Differential Regulation of Cardiac Actomyosin S-1 MgATPase by Protein Kinase C Isozyme-Specific Phosphorylation of Specific Sites in Cardiac Troponin I and Its Phosphorylation Site Mutants†

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ABSTRACT: The significance of site-specific phosphorylation by protein kinase C (PKC) isozymes α and δ and protein kinase A (PKA) of troponin I (TnI) and its phosphorylation site mutants in the regulation of Ca^{2+}-stimulated MgATPase activity of reconstituted actomyosin S-1 was investigated. The genetically defined TnI mutants used were T144A, S43A/S45A, S43A/S45A/T144A (in which the PKC phosphorylation sites Thr-144 and Ser-43/Ser-45 were respectively substituted by Ala) and N32 (in which the first 32 amino acids in the NH_2-terminal sequence containing Ser-23/Ser-24 were deleted). Although the PKC isozymes displayed different substrate phosphorylation kinetics, PKC-α phosphorylated equally well TnI wild type and all mutants, whereas N32 was a much poorer substrate for PKC-δ. Furthermore, the two PKC isozymes exhibited discrete specificities in phosphorylating distinct sites in TnI and its mutants, either as individual subunits or as components of the reconstituted troponin complex. Unlike PKC-α, PKC-δ favorably phosphorylated the PKA-preferred site Ser-23/Ser-24 and hence, like PKA, reduced the Ca^{2+} sensitivity of the reconstituted actomyosin S-1 MgATPase. In contrast, PKC-α preferred to phosphorylate Ser-43/Ser-45 (common sites for all isozymes) and thus reduced the maximal Ca^{2+}-stimulated activity of the MgATPase. In this respect, PKC-δ, by cross-phosphorylating the PKA sites, functioned as a hybrid of PKC-α and PKA. The site specificities and hence functional differences between PKC-α and -δ were most evident at low phosphorylation (1 mol of phosphate/mol) of TnI wild type and were magnified when S43A/S45A and N32 were used as substrates. The present study has demonstrated, for the first time, that distinct functional consequences could arise from the site-selective preferences of PKC-α and -δ for phosphorylating a single substrate in the myocardium, i.e., TnI.

Phosphorylation of myofibrillar proteins has been shown to play a regulatory role in cardiac contractility (Solaro, 1986). TnI[1] is one of the most thoroughly studied phosphoproteins in the thin filament. This protein is a component of the three-subunit Tn complex and, by binding to actin, inhibits actomyosin MgATPase activity when muscle is in the resting (low Ca^{2+}) state, but upon binding of Ca^{2+} to low-affinity site(s) of TnC, a conformational change occurs causing the affinity of TnI to now be greater for TnC than for actin. Thus, inhibition on actomyosin MgATPase is released and muscle contraction is allowed to occur (Zot & Potter, 1987). Phosphorylation *in situ* of TnI at Ser-23/Ser-24 by PKA, activated in response to β-adrenergic receptor stimulation, has been shown to result in decreased myofibrillar Ca^{2+} sensitivity (England, 1976; Kranias et al., 1985; Venema & Kuo, 1993) because of the ability of phosphorylated TnI to reduce the affinity of TnC for Ca^{2+} (Robertson et al., 1982). It has been suggested that the decreased Ca^{2+} responsiveness may function as a negative feedback mechanism (Solaro, 1986) or contribute to the increased relaxation rate of beating hearts under the influence of β-adrenergic stimulation (Zhang et al., 1995a). Phosphorylation of TnI *in vitro* at Ser-23/Ser-24 by PKA also reduced the Ca^{2+} sensitivity of MgATPase of reconstituted actomyosin (Ray & England, 1976; Solaro, 1986; Noland et al., 1995) or substituted myofibrils (Wattanapermpool et al., 1995).

PKC is another important signaling protein kinase in cardiac physiology and has been implicated in the regulation of ion channels, intracellular ion concentrations and pH, gene expression, and hypertrophic growth (Puceat & Brown, 1994). Activation of PKC has been shown to be associated with the stimulation of a number of receptors, including muscarinic cholinergic (Brown et al., 1985), α_1-adrenergic

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[1] Abbreviations: TnI, troponin I; TnT, troponin T; TnC, troponin C; Tn, troponin; Tm, tropomyosin; PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C; DTT, dithiothreitol; ME, β-mercaptoethanol; PS, phosphatidylserine; MOPS, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; MBP, myelin basic protein; MLC-2, myosin light chain-2.

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(Brown et al., 1985; Kaku et al., 1991), angiotensin II (Dosemeci et al., 1988), and endothelin (Kramer et al., 1991) receptors. Furthermore, phorbol esters (PKC activators) have been shown to cause predominantly negative (with some positive) inotropic effects on a variety of cardiac preparations, including isolated cardiomyocytes (Leatherman et al., 1987; Dosemeci et al., 1988; Capogrossi et al., 1990; Watson & Karmazyn, 1991; Ward & Moffiat, 1992; Karmazyn & Haist, 1993). We reported previously that PKC phosphorylated TnI at multiple sites (Noland et al., 1989; Noland & Kuo, 1991; Venema & Kuo, 1993), including the typical PKA sites Ser-23/Ser-24 under certain conditions, leading to modified myofilament properties such as reduced Ca^{2+}-stimulated actomyosin MgATPase activity (Noland & Kuo, 1991, 1992; Venema & Kuo, 1993). In addition to TnI, PKC readily phosphorylated TnT at multiple sites and attenuated the maximal activity of Ca^{2+}-stimulated actomyosin MgATPase (Noland et al., 1989, 1991). Modifications of myofilament properties brought about by phosphorylation of TnI and/or TnT were, in part, due to altered interactions among the contractile proteins (Noland & Kuo, 1992, 1993a). The *in vitro* phosphorylation of multiple sites by PKC is likely to be physiologically revelant, because the same sites in TnI (Venema & Kuo, 1993; Jideama et al., 1996) and TnT (Jideama et al., 1996) were similarly phosphorylated in adult rat cardiomyocytes incubated with phorbol ester or phenylephrine (an α_1-adrenergic agonist).

PKC is the product of a gene superfamily, and 13 subspecies have been identified to date (for a review, see Nishizuka (1995)). Expression of PKC isozymes in cardiomyocytes is developmentally regulated; i.e., a greater number of isozymes and higher levels of expression have been found in fetal/neonatal than in adult cells (Bogoyevitch et al., 1993; Disatnick et al., 1994; Puceat et al., 1994; Rybin & Steinberg, 1994). The current consensus is that adult cardiomyocytes express PKC isozymes α, δ, ϵ, and ζ (Bogoyevitch et al., 1993; Rybin & Steinberg, 1994; Disatnik et al., 1994; Puceat et al., 1994; Steinberg et al., 1995; Ventura et al., 1995). It has been hypothesized that the individual isozymes may have specific functions based upon, in part, phosphorylation of specific substrates (Kazanietz et al., 1993; Nishizuka, 1995; Dekker & Parker, 1994; Steinberg et al., 1995; Ventura et al., 1995). Because brain pan-PKC preparations (mixtures of isozymes) were used in our previous phosphorylation and functional studies (Noland et al., 1989, 1991, 1995; Noland & Kuo, 1992, 1993a,b; Venema & Kuo, 1993; Venema et al., 1993), the involvement of the individual isozymes remained unclear. Furthermore, since PKC phosphorylated multiple sites in TnI and TnT, it was impossible to assign specific changes in myofilament properties to phosphorylation of specific sites. We have begun to address these critical issues. We found recently, with the use of mouse and rat cardiac TnI mutants in which the identified phosphorylation sites were substituted by Ala residues or deleted, that phosphorylation of Ser-23/Ser-24 and Ser-43/Ser-45 by pan-PKC resulted in decreased Ca^{2+} sensitivity and maximal activity, respectively, of Ca^{2+}-stimulated MgATPase of reconstituted actomyosin S-1 (Noland, et al., 1995). In recent experiments, we observed that PKC isozymes (α, δ, ϵ, ζ) displayed distinct substrate specificities in phosphorylating multiple sites in bovine cardiac TnI and TnT (Jideama et al., 1996). In particular, PKC-α and -ϵ shared common site specificities whereas PKC-δ mimicked PKA in cross-phosphorylating Ser-23/Ser-24 in TnI and hence could function as if it were a hybrid of PKC-α and PKA. In the present study, we have chosen PKC-α and -δ as examples, systematically examined the specificities of the two PKC isozymes in phosphorylating specific sites as probed with TnI mutants, and explored the functional significance of isozyme-specific phosphorylation of specific sites in TnI.

EXPERIMENTAL PROCEDURES

Preparations of Cardiac Contractile Proteins, TnI Wild Type and Mutants, PKC Isozymes, and PKA. Bovine heart ventricles were used as the source of contractile proteins. TnC, TnI, and TnT were purified according to the method of Potter (1982) and stored at −70 °C in 50 mM Tris-HCl (pH 8.0) containing 6 M urea, 1 mM EDTA and 15 mM ME. Tm (Stull & Buss, 1977), F-actin (Pardee & Spudich, 1982), and myosin S-1 (Siemankowski & White, 1984) were prepared as previously described. In order to prevent oxidation of TnI and Tm, 1 mM DTT or 15 mM ME was added to all solutions throughout the preparation and reconstitution procedures. Recombinant mouse cardiac TnI wild type and mutants (T144A, S43A/S45A and S43A/S45A/T144A), in which the identified PKC phosphorylation sites Thr-144 and Ser-43/Ser-45 were respectively substituted by Ala residues (Noland et al., 1995), and a rat cardiac TnI mutant (N32), in which the first 32 residues in the NH_2-terminus containing the PKA phosphorylation sites Ser-23/Ser-24 were deleted (Guo et al., 1994), were expressed in bacterial cells and purified as reported (Guo et al., 1994; Noland et al., 1995). It should be noted that the positions of the amino acid residues referred to in this and an earlier paper (Noland et al., 1995) concerning mouse and rat TnI are derived from the published amino acid sequence of bovine cardiac TnI (Leszyk et al., 1988), which begins with a NH_2-terminal Ala, and from the amino acid sequence predicted by the nucleotide sequence of the cDNA clone of mouse cardiac TnI wild type (Guo et al., 1994), which begins with a NH_2-terminal Met. Recombinant PKC isozymes (α, δ, ϵ, ζ) were purified from insect cells transfected with baculovirus expression vectors (Martiny-Baron et al., 1993), whereas PKA was purified from bovine heart extracts (Kuo et al., 1970).

Phosphorylation of TnI Wild Type and Mutants, and Tryptic Phosphopeptide Analysis. Prior to phosphorylation, TnI was dialyzed (24 h, 4 °C) against 10 mM Tris-HCl (pH 7.5) containing 1 mM DTT with sequential KCl concentrations of 1, 0.7, and 0.3 M (Potter, 1982). The conditions for phosphorylation by PKC and PKA were essentially as described elsewhere (Noland et al., 1995), except that PKC-α was activated by PS (20 μg/mL)/$CaCl_2$ (100 μM)/diolein (5 μg/mL); PKC-δ and PKC-ϵ, by PS/diolein but omitting $CaCl_2$; PKC-ζ, by PS only. KCl (0.3 M) was included in all phosphorylation studies in order to keep TnI in solution. For phosphorylation kinetic studies (Figure 1), 5 μM [γ-^{32}P]-ATP (about (1−2) × 10^6 cpm) was used. A high concentration (400 μM) of the radioactive ATP (about (4−6) × 10^6 cpm) was used for the phosphorylation of preparations of TnI (1−6 μM) or Tn complex (2 μM, reconstituted as described below) that were subsequently analyzed by two-dimensional phosphopeptide mapping (Figures 2 and 3). The ^{32}P-labeled TnI (or Tn) preparations were electrophoresed

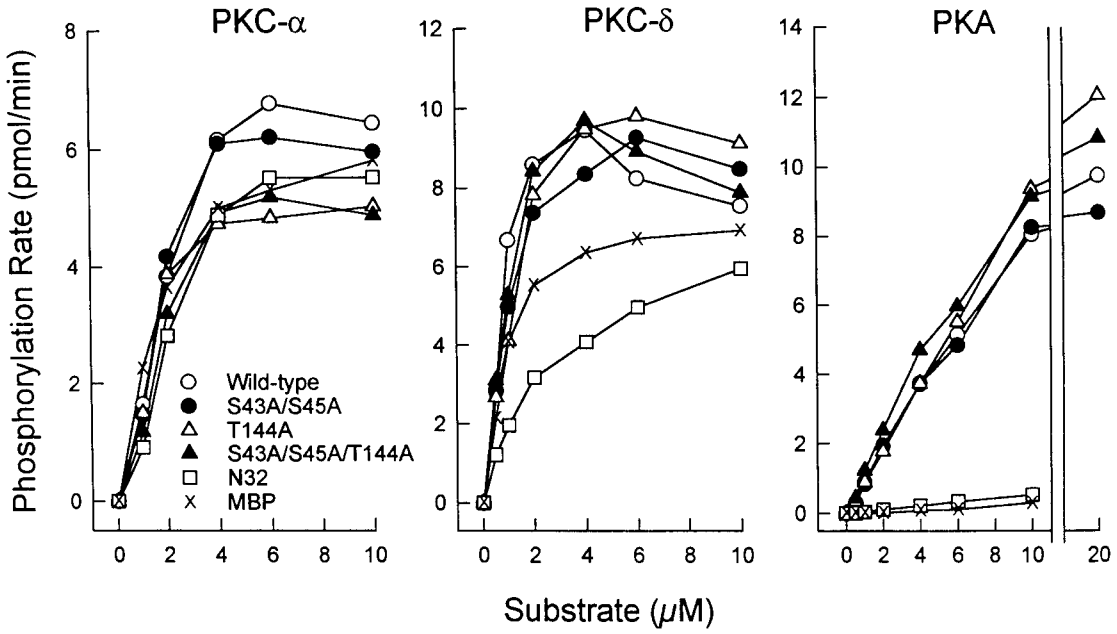

FIGURE 1: Concentration-dependent phosphorylation of MBP and cardiac TnI wild type and mutants by PKC-α, PKC-δ, or PKA. All phosphorylation reactions were carried out for 15 min in the presence of 5 μM [γ^{-32}P]ATP (2×10^6 cpm) and 0.3 M KCl, which is required for the solubility of TnI preparations. MBP was included as a reference substrate. The phosphorylation rate was linear as a function of incubation time and enzyme amount under the experimental conditions. The low phosphorylation rate of MBP by PKA was due to selective inhibition by 0.3 M KCl. The findings were confirmed in another set of experiments. See Experiment Procedures for further details.

on sodium dodecyl sulfate−polyacrylamide gels, and tryptic phosphopeptide maps of samples excised from the gels were prepared as previously described (Noland et al., 1995). For actomyosin S-1 MgATPase studies (Figures 4−6), TnI (6 μM) was phosphorylated with 400 μM nonradioactive ATP under identical conditions. Control incubations (unphosphorylated controls) were conducted in parallel experiments using heat-inactivated PKC or PKA.

Reconstitution of Tn and Thin Filament, and Assay for Ca^{2+}-Stimulated Actomyosin S-1 MgATPase. Reconstitution of Tn containing unphosphorylated or phosphorylated TnI wild type and mutants was carried out as in our recent study (Noland et al., 1995), essentially according to the procedure described for native TnI by Potter (1982). Briefly, the TnI samples (6 μM in 1.5 mL) were mixed with bovine cardiac TnT and TnC at equal molar ratios in 10 mM imidazole-HCl (pH 7.0) containing 6 M urea, 1 mM $CaCl_2$ and 1 mM DTT, and the mixtures were dialyzed (48 h, 4 °C) against 10 mM imidazole-HCl (pH 7.0) containing 1 mM magnesium acetate and 1 mM DTT with sequential KCl concentrations of 1.0, 0.7, 0.3, and 0.1 M. The resulting Tn complexes were concentrated by ultrafiltration and either stored at −70 °C or used immediately to reconstitute the thin filament. The thin filament (regulated actin) was reconstituted by mixing Tn, bovine Tm, and F-actin at molar ratios of 1:1:5, and the mixture was dialyzed (16 h, 4 °C) against 10 mM imidazole-HCl (pH 7.0) containing 10 mM KCl, 2 mM ATP, 1 mM magnesium acetate and 1 mM DTT. The resulting complex was stored at 4 °C and used within 48 h. Prior to assay for MgATPase, actomyosin S-1 was reconstituted from 4 μM regulated actin and 0.4 μM bovine cardiac myosin S-1. The resulting actomyosin S-1 was incubated (0.5−2 h, 4 °C) in the reaction mixtures (see below) without ATP. The Ca^{2+}-stimulated MgATPase activity was assayed at 30 °C for 10 min in reaction mixtures (0.25 mL) containing actomyosin S-1 (0.4 μM), 10 mM MOPS−KOH (pH 7.0), 4.1 mM magnesium acetate, 2.1 mM [γ-^{32}P]ATP ((5−8) × 10^6 cpm), 1 mM DTT, 1 mM EGTA, and 0−1.05 mM $CaCl_2$. $CaCl_2$, when present, was added as a mixture with EGTA and MOPS−KOH to give the calculated free Ca^{2+} concentrations of 0−100 μM in the assay mixtures. Appropriate volumes of 5 M KOH were added to adjust the pH to 7.0, and KCl was added to bring the final ionic strength to 18 mM. The reactions were initiated by addition of [γ-^{32}P]ATP and terminated by addition of 10% charcoal suspension, and the released ^{32}Pi was determined as described (Noland & Kuo, 1991; Noland et al., 1995). The free Ca^{2+} concentrations in incubation mixtures were calculated by the method of Fabiato and Fabiato (1979). MgATPase activity (expressed as s^{-1}; mol of ATP hydrolyzed (mol of myosin S-1)$^{-1}$ s^{-1}) was linear as a function of incubation time and amount of actomyosin S-1 under the assay conditions. Kinetic data for Ca^{2+}-stimulated actomyosin S-1 MgATPase (as a function of Ca^{2+} concentration) were calculated by nonlinear least-squares regression analysis using a modified Hill equation as previously reported (Noland & Kuo, 1991; Noland et al., 1995).

RESULTS

The initial rates of phosphorylation of mouse cardiac TnI wild type and mutants by PKC-α and -δ in comparison to those by PKA were studied (Figure 1), and the kinetic data are summarized (Table 1). It was found that PKC-α phosphorylated wild type and all mutants nearly equally well, as indicated by their similar V_{max}/K_m ratios. N32 (rat cardiac TnI from which a 32-amino acid NH_2-terminal sequence containing Ser-23/Ser-24 was deleted), however, was a much more inferior substrate for PKC-δ, which effectively phosphorylated the other mutants and wild type. In contrast, PKA phosphorylated wild type and all mutants but did not phosphorylate N32. These findings on N32 were particularly noteworthy, suggesting that PKC-α and PKC-δ possessed

Table 1: Summary of Kinetic Constants for Phosphorylation of MBP and Cardiac TnI Wild Type and Mutants by PKC-α, PKC-δ, or PKA[a]

enzyme and substrate	V_{max} (pmol/min)	K_m (μM)	V_{max}/K_m	Hill coeff
PKC-α				
wild type	6.7 ± 0.2	1.7 ± 0.1	3.9	2.7 ± 0.6
S43A/S45A	6.2 ± 0.2	1.6 ± 0.1	3.9	3.2 ± 0.7
T144A	4.9 ± 0.1	1.3 ± 0.1	3.8	3.0 ± 0.2
S43A/S45A/T144A	5.1 ± 0.2	1.6 ± 0.1	3.2	3.1 ± 0.7
N32	5.7 ± 0.1	2.0 ± 0.1	2.9	2.7 ± 0.3
MBP	6.2 ± 0.2	1.5 ± 0.1	4.1	1.4 ± 0.1
PKC-δ				
wild type	9.5 ± 0.1	0.7 ± 0.02	13.9	2.3 ± 0.1
S43A/S45A	9.0 ± 0.4	0.9 ± 0.09	10.0	1.7 ± 0.4
T144A	10.0 ± 0.3	1.1 ± 0.08	9.1	1.9 ± 0.3
S43A/S45A/T144A	10.0 ± 0.2	0.9 ± 0.06	11.1	1.9 ± 0.3
N32	9.5 ± 1.0	5.5 ± 0.85	1.7	0.8 ± 0.1
MBP	7.0 ± 0.1	0.8 ± 0.02	8.8	1.5 ± 0.1
PKA				
wild type	11.1 ± 0.6	6.1 ± 0.5	1.8	1.7 ± 0.2
S43A/S45A	9.4 ± 0.7	5.0 ± 0.6	1.9	2.1 ± 0.6
T144A	13.8 ± 0.7	7.0 ± 0.5	2.0	1.9 ± 0.5
S43A/S45A/T144A	12.6 ± 0.9	5.8 ± 0.7	2.2	1.5 ± 0.2
N32	ND[b]	ND	ND	ND
MBP	ND	ND	ND	ND

[a] The values (means ± standard errors) were calculated by nonlinear least-squares regression analysis using the Michaelis−Menton equation, based upon the data shown in Figure 1. [b] Not determined due to low phosphorylation.

low and high preferences, respectively, for phosphorylating Ser-23/Ser-24, the PKA-favored phosphorylation sites.

In order to uncover the specificities of PKC isozymes in differentially phosphorylating multiple sites in TnI, two-dimensional tryptic phosphopeptide analysis of TnI wild type and mutants was performed (Figure 2). The phosphopeptide maps of TnI preparations at about midpoint (1.0−1.9 mol of phosphate incorporated/mol of protein) of maximal phosphorylation by PKC-α and -δ are shown here as examples. The relative levels of phosphorylation of Ser-23/Ser-24 (spot 5) and Ser-43/Ser-45 (spot 2A), compared to Thr-144 (spot 3) and other sites, for TnI wild-type, S43A/S45A and N32 phosphorylated to various extents by PKC-α and -δ are also summarized (Table 2). PKC-α phosphorylated most of the same sites in TnI wild type (Figure 2) that were previously identified in bovine cardiac TnI phosphorylated by brain pan-PKC (Noland and Kuo, 1989), and their identities were verified by the absence of individual spots from maps of the corresponding mutants, as noted in our recent work (Noland et al., 1995). The most noticeable differences were that PKC-α and -δ preferentially phosphorylated Ser-43/Ser-45 and Ser-23/Ser-24, respectively, in TnI wild type. Cross-phosphorylation of Ser-23/Ser-24 by PKC-α, however, became obvious when the isozyme either exhaustively phosphorylated TnI wild type to 3.2 mol/mol (Table 2) or phosphorylated the three substitution mutants (T144A, S43A/S45A, S43A/S45A/T144A), in which the preferred sites for PKC-α were respectively substituted by Ala (Figure 2). Although PKC-δ also phosphorylated other sites (spots 1, 3, and 4) in TnI wild type and the deletion mutant (N32) when these TnI preparations were extensively phosphorylated (Figure 2, Table 2), the selectivity of the isozyme in phosphorylating Ser-23/Ser-24 was particularly clear for the three substitution mutants (Figure 2). We also found that PKC-ε, (another major isozyme in adult cardiomyocytes) phosphorylated all the TnI preparations with similar specificity and effectiveness as did PKC-α, but PKC-ζ (a minor isozyme in myocytes) only very poorly phosphorylated them (data not shown). It should be noted that, in contrast to the PKC isozymes, PKA phosphorylated only Ser-23/Ser-24 in TnI wild type and all mutants except N32 (data not shown), as we reported recently (Noland et al., 1995).

We next examined whether the same sites phosphorylated in the individual TnI wild type and mutants would also be phosphorylated in the reconstituted Tn complex. Analysis of the resulting phosphopeptide maps (Figure 3) obtained from phosphorylated Tn complex (with 0.6−1.0 mol of phosphate/mol of TnI) revealed that the preference of PKC-δ, relative to PKC-α, for phosphorylating Ser-23/Ser-24 was retained. However, PKC-δ no longer exclusively phospho-

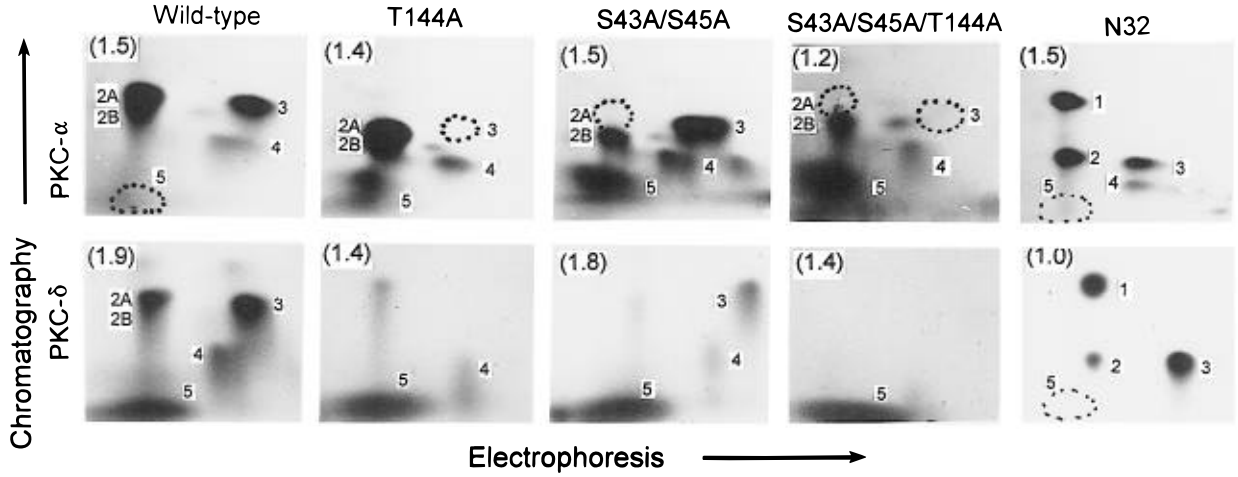

FIGURE 2: Autoradiograms showing site-specific phosphorylation of TnI wild type and mutants by PKC-α and PKC-δ. TnI preparations (6 μM) were phosphorylated for 0.5−1 h with 400 μM [γ-^{32}P]ATP, and two-dimensional tryptic phosphopeptide mapping was performed. The extents of phosphorylation (mole of phosphate incorporated/mole of protein) are shown in parentheses. The findings were confirmed in whole or in part, in four to six other experiments. See Experimental Procedures for further details. The autoradiograms shown were from representative experiments with different exposure times on X-ray film. They are, therefore, not indicative of the absolute extent of phosphorylation. Comparisons between different TnI preparations and PKC isozymes should be based on the relative phosphorylation of sites within each TnI preparation phosphorylated by an individual PKC isozyme. Phosphorylation sites: spot 1, Ser-78; spot 2A (formerly 2), Ser-43/Ser-45; spot 3, Thr-144; spot 5, Ser-23/Ser-24; spots 2B and 4, unidentified (see text).

Table 2: Site-Specific Phosphorylation of TnI Wild Type and Mutants by PKC-α and PKC-δ[a]

TnI preparation and PKC isozyme	phosphorylation extent (mol/mol)	site-specific phosphorylation (% of total)		
		Ser-23/Ser-24 (spot 5)	Ser-43/Ser-45 (spot 2A)	Thr-144 (spot 3) and others
wild type				
PKC-α	0.8	4	35	62
	1.5	8	41	51
	3.2	25	25	51
PKC-δ	0.6	19	11	70
	1.9	38	18	44
	2.8	55	18	27
S43A/S45A				
PKC-α	0.8	2	0	98
	1.5	22	0	78
	2.3	32	0	68
PKC-δ	0.7	27	0	73
	1.8	72	0	28
	2.5	79	0	21
N32				
PKC-α	0.9	0	39	61
	1.5	0	34	66
	2.6	0	30	70
PKC-δ	0.3	0	10	90
	1.0	0	13	87
	2.2	0	32	68

[a] TnI wild type and mutants were phosphorylated by PKC-α and PKC-δ to various extents as described in the legend to Figure 2, and the autoradiograms of the tryptic phosphopeptide maps were analyzed by densitometry (Lynx video densitometer).

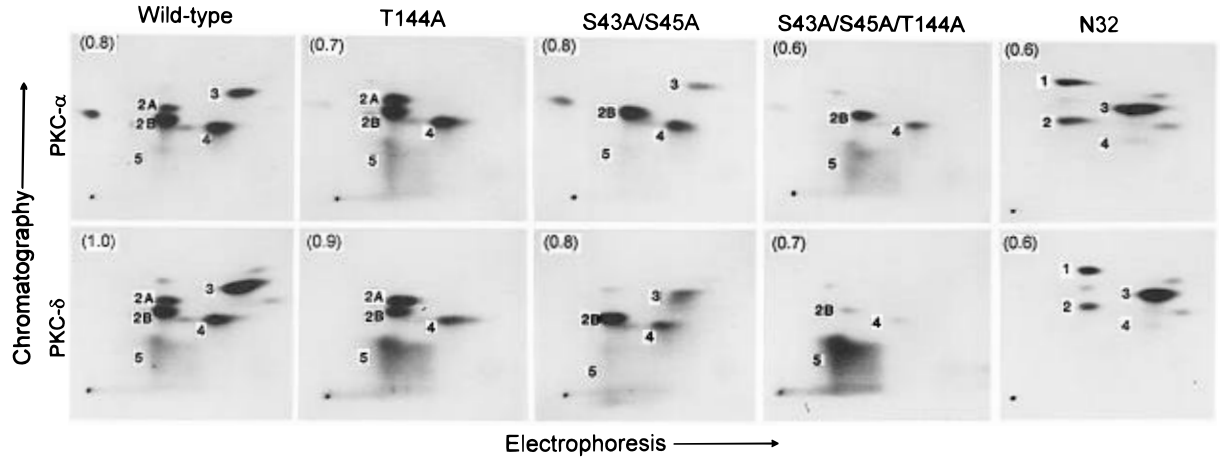

FIGURE 3: Autoradiograms showing site-specific phosphorylation by PKC-α and PKC-δ of TnI wild type and mutants present in reconstituted Tn complexes. Reconstituted Tn preparations (2 μM) were phosphorylated for 3 h, and phosphopeptide maps were prepared as described in Experimental Procedures. The extents of phosphorylation (mol phosphate incorporated/mol TnI) are shown in parentheses.

rylated Ser-23/Ser-24 in the T144A, S43A/S45A, and S43A/S45A/T144A mutants in the Tn complex (Figure 3), as it did when the mutants were in the form of free subunits (Figure 2). One reason for this discrepancy might be that the structures of Tn complexes reconstituted from the TnI mutants decreased the accessibility of Ser-23/Ser-24 for phosphorylation; thus, sites favored less by PKC-δ were phosphorylated. We previously reported that the same sites in bovine cardiac TnI, present as a free subunit or as a component of the Tn complex, were phosphorylated by brain pan-PKC (Noland et al., 1989) or in adult rat cardiac myocytes (Venema & Kuo, 1993; Jideama et al., 1996).

We also examined the temporal effects of phosphorylation of TnI wild type and mutants by PKC isozymes and PKA on the Ca^{2+}-stimulated MgATPase activity of reconstituted actomyosin S-1 (Figures 4−6), and the kinetic constants are summarized (Table 3). Phosphorylation by PKC-α of TnI wild type primarily caused reductions in the maximal Ca^{2+}-stimulated MgATPase activity with only minor decreases in the Ca^{2+} sensitivity (increases in the EC_{50} for Ca^{2+}) of the enzyme (Figure 4, Table 3). It was noted that the decrease in MgATPase was near maximal at a low level of phosphorylation (1.0 mol/mol) by PKC-α and that a higher phosphorylation (1.9 mol/mol) failed to appreciably further decrease the activity (Figure 4). The reason that could account for this phenomenon might be that a near-maximal phosphorylation of Ser-43/Ser-45 was already achieved at the low level of phosphorylation of TnI wild type, as supported by the data on site-specific phosphorylation shown above (Table 2). Phosphorylation by PKA, on the other hand, exclusively caused reductions in Ca^{2+} sensitivity that were positively related to the extent of TnI phosphorylation at Ser-23/Ser-24, in agreement with the observations of Zhang et al. (1995b). In contrast, PKC-δ had dual actions in that it affected both maximal activity and Ca^{2+} sensitivity, depending upon the extent of phosphorylation of TnI. Thus, decreased Ca^{2+} sensitivity was observed at both low (1.0 mol/mol) and high (2.2 mol/mol) phosphorylation, whereas

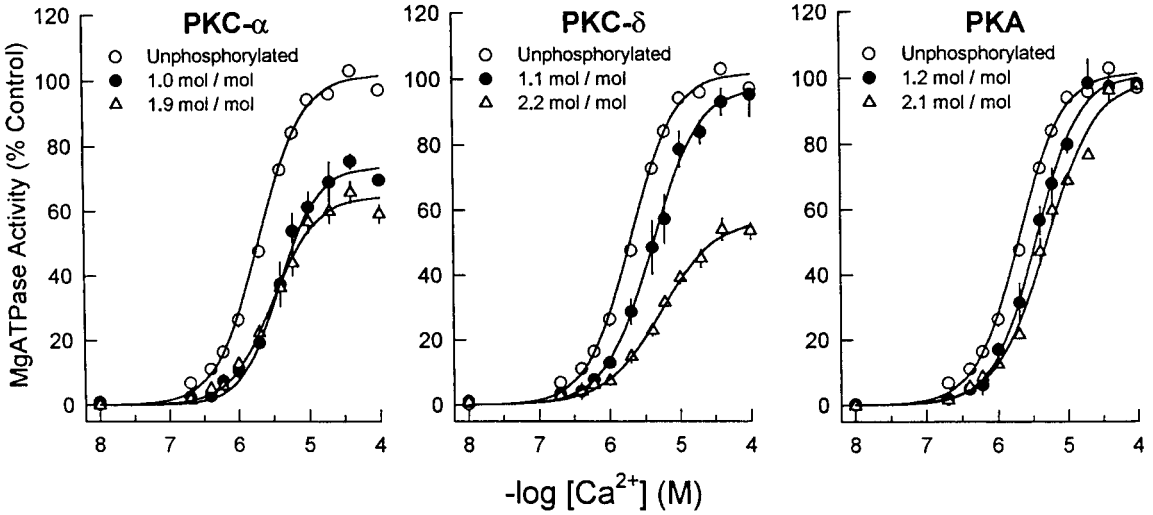

FIGURE 4: Effects of phosphorylation by PKC-α, PKC-δ, or PKA of TnI wild type on the Ca^{2+}-stimulated MgATPase activity of reconstituted actomyosin S-1. Unphosphorylated and phosphorylated (1.0−2.2 mol/mol) TnI wild-type preparations were used for reconstitution, and the enzyme activity was assayed in the presence of varying Ca^{2+} concentrations. Actomyosin S-1 MgATPase activity in the absence of Ca^{2+} was subtracted from values obtained in the presence of Ca^{2+} and was less than 10% of the total MgATPase activity. The maximal Ca^{2+}-stimulated activity (0.75 ± 0.01 s^{-1}) for reconstituted actomyosin containing unphosphorylated TnI wild type (control) was taken as 100%. The data points shown are means ± standard errors for unphosphorylated ($N = 10$) and phosphorylated ($N = 3-6$) TnI. In this figure and subsequent figures, data points without error bars indicated standard errors less than the size of the symbols. The curves drawn are the "best fits" of the data to Hill's equation using nonlinear regression.

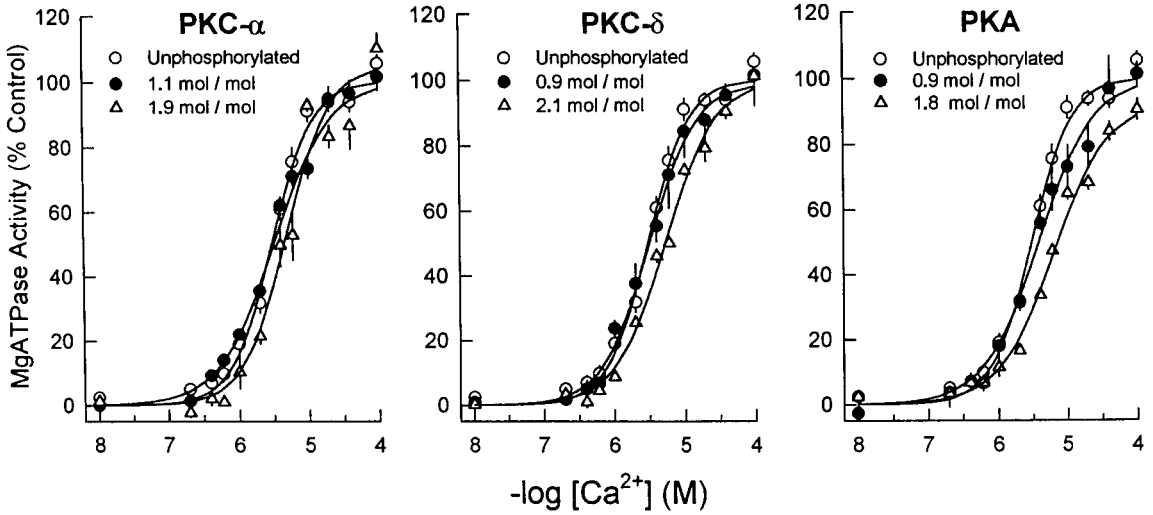

FIGURE 5: Effects of phosphorylation by PKC-α, PKC-δ, or PKA of the TnI S43A/S45A mutant on the Ca^{2+}-stimulated MgATPase activity of reconstituted actomyosin S-1. The experimental details are as described in the legend of Figure 3 except that S43A/S45A replaced TnI wild type. The MgATPase activity for the unphosphorylated mutant (0.41 ± 0.01 s^{-1}) was taken as 100%. The data points shown are means ± standard errors ($N = 3$).

decreased maximal MgATPase activity was observed only at higher phosphorylation (Figure 4, Table 3). The dual effects of PKC-δ were consistent with its unique ability to readily phosphorylate the typical PKA phosphorylation sites Ser-23/Ser-24 (Figure 2, Table 2) and caused reduced MgATPase Ca^{2+} sensitivity, whereas the reduction in MgATPase activity (Figure 4, Table 3) paralleled the subsequent phosphorylation at the common PKC phosphorylation sites such as Ser-43/Ser-45 (Table 2).

We also found that phosphorylation of the S43A/S45A TnI mutant by either PKC-α or -δ could not produce a significant reduction in the maximal Ca^{2+}-stimulated MgATPase activity, regardless of the phosphorylation extent (Figure 5, Table 3), consistent with our earlier conclusion that phosphorylation of Ser-43/Ser-45 was primarily responsible for reduced MgATPase activity caused by pan-PKC (Noland et al., 1995). In contrast, the Ca^{2+} sensitivity was reduced in concert with the degree to which each isozyme could phosphorylate Ser-23/Ser-24 (Figure 2, Table 2), relative to the other sites in the mutant. In similarity to TnI wild type, the phosphorylation of the S43A/S45A mutant by PKA caused reductions in the Ca^{2+} sensitivity that were positively correlated to the extent of phosphorylation at Ser-23/Ser-24. Phosphorylation of the TnI deletion mutant (N32) by either PKC-α or -δ (to 1.1 and 1.2 mol/mol, respectively) or PKA (to 0.2 mol/mol) did not lead to reductions in the Ca^{2+} sensitivity (Figure 6, Table 3), in line with earlier observations with pan-PKC (Noland et al., 1995) or with PKA (Guo et al., 1994; Noland et al., 1995; Wattanapermpool et al., 1995). However, differences in the degree of reduction of the maximal Ca^{2+}-stimulated MgATPase activity caused by the two PKC isozymes were quite apparent, with PKC-α causing a greater reduction (25%) in contrast to the smaller reduction (3%) caused by PKC-δ, consistent with

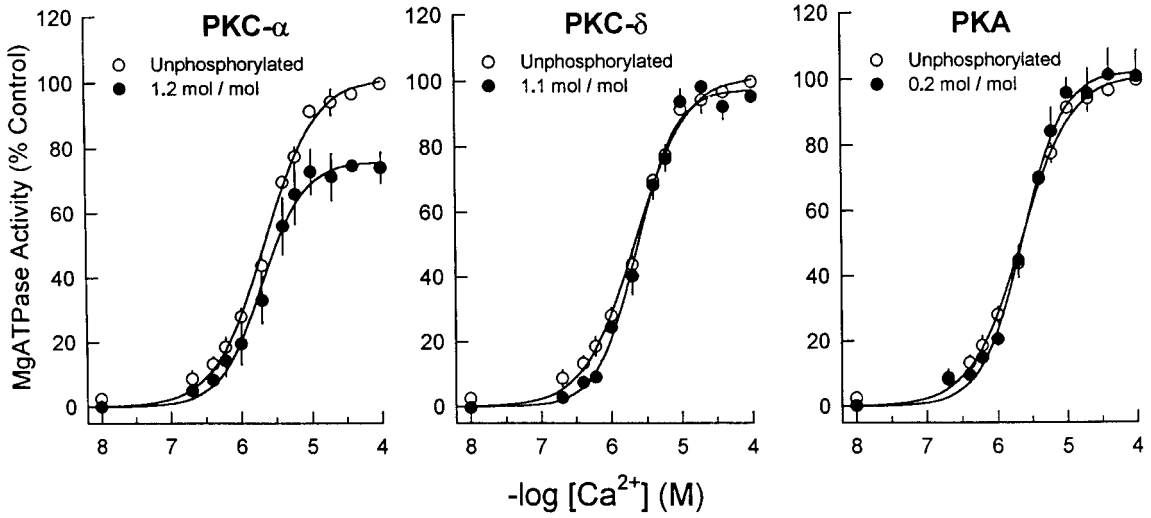

FIGURE 6: Effects of phosphorylation by PKC-α, PKC-δ, or PKA of the TnI N32 mutant on the Ca^{2+}-stimulated MgATPase activity of reconstituted actomyosin S-1. The experimental details are as described in the legend of Figure 3 except that N32 replaced TnI wild type. The MgATPase activity for the unphosphorylated mutant (0.61 ± 0.01 s^{-1}) was taken as 100%. The data points shown are means ± standard errors ($N = 3-6$).

Table 3: Summary of Kinetic Constants for Ca^{2+}-Stimulated MgATPase of Reconstituted Actomyosin S-1, Containing TnI Wild Type and Mutants Phosphorylated to Various Extents by PKC-α, PKC-δ, or PKA[a]

TnI preparation and enzyme	phosphorylation extent (mol/mol)	max Ca^{2+}-stimulated activ (s^{-1})	EC_{50} for Ca^{2+} (μM)	Hill coeff
wild-type				
none (control)	0.0	0.75 ± 0.01	2.0 ± 0.1	1.4 ± 0.1
PKC-α	1.0	0.55 ± 0.02^b	3.6 ± 0.3^b	1.6 ± 0.2
	1.9	0.48 ± 0.02^b	3.2 ± 0.3^b	1.4 ± 0.1
PKC-δ	1.1	0.73 ± 0.03	4.0 ± 0.4^b	1.3 ± 0.1
	2.2	0.43 ± 0.02^b	5.3 ± 0.6^b	1.1 ± 0.1^b
PKA	1.2	0.75 ± 0.02	3.5 ± 0.3^b	1.4 ± 0.1
	2.1	0.74 ± 0.02	5.0 ± 0.4^b	1.2 ± 0.1
S43A/S45A				
none (control)	0.0	0.41 ± 0.01	2.9 ± 0.2	1.6 ± 0.1
PKC-α	1.1	0.41 ± 0.01	3.4 ± 0.4	1.3 ± 0.1
	1.9	0.43 ± 0.02	3.9 ± 0.5^b	1.4 ± 0.2
PKC-δ	0.9	0.40 ± 0.01	3.1 ± 0.2	1.3 ± 0.1
	2.1	0.41 ± 0.01	5.0 ± 0.4^b	1.3 ± 0.1^b
PKA	0.9	0.45 ± 0.02	3.4 ± 0.3	1.3 ± 0.1^b
	1.8	0.38 ± 0.01	6.2 ± 0.5^b	1.1 ± 0.1^b
N32				
none (control)	0.0	0.61 ± 0.01	2.2 ± 0.1	1.3 ± 0.1
PKC-α	1.2	0.46 ± 0.02^b	2.0 ± 0.3	1.4 ± 0.2
PKC-δ	1.1	0.59 ± 0.01	2.3 ± 0.1	1.6 ± 0.1^b
PKA	0.2	0.62 ± 0.02	2.3 ± 0.2	1.5 ± 0.1

[a] The values (± standard errors) were calculated by nonlinear least-squares regression analysis using a modified form of the Hill equation, based upon the data shown in Figures 4−6. The maximal activity is expressed as reciprocal seconds (mol of ATP hydrolyzed (mol of myosin S-1)$^{-1}$ s^{-1}). [b] Significantly different from the respective control (Student's *t*-test, $p < 0.05$).

the preference of PKC-α, compared to PKC-δ, for phosphorylation of Ser-43/Ser-45 shown earlier (Figure 2, Table 2).

DISCUSSION

It is well documented that the PKC isozymes (α, δ, ϵ, ζ), expressed in adult cardiomyocytes, differ substantially not only in their modes of activation but also in their ligand and substrate (both synthetic and natural) specificities (Kazanietz et al., 1993; Nishizuka, 1995; Dekker & Parker, 1994; Steinberg et al., 1995). In the present study, we have extended these studies by presenting evidence showing that the PKC isozymes α and δ displayed distinct specificities in phosphorylating multiple sites in recombinant cardiac TnI wild type and phosphorylation site mutants. The site-specific phosphorylation catalyzed by the two isozymes also specifically altered the functional properties of reconstituted actomyosin S-1. We had previously determined, by using the genetically defined TnI phosphorylation site mutants S43A/S45A, T144A, S43A/S45A/T144A, S23A/S24A, and N32, that phosphorylated Ser-43/Ser-45 and Ser-23/Ser-24 were primarily responsible for the reduced MgATPase activity and Ca^{2+} sensitivity, respectively (Noland et al., 1995). However, because pan-PKC (consisting of a mixture of isozymes) was used, the contributions of individual isozymes in causing these effects were unclear. This question was answered in the present experiments where we found that PKC-α, by preferentially phosphorylating Ser-43/Ser-45 in TnI, decreased the Ca^{2+}-stimulated MgATPase activity of reconstituted myofilaments. In contrast, PKC-δ

effectively phosphorylated the PKA phosphorylation sites Ser-23/Ser-24, in addition to Ser-43/Ser-45 and other sites common to all PKC isozymes (Noland et al., 1995), therefore, behaving as if it were a functional hybrid of typical PKC and PKA in causing decreased Ca^{2+} sensitivity of reconstituted actomyosin. By using the TnI S43A/S45A and N32 mutants in the present experiments, we also confirmed the functional importance for phosphorylation at Ser-43/Ser-45 and Ser-23/Ser-24. Moreover, the differences between PKC-α and -δ, with respect to their site-specific phosphorylation and the functional consequence thereof, were magnified when the two mutants were used as substrates and for reconstitution.

Our findings, that PKC-α and -δ have distinct specificities for phosphorylating sites in TnI with unique consequences, suggest functionally divergent roles for the two PKC isozymes in the myocardium. This notion is supported by the extensive evidence showing differential developmental and signaling pathway regulation of the localization of selective PKC isozymes with the contractile apparatus (Bogoyevitch et al., 1993, 1994; Disatnik et al., 1994; Puceat et al., 1994; Clerk et al., 1995; Johnson & Mochly-Rosen, 1995; Johnson et al., 1995). Furthermore, *in situ* phosphorylation of TnI and other myocardial contractile proteins, including TnT, MLC-2, and C-protein, has been shown to occur upon activation of PKC in isolated or cultured cardiomyocytes following treatments with phorbol esters, α_1-adrenergic agonists, arachidonic acid, or endothelin (Liu et al., 1988; Venema & Kuo, 1993; Venema et al., 1993; Damron et al., 1995; Jideama et al., 1966). Both activation of α_1-adrenergic receptors in isolated myocytes and exposure of these cells to phorbol ester have resulted in the PKC-mediated phosphorylation of TnI (to 0.8 mol/mol) at sites corresponding to Ser-43/Ser-45 and Ser-23/Ser-24 (spots 5 and 9, respectively, in Venema & Kuo, (1993)), and moreover, the phorbol ester-stimulated phosphorylation of TnI was positively correlated to decreased MgATPase activity of isolated myofibrils (Venema & Kuo, 1993). Thus, the extent of the *in situ* phorbol ester-stimulated TnI phosphorylation in cardiac myocytes was consistent with the levels of *in vitro* (1 mol/mol) phosphorylation associated with the greatest differences in PKC isozyme specificities and subsequent functional effects (Tables 2 and 3). It is quite likely that PKC-α and/or -ϵ could have been responsible for the phorbol ester stimulated phosphorylation of TnI in these myocytes. Although the phosphorylation at Ser-23/Ser-24 was minimal in these experiments (Venema & Kuo, 1993), we recently observed significant phosphorylation of this site, following phorbol ester treatments of cardiac myocytes, suggesting a possible contribution by PKC-δ to the TnI phosphorylation (Jideama et al., 1966). The precise conditions whereby the individual PKC isozymes can be selectively activated in myocytes have not yet been determined. In addition, consideration must also be given to the interactions of phosphorylated TnI with other PKC-phosphorylated myofilament proteins such as TnT, which can markedly decrease actomyosin MgATPase activity (Noland & Kuo, 1991, 1992), and MLC-2, which can increase actomyosin MgATPase activity and Ca^{2+} sensitivity (Noland & Kuo, 1993b). The use of phosphorylation site mutants in *in vitro* experiments, such as reported here, and expression of the mutants in cardiac myocytes could help to isolate effects due to activation of individual PKC isozymes as well as help determine the functional roles of myofilament protein phosphorylation.

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