein kinase C regulation of the activity and Ca2+-sensitivity, respectively, of actomyosin S-1 MgATPase.

In cardiac myocytes, the activation of several types of receptors, such as α1-adrenergic (1-5), muscarinic (1-6), and purinergic (6) dynorphin A (7), endothelin-1 (8, 9), and angiotensin II (10-12) receptors, stimulates the hydrolysis of membrane phosphoinositides leading to the generation of two classes of second messengers, diacylglycerol and inositol trisphosphate. In many tissues diacylglycerol directly activates both the conventional Ca2+-dependent group of PKC isozymes (α, β1, β11, and γ) and the novel Ca2+-independent group of PKC isozymes (δ, ε, η, and θ), whereas inositol trisphosphate, by increasing intracellular Ca2+, indirectly activates Ca2+-independent PKC isozymes (for a review, see Ref. 13). It is worth noting that PKC-δ and PKC-ε, atypical members of the Ca2+-independent group, are activated by neither diacylglycerol nor Ca2+ (13). Several lines of recent evidence indicate involvement of PKC in cardiac function and development (14, 15) as well as differential expression of the PKC isozymes in cardiac myocytes and tissue (14-18). However, the complex molecular events mediated by PKC (or more precisely, by the individual isozymes) that are responsible for cardiac contractility regulation, for example, remain largely unclear. It has been reported that phenylephrine (α1-adrenergic receptor agonist) elicited transient negative inotropy followed by sustained positive inotropy (3, 19-21), endothelin-1 caused monotonic positive inotropy (22), whereas dynorphin A (κ-opioid receptor agonist) induced negative inotropy (7). All three of these distinct receptor agonists are believed to act, at least in part, through PKC activation. Furthermore, phorbol esters (such as TPA), potent and long-acting PKC activators, produced predominantly negative inotropic effects in various cardiac preparations (23-27). These seemingly paradoxical observations might reflect certain opposing factors of PKC activation which include the net effects of intracellular pH change, the size of intracellular Ca2+ transient, and the states and species of cellular proteins being phosphorylated.

One target for PKC in the heart is the contractile apparatus itself. Cardiac Tni and TnT from the thin filament have been shown to be effective substrates for PKC (28), and some of the phosphorylation sites in these proteins have been determined (29, 30). Phosphorylation of Tni and/or TnT by PKC resulted in an inhibition of Ca2+-stimulated MgATPase of the reconstituted actomyosin complex (31-33) or in native myofilibr preparations (32, 34), an effect associated with altered interactions among the contractile protein components (32, 33). PKC also phosphorylated MLC2 (34-36) and C-protein (34-36) in myofibrillar and thin filament preparations. Phosphorylation of

1 The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbal-13-acetate; Tn, troponin; TnI, troponin I; N32, cardiac Tni mutant with a deletion of the first 32 amino acid residues from the NH2 terminus; TnT, troponin T; TnC, troponin C; Tm, tropomyosin; MLC, myosin light chain; PKA, protein kinase A; PCR, polymerase chain reaction; DTT, dithiothreitol; ET-18-OCH3, 1-O- octadecyl-2,6-dimethyl glycerol-3-phosphate; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; MBP, myelin basic protein.
MLC2 by PKC or MLCK kinase has been shown to cause further activation of Ca$^{2+}$-stimulated MgATPase of thick filament-substituted myofibrils (36). Because the overall effect of PKC phosphorylation of myofibrils (which caused phosphorylation of Tnl, TnT, C-protein, and MLC2) was predominantly an inhibition of Ca$^{2+}$-stimulated MgATPase, it was suggested that the inhibitory effect of Tnl/TnT phosphorylation could override the stimulatory effect of MLC2 phosphorylation (35, 36). Therefore, it appears that the actual activity (inhibition or activation) of Ca$^{2+}$-stimulated myofibrillar MgATPase could be regulated by the relative phosphorylation states of these proteins (i.e. Tnl/TnT versus MLC2). The biological significance of PKC phosphorylation of contractile proteins has been substantiated by the findings that in vitro phosphorylation studies were found to be the same as those in situ as determined by using living cardiomyocytes incubated with TPA or $\alpha_2$-adrenergic agonist (34, 35).

Previously, we demonstrated that PKC phosphorylated bovine cardiac TnI at Ser-43/Ser-45 and that PKC phosphorylated bovine cardiac TnI at Ser-23/Ser-24 (40, 41) suggested that the NH2-terminal domain surrounding Ser-43/Ser-45 (of the cardiac sequence) functions to anchor TnI to TnC and other components of the thin filament and thus represents an important site for possible regulation by phosphorylation.

**EXPERIMENTAL PROCEDURES**

Materials—Phosphatidylinositol, sn-1,2-dioleoyl, and TPA were purchased from Sigma; [gamma-32P]ATP was from Amersham Corp., ET-18-OCH$_3$ was from Calbiochem; fresh bovine hearts and porcine brains containing 6M urea, 1 mM EDTA, and 15 mM 2-mercaptoethanol. Tm was prepared by the method of Stull and Buss (45) and F-actin by the procedures of Pardee and Spudich (46). Myosin and its S-1 fragment were prepared according to the method of Siemankowski and White (47). In order to prevent oxidation of Tnl and Tm, 1.0 mM DTT or 15 mM 2-mercaptoethanol were added to all solutions throughout the purification and reconstitution procedures. Expression and purification of contractile proteins were determined using molecular weights and extinction coefficients provided by Tobacman (48).

Construction of the Plasmids Expressing Cardiac Wild-type Tnl and Mutants—Mouse cardiac wild-type Tnl was done by reverse transcriptase-PCR as described elsewhere (39). Construction of the T144A, S43A/S45A, and S23A/S24A mutants of mouse cardiac Tnl was based on the methodology of recombination PCR described by Higuchi et al. (49). The DNA template for recombinant PCR reactions was the mouse wild-type cardiac Tnl cDNA done into the pET-3d vector at NcoI and BamHI sites (39). The two "inside" primers which direct Thr-144 to Ala-144 were 5' TTAAGGCGGCGGTCTCTCGAGAG-3' and 5' CTCTTGGAGAGGGCGCCGGCTTTA-3'. The two "inside" primers converting Ser-43 and Ser-45 to Ala-43 and Ala-45 were 5' GAAACTCTAGTTG-3' and 5' CAACCTGAAGTTTCTTGCGGCCG-3', respectively. The proteins were gel-purified and cloned into pET-3d vector at NcoI and BamHI sites. Several days (for DNA selection) were selected by sequencing analysis, and one clone from each construct was used to transform BL21(DE3) (Novagen) for expression. To construct the S43A/S45A/T144A triple mutation, clone S43A/S45A was digested by NcoI and PstI (a unique site in the gene) restriction enzymes and the T144A clone was digested by PstI and BamHI. The NcoI/PstI fragment of S43A/S45A and the PstI/BamHI fragment of T144A were gel-purified and ligated into the NcoI/PstI-digested pET-3d vector. The resulting clone containing the triple mutation was analyzed by nucleotide sequencing and was used to transform BL21(DE3) for expression. The N32 (formerly referred to as Tnl/NH2) mutant of rat cardiac Tnl, in which the first 32 amino acid residues in the NH2 ter选用的部分。
reaction mixtures (2.0 ml) contained 0.4 mM nonradioactive ATP and 6.0 μM TnI. Reactions were initiated by addition of ATP, carried out for up to 3–4 h at 30 °C for maximal phosphorylation, and terminated by addition of trichloroacetic acid (10 M) containing 10 mM EDTA, 150 mM NaF, and 1.0 mM DTT. To determine the extent of phosphorylation or for thin film sedimentation analysis (29), parallel reaction mixtures (2.0 ml) contained 0.4 mM [32P]ATP (4–5 × 10^6 cpm). The 32P-TnI was separated using 6–15% linear gradient SDS-polyacrylamide gels and used within 48 h.

**Reconstitution of Tn and Thin Filament—Reconstituted Tn (30)** was prepared by mixing (in 10 mM imidazole-HCl, pH 7.0, containing 6 mM urea, 1.0 mM CaCl2, and 1.0 mM DTT) cardiac TnT and TnC and phosphorylated or unphosphorylated TnI at a molar ratio of 1:1:1. The mixture was then dialyzed (48 h, 4 °C) against 10 mM imidazole-HCl (pH 7.0) containing 1.0 mM magnesium acetate, and 1.0 mM DTT with 1.0 M KCl to give the calculated free Ca2+ concentrations of 0.01–100 M. The reaction mixtures (see below) without ATP. The Ca2+–stimulated MgATPase activity was assayed at 30 °C for 10 min in reaction mixtures (0.5 ml) containing actomyosin S-1 (0.3–0.4 μM), 10 mM MOPS-KOH (pH 7.0 or 6.5), 4.1 mM magnesium acetate, 2.1 mM CaCl2 (the free Ca2+ concentration was calculated to be 40 μM), CaCl2, when present, was added as a mixture with EDTA and MOPS-KOH to give the calculated free Ca2+ concentrations of 0.01–100 μM in the reaction mixtures. Appropriate volumes of 5 M KOH were added to adjust the pH. CaCl2 was added to bring the final ionic strength to 18 mM. The reactions were initiated by addition of [γ-32P]ATP and terminated by addition of 10% charcoal suspension, and the released 32P, as determined as described previously (31, 32). Stabilizing constants used in the calculation of the total concentrations of reaction constituents required to give the necessary free Ca2+ concentrations were determined using the procedures of Fabiato and Fabiato (54) as described previously (31). MgATPase activity, reported as mol of P/mol of S-1/s (s−1), was linear as a function of time or amount of reconstituted actomyosin S-1 under the assay conditions. Kinetic data for Ca2+ stimulation of actomyosin S-1 MgATPase (as a function of Ca2+ concentration) were calculated by nonlinear least-squares regression analysis using a modified form of the A. V. Hill equation as described previously (31, 32, 36). Similarly, the kinetic data for Ca2+-stimulated actomyosin S-1 activity, as a function of thin film concentration, were analyzed using the A. V. Hill equation where K_Hill is defined as the concentration of thin film producing 50% maximal Ca2+-stimulated MgATPase activity, and Vmax is the estimated maximal thin film-stimulated activity. The binding of bovine cardiac myosin S-1 (in the presence of ATP) to reconstituted thin films containing various TnI preparations was assessed, as described previously (32), by separating free S-1 from actin-bound S-1 using the ultracentrifugation techniques described by Chalovich and Eisenberg (55), as modified by Tobacman and Adelstein (56).

**RESULTS**

The abilities of recombinant mouse cardiac wild-type TnI and various mutants, in which the identified phosphorylation sites for PKC and PKA were either substituted or deleted, compared to native bovine cardiac TnI, MBP, and histone H1, to serve as substrates for PKC and PKA were examined, and the kinetic constants are summarized (Table I). It was found that the mutations caused subtle changes in the substrate activities (indicated by the Vmax/Km ratios) of the resulting proteins for PKC, i.e. in a decreasing order, wild-type TnI > T144A > N32 > S43A/S45A > S43A/S45A/T144A > S23A/S24A > MBP. The mutations on PKC phosphorylation sites, in contrast, caused little or no change in substrate activities for PKA, i.e. wild-type > T144A = S43A/S45A ~ S43A/S45A/T144A ~ S23A/S24A. As expected, mutants S23A/S24A and N32, in which PKA phosphorylation sites were, respectively, substituted and deleted, were not significantly phosphorylated by PKA, indicating that PKA was essentially unable to phosphorylate the PKC sites. Bovine TnI was an inferior substrate, compared to mouse wild-type TnI, for both PKC and PKA. Although MBP and histone H1 were effective substrates for PKC in the presence of 0.3 mM KCl (which was required for keeping TnI preparations in the soluble form), they were not appreciably phosphorylated by PKA under the same conditions. Because MBP and histone H1 were effectively phosphorylated by PKA in the absence of 0.3 mM KCl (albeit lower than by PKC), it seemed that PKA was more sensitive to salt inhibition than PKC.

The time-dependent phosphorylation of TnI preparations and MBP by PKC indicated that the initial reaction rates for these proteins were somewhat different, with S23A/S24A being the least effective substrate (Fig. 1). If the reactions were carried out for an extended time (3 h) with high...
amounts of PKC and ATP, higher phosphorylation extents of 1.3–2.7 mol of phosphate/mol of protein were obtained for all TnI preparations (Fig. 1). The initial phosphorylation rate by PKA was found to be similar for wild-type, S43A/S45A and T144A, but was slightly higher for S43A/S45A/T144A (Fig. 2). For these TnI preparations, phosphorylation of about 0.8–1.4 mol of phosphate/mol of protein was obtained after prolonged incubation with high amounts of PKA and ATP. As expected, S23A/S24A and N32 were not appreciably phosphorylated by PKA.

Two-dimensional tryptic peptide maps of various TnI preparations phosphorylated by PKC (Fig. 3) and PKA (Fig. 4) were examined. A pattern of five 32P-phosphopeptides was readily detected for bovine TnI phosphorylated by PKC (Fig. 3). We previously determined, using bovine cardiac TnI, that the phosphorylated residues were Ser-78 in peptide 1, Ser-43/Ser-45 in peptide 2, and Thr-144 in peptide 3 (29). Our previous evidence also suggested that peptide 5 contains phosphorylated Ser-23/Ser-24 (34). The identity of the phosphorylated residue in peptide 4 is unknown. A similar peptide map was obtained for mouse wild-type TnI phosphorylated by PKC. Phosphopeptide 1 was absent from maps of mouse TnI preparations because Ser-78 in the bovine and rat sequences (50) is replaced by a nonphosphorylatable Arg residue in the mouse sequence (39).

Substitutions of Ala for the phosphorylatable residues in the mouse TnI mutants T144A, S43A/S45A, and S43A/S45A/T144A were confirmed by the absence of corresponding phosphopeptides in their tryptic peptide maps (Fig. 3). For the mouse TnI mutant S23A/S24A, phosphopeptide 5 was absent while phosphorylation at Ser-43/Ser-45 (peptide 2) was predominant. Similarly, peptide 5 was absent while phosphopeptides 1, 2, 3, and 4 were present of the rat TnI mutant N32. Rat cardiac TnI (50) has Val at position 76 instead of Ala in the bovine sequence and thus the N32 mutant would produce a tryptic phosphopeptide (containing Ser-78) with a different mobility than that from bovine TnI (Fig. 3). In comparison to PKC, PKA almost exclusively and preferentially phosphorylated Ser-23/Ser-24 in all TnI preparations tested, as confirmed by the lack of phosphopeptide 5 in maps for N32 (Fig. 4) and S23A/S24A (data not shown). A very minor phosphorylation at Thr-144 (phosphopeptide 3) by PKA was also noted in all TnI preparations containing that amino acid residue (Fig. 4).

We reported previously that PKC phosphorylation of bovine cardiac TnI at multiple sites resulted in a reduced maximal Ca\(^{2+}\)-stimulated MgATPase activity of reconstituted actomyosin and actomyosin S-1 (31). The TnI preparations (6 μM) were exhaustively phosphorylated by PKC for 3 h and the phosphorylation values (moles of phosphate/mol of protein) are shown in parentheses. The findings were confirmed, in entirety or in part, in three to six other experiments. See “Experimental Procedures” for further details.

We therefore examined the MgATPase activity of reconstituted actomyosin S-1 at pH 6.5 in addition to the standard pH 7.0 (Fig. 5), and the kinetic constants are summarized (Table II). Phosphorylation by PKC of wild-type resulted in 62 and 85% reductions in the maximal Ca\(^{2+}\) stimulation when the MgATPase activity was assayed at pH 7.0 and 6.5, respectively.
Similarly, PKC-phosphorylated T144A caused 78 and 80% reductions of the corresponding values, whereas phosphorylation of S43A/S45A led to much smaller reductions of 25 and 24%, suggesting that phosphorylation at Ser-43/Ser-45, but not Thr-144, was primarily responsible for the reduced Ca\(^{2+}\)-stimulated activity. Phosphorylation of the S43A/S45A/T144A produced intermediate reductions, i.e. 34 and 48% at pH 7.0 and 6.5. As with Thr-144, phosphorylation at Ser-23/Ser-24 (the PKA-preferred sites) could not account for the reduced maximal Ca\(^{2+}\)-stimulated activity, because PKC phosphorylation of S23A/S24A and N32 still resulted in marked reductions (51–144, was primarily responsible for the reduced Ca\(^{2+}\) sensitivity of this enzyme. PKC phosphorylation at Ser-23/Ser-24, resulted in a 2.9-fold increase in the EC\(_{50}\) for Ca\(^{2+}\) compared to that reported previously (31), which was less than that for phosphorylated mouse wild-type TnI (Table II).

The mutations at the phosphorylation sites also affected the Ca\(^{2+}\) sensitivity of the actomyosin MgATPase (Fig. 5 and Table II). All of the reconstituted actomyosin S-1 preparations, containing unphosphorylated TnI mutants as well as wild-type or bovine TnI, exhibited decreased Ca\(^{2+}\) sensitivity at pH 6.5 compared to pH 7.0, although the extent of the decrease varied somewhat among the TnI preparations. However, when assayed at pH 7.0, higher EC\(_{50}\) values for Ca\(^{2+}\) (i.e. decreased Ca\(^{2+}\) sensitivity) of 1.6–2.7 \(\mu\)M were noted for reconstituted actomyosin S-1 preparations containing unphosphorylated S43A/S45A, S43A/S45A/T144A, S23A/S24A, and N32, but not T144A (1.1 \(\mu\)M), compared to mouse wild-type (1.2 \(\mu\)M) and bovine TnI (1.1 \(\mu\)M). Qualitatively similar differences in Ca\(^{2+}\) sensitivity were also observed when the preparations were assayed at pH 6.5. The findings suggested the importance of Ser-23/Ser-24 and Ser-43/Ser-45 in the regulation of the thin filament Ca\(^{2+}\) sensitivity. PKC phosphorylation of wild-type, T144A, and bovine TnI caused up to 3-fold decreases in Ca\(^{2+}\) sensitivity at pH 7.0 and 6.5, but the effect was less pronounced or even undetectable for other TnI mutants, particularly N32 and S43A/S45A, further supporting the importance of Ser-23/Ser-24 and Ser-43/Ser-45 in Ca\(^{2+}\) sensitivity. No apparent differences were observed in the basal MgATPase activities (in the absence of added Ca\(^{2+}\)) among the actomyosin S-1 preparations containing the reconstituting TnI proteins, whether phosphorylated or unphosphorylated (data not shown).

Because PKC cross-phosphorylated PKA sites (Ser-23/Ser-24) in TnI (Fig. 3) and phosphorylation at these sites led to a decreased Ca\(^{2+}\) sensitivity of MgATPase (Fig. 5 and Table II), we directly compared the effects of PKC and/or PKA phosphorylation of wild-type TnI (Fig. 6). The kinetic data are summarized (Table III). A short (15-min) exposure of TnI to PKC caused the incorporation of 1.1 mol of phosphate/mol of TnI and phosphorylation of all PKC sites, but only minor phosphorylation of PKA sites Ser-23/Ser-24 (phosphopeptide 5) was noted. This short exposure to PKC produced a 32% decrease in maximal Ca\(^{2+}\) stimulation and a 2.2-fold increase in the EC\(_{50}\) for Ca\(^{2+}\). In contrast, a brief exposure of TnI to PKA, which allowed exclusive phosphorylation (1.3 mol of phosphate/mol of protein) at Ser-23/Ser-24, resulted in a 2.9-fold increase in the EC\(_{50}\) for Ca\(^{2+}\) without appreciably affecting the maximal Ca\(^{2+}\) activation. When all sites were phosphorylated (2.3 mol of phosphate/mol of TnI) due to a short exposure to both PKA and PKC, there was 55% reduction in this activation, accompanied by a 3.9-fold increase in the EC\(_{50}\) for Ca\(^{2+}\). The combined effects of the two enzymes were similar to those produced by a long (2-h) incubation with PKC, yielding phosphorylation (2.3 mol of phosphate/mol of TnI) at all sites, a 56% reduction of the maximal Ca\(^{2+}\)-stimulated activity, and 6-fold increase in the EC\(_{50}\) for Ca\(^{2+}\). These findings are consistent with the idea that PKC decreased Ca\(^{2+}\) sensitivity by cross-phosphorylating the typical PKA sites (Ser-23/Ser-24) or by phosphorylating certain PKC sites. Moreover, the actions of the two enzymes could be additive under certain conditions.
The results from the above experiments (Figs. 5 and 6) suggested that PKC phosphorylation of TnI affected its interactions with other components of the thin filament, preventing full Ca\(^{2+}\)-dependent activation of the complex. We were also interested in determining if phosphorylation altered interactions of the thick and thin filaments. Therefore, we next examined the concentration-dependent effects of the thin filament on the Ca\(^{2+}\)-stimulated MgATPase activity of myosin S-1 (Fig. 7). The kinetic data are summarized (Table IV). PKC phosphorylation of all of the TnI preparations, except S43A/S45A and S43A/S45A/T144A, markedly increased (up to 4-fold) the \(K_{\text{app}}\) (i.e. decreased apparent affinity) for the thin filament. Phosphorylation of all of the TnI preparations except N32 and S43A/S45A also caused a reduction in the \(V_{\text{max}}\) with T144A and S23A/S24A providing the greater (55 and 43%, respectively) reductions compared to other TnI preparations (2-33%). This evidence suggested that, while phosphorylation at Ser-43/Ser-45 was primarily responsible for the majority of the apparent decreased \(V_{\text{max}}\) and affinity of myosin S1 for regulated actin, phosphorylation of TnI at other sites (excluding Thr-144) may also affect these parameters. We also noted that the \(K_{\text{app}}\) of myosin S-1 for thin filaments containing either unphosphorylated S43A/S45A, S43A/S45A/T144A, S23A/S24A, or N32 was greater than that containing wild-type or T144A. These findings suggested that substitution of Ser-43/45 or Ser-23/Ser-24 by Ala residues or deletion of the NH\(_2\)-terminal sequence containing Ser-23/24 caused a decreased affinity of S-1 for the thin filament. In agreement with the observations of Tobacman and Adelstein (56), we also observed that, for all preparations of regulated actin studied, the addition of Ca\(^{2+}\) altered primarily the \(V_{\text{max}}\) of the MgATPase activity and not the \(K_{\text{app}}\) of myosin S-1 for regulated actin (data not shown). In additional experiments, we found that the binding of 0.3 \(\mu M\) myosin S-1 to 20 \(\mu M\) regulated actin, in the presence of 2.1 \(mM\) ATP, was reduced 26-30% in preparations containing PKC-phosphorylated bovine TnI, wild-type TnI, and T144A, compared to a 10% reduction for the phosphorylated S43A/S45A (data not shown). These findings underscored the importance of phosphorylation at Ser-43/Ser-45 in regulating the interactions of TnI with other components of the thin filament and ultimately interactions of the thick and thin filaments.

### DISCUSSION

Point mutations of PKC and PKA phosphorylation sites and a deletion of PKA phosphorylation sites in cardiac TnI did not bring about gross changes in the general properties of the mutants. The mutants retained their abilities to reconstitute into functional actomyosin S-1, which demonstrated typical activation by Ca\(^{2+}\) and pH-dependent Ca\(^{2+}\)-sensitivity of MgATPase. Certain observations concerning phosphorylation of the mutants by PKC seemed noteworthy. Although mutations altered phosphorylation kinetics as expected, the extents of phosphorylation that we observed for the mutants were quite surprising. If one assumed Ser-23/Ser-24, Ser-43/Ser-45, Thr-144, and the unidentified site in phosphopeptide 4 in mouse wild-type TnC could be exhaustively phosphorylated, a stoichiometry of six could be expected. However, we failed to obtain a maximal value that was higher than three. On the other hand, a stoichiometry similar to that for wild-type was noted for the triple mutant S43A/S45A/T144A (eg. Fig. 1). These findings seemed to support a hypothesis that phosphorylation of multi-
ple sites in TnI might be mutually regulating. It is conceivable, therefore, that phosphorylation of certain sites in wild-type could negatively regulate or even restrict further phosphorylation of other sites, resulting in phosphorylation that was much lower than the theoretical value. In contrast, absence of preferred sites such as in mutant S43A/S45A/T144A could lead to an extensive and rapid cross-phosphorylation of the PKA sites (Ser-23/Ser-24) (eg. Fig. 3), resulting in phosphorylation that was higher than expected. This “plasticity” concerning multisite phosphorylation might be of special significance, in light of the complex roles played by TnI (and perhaps TnT as well) in the regulation of contractile activity.

The PKC phosphorylation sites in cardiac TnI are located in specific functional domains of the protein. Studies on fast skeletal muscle TnI have indicated that two functional domains exist in the protein (40, 41, 59–71). The NH₂-terminal domain of TnI binds strongly to the COOH-terminal domain of TnC and serves to anchor TnI to the other components of the Tn complex. The COOH-terminal half of TnI contains a region, residues 96–116, corresponding to residues 130–150 in the bovine and mouse cardiac sequences (39, 50), that can bind to Tm-actin and inhibit the actomyosin MgATPase activity, as well as bind to TnC (40, 41, 59–71). Talbot and Hodges (61) identified the minimal “inhibitory” sequence within this region to be comprised of residues 105–114, corresponding to residues 139–148 in the bovine cardiac sequence (50). In the absence of Ca²⁺, this inhibitory domain of TnI remains bound to Tm-actin. When Ca²⁺ binds to TnC, a conformational change in the Tn complex occurs causing the inhibitory region, along with the remainder of the COOH-terminal region of TnI, to bind to certain sites in the NH₂- and COOH-terminal domains of TnC, leading to removal of inhibition by TnI (40, 41, 67). Within this inhibitory sequence, cardiac TnI has Thr at position 144 in place of Pro in the skeletal muscle sequences. Using synthetic peptides corresponding to residues 104–115 of the skeletal muscle sequence and residues 138–149 of the cardiac sequence, Talbot and Hodges (62) showed that Pro or Thr substitutions had no effect on the inhibitory activity of the peptides. In contrast, Van Eyk and Hodges (64) demonstrated that replacement of these polar residues with the neutral amino acid Gly caused a 38% loss of inhibitory activity for the synthetic peptide. We, however, mutated this site in cardiac TnI to Ala (T144A) and found little or no effects on MgATPase activity. Because the inhibitory region of TnI contains several basic amino acids, we suspected that adding a negatively charged phosphate group on Thr-144 would affect TnI-thin filament interactions sufficiently to account for the PKC-mediated inhibition of the Ca²⁺-stimulated MgATPase activity. This, however, was not the case in that a full inhibitory effect due to phosphorylation by PKC was retained with the T144A mutant. Because none of our mutants could be phosphorylated only at Thr-144, we were not able to ascertain the direct effects of phosphorylation at this site.

Cardiac TnI differs from fast and slow skeletal muscle TnI primarily in the NH₂-terminal region. The presence of a cardiac-specific extension of 24–36 amino acids at the NH₂-terminus suggests that an additional functional domain exists in cardiac TnI (50, 72). However, deletion of this region (the N32 mutant) conferred little or no changes to the inhibition by TnI of actomyosin MgATPase activity or to overall regulation by Ca²⁺ or pH of myofilament function (39) (Table II). Reconstituted actomyosin S-1, containing the unphosphorylated S23A/S24A or N32 mutants did, however, display decreased Ca²⁺ sensitivity (Fig. 5, Table II), suggesting that the serine residues may be

### Table III

| Phosphorylation conditions and extent | Maximal Ca²⁺-stimulated activity | EC₅₀ for Ca²⁺ | Hill coefficient |
|-------------------------------------|----------------------------------|---------------|-----------------|
| Short (15 min) incubation            |                                  |               |                 |
| Unphosphorylated                     | 1.47 ± 0.05                      | 1.3 ± 0.1     | 1.4 ± 0.2       |
| + PKC (1.1)                         | 1.00 ± 0.04                      | 2.8 ± 0.3     | 1.5 ± 0.2       |
| + PKA (1.3)                         | 1.37 ± 0.09                      | 3.7 ± 0.7     | 1.3 ± 0.3       |
| + PKC + PKA (2.3)                   | 0.67 ± 0.02                      | 5.0 ± 0.4     | 1.6 ± 0.1       |
| Long (2 h) incubation               |                                  |               |                 |
| Unphosphorylated                     | 1.35 ± 0.03                      | 1.2 ± 0.1     | 1.6 ± 0.2       |
| + PKC (2.3)                         | 0.59 ± 0.03                      | 7.2 ± 1.0     | 1.1 ± 0.1       |

* Molar phosphate/mol of protein.
important for normal Ca\(^{2+}\) sensitivity of the actomyosin complex. A similar effect was apparent when wild-type TnI was replaced with the N32 mutant in cardiac myofibril preparations (39). Furthermore, phosphorylation by PKA of Ser-23/Ser-24 produced a decreased Ca\(^{2+}\) sensitivity of reconstituted actomyosin and native myofibrils (34, 37, 38) (Fig. 6). These results suggest that phosphorylation of Ser-23/Ser-24 alters the interaction of the NH\(_2\)-terminal extension with other functional domains of TnI which regulate the TnI-TnC or TnI-actin interactions. Although PKA is considered the primary protein kinase for phosphorylation at these Ser residues, our studies indicated that PKC can cross-phosphorylate Ser-23/Ser-24 and decrease the Ca\(^{2+}\) sensitivity of the resulting reconstituted actomyosin complex (Figs. 5 and 6, Tables II and III). Since both PKA and PKC can phosphorylate TnI at Ser-23/Ser-24, it was suggested that PKC, in addition to PKA, could regulate the Ca\(^{2+}\) sensitivity of the myofilaments. Simply put, PKA appeared to regulate primarily the Ca\(^{2+}\) sensitivity, whereas PKC modulated both the Ca\(^{2+}\) sensitivity and activity of the myofilament MgATPase. It appeared that PKC phosphorylated more readily Ser-23/Ser-24 (phosphopeptide 5) in mouse TnI than in bovine TnI (Fig. 3). This difference may account for the more pronounced decrease in Ca\(^{2+}\) sensitivity of MgATPase of actomyosin-containing mouse TnI, compared to bovine TnI, phosphorylated by PKC when the enzyme was assayed at pH 7.0 (Table II), and our earlier findings that PKC phosphorylation of bovine TnI resulted primarily in inhibition of the activity without consistently affecting the Ca\(^{2+}\) sensitivity of actomyosin MgATPase (31–33).

As suggested from studies on skeletal muscle TnI (40–41, 59, 65–69), Ser-43/Ser-45 are located in the "anchor" region of cardiac TnI, residues 33–80 (50), that binds to TnC. Furthermore, these residues are located in the highly basic segment comprising residues 37–51 (50) that probably directly interacts with the acidic protein TnC (59, 68, 69). The NH\(_2\)-terminal region of skeletal muscle TnI also interacts with TnT (70, 71), implying that this region of TnI may not be accessible to PKC. However, by using reconstituted TnI, we demonstrated that these Ser residues were phosphorylated by PKC, even when TnI was complexed with TnC (29). Moreover, analysis of tryptic phosphopeptide maps of TnI from \(^{32}\)P-labeled rat cardiac myocytes treated with phorbol ester or phenylephrine revealed the presence of a phosphopeptide corresponding to that containing Ser-43/Ser-45 (34). These results suggested that Ser-43/Ser-45 could be phosphorylated in intact myocytes upon activation of PKC. Due to the location of these amino acids, it was not surprising to find that phosphorylation at Ser-43/Ser-45 was responsible for the major portion of the PKC-mediated inhibition of the maximal Ca\(^{2+}\)-stimulated MgATPase activity of reconstituted actomyosin S-1 complex (Figs. 5 and 6, Tables III and IV). Furthermore, simple substitution of these Ser residues with

| TnI preparation | Phosphorylation | \(V_{\text{max}}\) \(\text{M}\) | \(K_{\text{app}}\) for regulated actin \(\mu\text{M}\) | Hill coefficient |
|----------------|----------------|-----------------|-----------------|----------------|
| Wild-type TnI  | –              | 4.6 ± 0.1        | 7.5 ± 0.3        | 1.5 ± 0.1      |
|                | +              | 3.1 ± 0.4        | 22.0 ± 6.3       | 1.3 ± 0.2      |
| T144A          | –              | 4.7 ± 0.1        | 8.0 ± 0.2        | 1.6 ± 0.1      |
|                | +              | 2.1 ± 0.4        | 22.0 ± 7.7       | 1.4 ± 0.3      |
| S43A/S45A      | –              | 4.4 ± 0.2        | 12.0 ± 2.6       | 1.3 ± 0.2      |
|                | +              | 4.3 ± 0.3        | 16.0 ± 2.0       | 1.4 ± 0.1      |
| S43A/S45A/T144A| –              | 5.1 ± 0.4        | 20.0 ± 3.0       | 1.4 ± 0.1      |
|                | +              | 3.6 ± 0.4        | 20.0 ± 3.0       | 1.8 ± 0.3      |
| S23A/S24A      | –              | 4.7 ± 0.3        | 15.7 ± 2.0       | 1.4 ± 0.2      |
|                | +              | 2.7 ± 0.5        | 26.0 ± 4.0       | 1.0 ± 0.2      |
| N32            | –              | 5.0 ± 0.1        | 12.3 ± 0.4       | 1.8 ± 0.1      |
|                | +              | 4.7 ± 0.3        | 27.6 ± 2.7       | 1.4 ± 0.1      |
| Bovine TnI     | –              | 4.1 ± 0.2        | 7.6 ± 0.8        | 1.6 ± 0.3      |
|                | +              | 3.7 ± 0.3        | 30.2 ± 4.2       | 1.2 ± 0.1      |
Ala residues resulted in a significant decrease in the maximal Ca$^{2+}$-stimulated MgATPase activity (Table II). We can therefore hypothesize that Ser-43/Ser-45 appeared to be critical for maintaining the maximal Ca$^{2+}$-stimulated actomyosin MgATPase activity and that phosphorylation of these residues represents an important mechanism for regulation (inhibition) of the enzyme activity and, hence, the function of the contractile apparatus. In addition, the Ala for Ser substitutions resulted in a decreased Ca$^{2+}$ sensitivity for the reconstituted actomyosin, and phosphorylation of Ser-23/Ser-24 in S43A/S45A could not further decrease the Ca$^{2+}$ sensitivity (Fig. 5 and Table II). This suggested that Ser-43/Ser-45 may be in the region of TnI which interacts with the phosphorylated Ser-23/Ser-24 and ultimately, through interactions with either TnC or TnT, may influence the Ca$^{2+}$ sensitivity of the contractile complex.

With the use of additional mutants, we are currently investigating the more discrete effects of phosphorylation at Ser-43/Ser-45 on interactions of TnI with other components of the thin filament, such as binding of TnI to F-actin, TnT, and TnC.

Phosphorylation of TnI at Ser-43/Ser-45 also caused a decrease in the apparent affinity (increased $K_{d}$) of S-1 for the thin filament, leading to MgATPase inactivation (Fig. 7 and Table III) and a decrease in the actual binding of S-1-ATP to the thin filament (data not shown). It seemed that phosphorylation at Ser-43/Ser-45 would cause a conformational change in TnI such that TnCCa$^{2+}$ is prevented from completely "pulling" the inhibitory TnI away from certain binding sites on F-actin, with which myosin interacts. According to the model of Hill et al. (73), as modified by Lehrer (74), we hypothesize that phosphorylated TnI would then continue to stabilize a portion of the thin filament in the "off" state, preventing the binding of additional S-1-ATP (i.e. weak binding cross-bridges), thereby reducing the number of cross-bridges able to isomerize to "strongly bound" cross-bridges, and ultimately preventing the complete myosin head-induced "off to on" transition of Tm. Under the low ionic strength conditions (18 mM KCl) used in the present study, the binding of S-1-ATP to regulated actin is only modestly sensitive to Ca$^{2+}$ (56, 75) and not inhibited by unphosphorylated TnI (75). We predict, however, that the effects of phosphorylated TnI would be more pronounced under conditions of physiological ionic strength (120 mM KCl) where even unphosphorylated TnI has been shown to affect the binding of S-1-ATP to the thin filament (76). We are currently investigating such possibilities as well as other effects of phosphorylated TnI on the interactions of the thick and thin filaments.

Adult cardiac tissue is uniquely sensitive to the effects of pH. Acidotic conditions cause greater reductions in the maximal force, shortening velocity, and Ca$^{2+}$ sensitivity of fibers from adult cardiac muscle, compared to fibers from neonatal cardiac and slow and fast skeletal muscles (39, 57–58, 77–79). These functional effects have been proposed to be the consequence of pH-sensitive alterations in the binding of Ca$^{2+}$ to TnC, and the differential transmission of the Ca$^{2+}$ binding signal has been suggested to be dependent upon the isoforms of TnI and TnC (57, 58, 78, 79). In the present studies, we found that at pH 6.5, compared to pH 7.0, TnI phosphorylated by PKC caused greater reductions in the Ca$^{2+}$-stimulated MgATPase activity of reconstituted actomyosin S-1 (Fig. 5 and Table II). This suggested that effects on myocardial contractility due to phosphorylation of TnI by PKC may be more pronounced during acidosis.

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Cardiac Troponin I Mutants

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