Phosphorylation Specificities of Protein Kinase C Isozymes for Bovine Cardiac Troponin I and Troponin T and Sites within These Proteins and Regulation of Myofilament Properties*

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Protein kinase C (PKC) isozymes α, δ, ε, and ζ, shown to be expressed in adult rat cardiomyocytes, displayed distinct substrate specificities in phosphorylating troponin I and troponin T subunits in the bovine cardiac troponin complex. Thus, because they have different substrate affinities, PKC-α, δ, and ε phosphorylated troponin I more than troponin T, but PKC-ζ conversely phosphorylated the latter more than the former. Furthermore, PKC isozymes exhibited discrete specificities in phosphorylating distinct sites in these proteins as free subunits or in the troponin complex. Unlike other isozymes, PKC-δ was uniquely able to phosphorylate Ser-23/Ser-24 in troponin I, the bona fide phosphorylation sites for protein kinase A (PKA); and consequently, like PKA, it reduced Ca\(^{2+}\) sensitivity and maximal activity of MgATPase of reconstituted actomyosin S-1. In addition, PKC-δ, like PKC-α, readily phosphorylated Ser-43/Ser-45 (sites common for all PKC isozymes) and reduced maximal activity of MgATPase. In this respect, PKC-δ functioned as a hybrid of PKC-α and PKA. In contrast to PKC-α, δ, and ε, PKC-ζ exclusively phosphorylated two previously unknown sites in troponin T. Phosphorylation of troponin T by PKC-α resulted in decreases in both Ca\(^{2+}\) sensitivity and maximal activity, whereas phosphorylation by PKC-ζ resulted in a slight increase of the Ca\(^{2+}\) sensitivity without affecting the maximal activity of MgATPase. Most of the in vitro phosphorylation sites in troponin I and troponin T were confirmed in situ in adult rat cardiomyocytes.

The present study has demonstrated for the first time distinct specificities of PKC isozymes for phosphorylation of two physiological substrates in the myocardium, with functional consequences.

Actomyosin MgATPase, the molecular motor of cardiac muscle contraction, is regulated by Tn\(^{1}\) and Tm. The Tn complex consists of three subunits, i.e. the Ca\(^{2+}\)-binding TnC, the ATPase-inhibiting TnI, and the Tm-binding TnT. The current understanding of Ca\(^{2+}\)-stimulated cardiac contraction at the level of contractile apparatus, based largely on evidence obtained from skeletal muscle studies, can be summarized as follows. In the relaxed state (in the absence of Ca\(^{2+}\)), TnI and Tm prevent cross-bridge cycling between actin and myosin heads through steric blocking of myosin-binding sites on actin (1–3) and possibly by inhibiting a kinetic step in the actomyosin ATP hydrolysis cycle, such as release of P\(_i\) (4–6) or ADP (7) from the actin-myosin complex. The contractile apparatus becomes activated in the presence of Ca\(^{2+}\), which is a process beginning with a conformational change in TnC upon binding of Ca\(^{2+}\) (8), resulting in an increased affinity of TnC for the ATPase-inhibiting region of TnI (9) and eventually functional detachment of TnI from actin (10). This allows Tm to roll toward the center of the actin helix groove and unmask myosin-binding sites on actin and ultimately leads to MgATPase activation and muscle contraction (11, 12).

In addition to direct activation by Ca\(^{2+}\) through its binding to TnC, actomyosin MgATPase can be further modulated by phosphorylation of contractile proteins (13). It has been reported that phosphorylation of TnI by PKA resulted in decreased affinity of Ca\(^{2+}\) for TnC without affecting the maximal activity of MgATPase (13–16). We reported earlier that TnI was also effectively and stoichiometrically phosphorylated by PKC in vitro (16–19) and in situ in adult rat cardiomyocytes (20) at multiple and similar sites, resulting in decreased maximal activity of the enzyme (16, 19–22) that was accompanied by altered interactions of phosphorylated TnI with other contractile protein components (21, 22). More recently, with the use of TnI mutants in which several identified phosphorylation sites (18) were substituted by Ala or deleted, we have identified that phosphorylation of Ser-43/Ser-45 by PKC was largely responsible for the reduced ATPase activity (16). We further observed that Ser-23/Ser-24, the bona fide PKA phosphorylation sites in TnI (13), could be cross-phosphorylated by PKC when TnI was exhaustively phosphorylated, leading to decreases in both Ca\(^{2+}\) sensitivity and maximal activity of the enzyme (16). Like TnI, TnT has been shown to be phosphorylated stoichiometrically and at multiple sites by PKC in vitro (17–19), leading to reduced Ca\(^{2+}\)-stimulated actomyosin MgATPase activity (19, 21). This effect was characterized by reduced affinity of the phosphorylated TnT toward Tm-actin tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.

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§ The abbreviations used are: Tn, troponin; Tm, tropomyosin; TnC, TnI, and TnT, troponin C, I, and T subunits, respectively; PKC, protein kinase C; PKA, protein kinase A; PS, phosphatidylserine; TPA, 12-O-tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.

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and accordingly reduced affinity of the thin filament (regulated actin) toward myosin (21). Although TnT was also phosphorylated by PKC in adult rat cardiomyocytes (20, 23), its in situ phosphorylation sites have not yet been determined.

PKC is the product of a gene superfamily, and 13 subspecies have been identified (24). Expression of PKC isoforms in cardiomyocytes is developmentally regulated, i.e., higher numbers and levels of the isoforms have been found in fetal/neonatal than in adult cells (25–28). The current consensus is that in adult cardiomyocytes PKC-ε is abundantly expressed (25–29); PKC-δ and -ζ are readily detectable (25–29); PKC-α is either absent (25–27) or present (28, 29); and PKC-β and -γ are not expressed (25–28). In agreement with Puceat et al. (28) and Ventura et al. (29), we could readily detect PKC-α by Western blots and the PS/Ca2+/diacylglycerol-dependent protein kinase activity (most likely due to PKC-α) in the homogenates of adult rat cardiomyocytes. It has been hypothesized that the individual PKC isoforms may have specific cellular functions as a consequence of, in part, phosphorylation of specific substrate proteins (24, 30). Because brain pan PKC preparations (mixtures of isoforms) were used in all of our earlier phosphorylation and functional studies of contractile proteins (16–22), involvements of the individual isoforms remain unclear. In the present studies, we have investigated these crucial issues. We found that PKC isoforms exhibited distinct phosphorylation specificities with certain functional consequences.

**EXPERIMENTAL PROCEDURES**

**Preparations of Cardiac Contractile Proteins, Synthetic Peptides, Recombinant 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 (31) and stored at −70 °C in 50 mM Tris-HCl (pH 8.0) containing 6 mM urea, 1 mM EDTA, and 15 mM 2-mercaptoethanol. Tn complex was reconstituted from the three individual subunits (31). Tm (32), F-actin (33), and myosin S-1 (33) were prepared as described by others. In order to prevent oxidation of TnI and Tm, 1 mM DTT or 15 mM 2-mercaptoethanol was added to all solutions throughout the preparation and reconstitution procedures. TnI and TnT peptides containing phosphorylation sites were synthesized at the Emory University Microchemical Facility. Recombinant PKC isoforms (ε, δ, ε, and ζ) were purified from baculovirus-infected Sf9 insect cells (34–36). PKA was purified from bovine heart extracts (37).

**Phosphorylation of Contractile Proteins, TnI and TnT Peptides, and Phosphopeptide Analysis—**Prior to phosphorylation, TnI and TnT were dialyzed against 10 mM Tris-HCl (pH 7.5) containing 1 mM DTT with sequential KCl concentrations of 0.1, 0.7, and 0.3 M (16). The conditions of phosphorylation by PKC and PKA were essentially as described elsewhere (16, 19, 21, 22) except that PKC-α was activated by PS (20 μg/ml)/CaCl2 (100 μM)/diolene (5 μg/ml); PKC-δ and -ε were activated by PS/oligene but omitting CaCl2, and PKC-ζ was activated by PS only. KCl (0.3 M) was included in all phosphorylation studies in order to keep TnI and TnT in solution. The apparent Km values were determined by densitometric analysis (Lynx 400 video densitometer) of the autoradiograms. The findings were confirmed in three other experiments.

**RESULTS**

Distinct substrate specificities of the four PKC isoforms (ε, δ, ε, and ζ) shown to be expressed in adult rat cardiomyocytes (25–29) for phosphorylation of TnI and TnT in the bovine cardiac Tn complex (0.5–20 μM) were observed (Fig. 1). Thus, because they display different substrate affinities (indicated by apparent Km values), PKC-α phosphorylated TnI considerably more than TnT; PKC-δ and PKC-ε phosphorylated TnT much more than TnI, and PKC-ζ phosphorylated TnT much more than TnI. Furthermore, differential substrate inhibitions of the isoforms were also noted at high Tn concentrations. Notably, at 20 μM TnI, phosphorylation of TnI by PKC-α was markedly reduced, that by PKC-δ was moderately attenuated, and that by PKC-ζ was nearly completely diminished; phosphorylation of TnT by PKC-δ or PKC-ε was also nearly completely inhibited. These findings indicated that PKC isoforms displayed discrete specificities for the two proteins present in the Tn complex with respect to phosphorylation extents and substrate affinities or inhibitions. The above findings were confirmed in parallel, time-dependent (5–120 min) experiment where 2 μM Tn complex was phosphorylated by the isoforms (Fig. 2). The
same conclusion was reached in separate experiments where adult rat cardiac Tn, instead of bovine Tn, was used as substrate.\(^2\) As previously reported (13, 15, 20, 39), PKA phosphorylated only TnI in the bovine or rat cardiac Tn complex (data not shown).

It was suspected that PKC isozymes might exhibit further defined substrate specificities in that they would differentially phosphorylate multiple sites in TnI. We found that this was indeed the case (Fig. 3). At about the midpoint (0.2–1.0 mol of P/mol) of maximal phosphorylation of TnI by the individual isozymes and PKA, the relative extents of \(^32\)P incorporation into the individual tryptic phosphopeptide spots (in decreasing order) were 2 >> 3A >> 3B >> 4 >> 5–6 for PKC-\(\alpha\), 1–3A >> 2 >> 3B >> 6–4 for PKC-\(\delta\), 2 >> 4–3A >> 5–6 for PKC-\(\varepsilon\), 4–2 >> others for PKC-\(\zeta\), and 5 >> others for PKA. The most noteworthy finding was that \(^32\)P incorporation into spot 5 was uniquely and preferentially catalyzed by PKC-\(\delta\), mimicking the action of PKA. Minor labeling of spot 5 was observed only when TnI was exhaustively phosphorylated by other PKC isozymes, such as that shown for PKC-\(\alpha\) (with an extent of phosphorylation of 2.5 mol of P/mol). It was also observed that \(^32\)P labeling of spot 4 was selectively catalyzed by PKC-\(\varepsilon\) and -\(\zeta\) and that labeling of spots 1 and 3A by PKC-\(\varepsilon\) was the lowest. We have determined earlier (16, 18) that phosphopeptide spot 1 contained phosphorylation site Ser-194 in TnI that was phosphorylated by brain pan PKC,\(^3\) a site previously identified by others (40). It was intriguing that PKC-\(\zeta\) exclusively catalyzed \(^32\)P incorporation into spots 8 and 9, previously unrecognized major phosphopeptides containing unknown phosphorylation sites. The phosphorylation sites in spots 2 and 7 are also unknown at present.

In order to establish the physiological relevance of sites phosphorylated in the free TnI and TnT subunits, we examined whether the same sites in the respective subunits would also be phosphorylated in the Tn complex or in cardiomycocytes. Phosphopeptide maps, essentially the same as those shown above for the free subunits (Figs. 3 and 4), were observed for phosphorylation (approximately 1 mol/mol) of the Tn complex by the respective PKC isozymes (data not shown). A minor difference, however, was noted for the latter. PKC-\(\delta\) and -\(\varepsilon\) (compared with -\(\alpha\)) preferentially phosphorylated spot 3A (compared with spots 1 and 2) in TnI. Certain in vitro phosphorylation sites in the free TnI subunit shown in Fig. 3 were also identified in cardiomycocytes incubated under the basal condition, and phosphorylation of these sites was stimulated by TPA (Fig. 5). These findings, while confirming an earlier study (20), also showed that phosphorylation in spot 5 (Ser-23/Ser-24) that was enhanced by TPA was likely due to activation of PKC-\(\delta\). It was surprising that the site in spot 8 in TnT was principally phosphorylated in cardiomycocytes and that this phosphorylation was not stimulated by TPA (Fig. 5). Because this site was specific for PKC-\(\zeta\) (Fig. 4) and this atypical isozyme is not activated by diacylglycerol (or TPA) and Ca\(^{2+}\) (24), it seemed most noteworthy finding was that \(^32\)P labeling of spot 4 was selectively catalyzed by PKC-\(\delta\), mimicking the action of PKA. Minor labeling of spot 5 was observed only when TnI was exhaustively phosphorylated by other PKC isozymes, such as that shown for PKC-\(\alpha\) (with an extent of phosphorylation of 2.5 mol of P/mol). It was also observed that \(^32\)P labeling of spot 4 was selectively catalyzed by PKC-\(\varepsilon\) and -\(\zeta\) and that labeling of spots 1 and 3A by PKC-\(\varepsilon\) was the lowest. We have determined earlier (16, 18) that phosphopeptide spot 1 contained phosphorylation site Ser-194 in TnI that was phosphorylated by brain pan PKC,\(^3\) a site previously identified by others (40). It was intriguing that PKC-\(\zeta\) exclusively catalyzed \(^32\)P incorporation into spots 8 and 9, previously unrecognized major phosphopeptides containing unknown phosphorylation sites. The phosphorylation sites in spots 2 and 7 are also unknown at present.

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that PKC-\(\zeta\) is likely to be the major PKC subspecies that phosphorylates TnT in vivo in cardiomyocytes. Other in vitro phosphorylation sites in TnT seen in Fig. 4, however, were slightly stimulated by TPA (Fig. 5).

The phosphorylation kinetics for bovine cardiac TnI and synthetic TnI peptides were studied in order to further examine the substrate specificities of PKC isozymes and PKA (Fig. 6). The representative peptides used were TnI-(17–30), TnI-(33–53), and TnI-(134–154), which contain phosphorylation sites Ser-23/Ser-24, Ser-43/Ser-45, and Thr-144, respectively. It was found that PKC-\(\alpha\), -\(\delta\), and -\(\epsilon\), but not PKC-\(\zeta\), effectively phosphorylated TnI and that a marked substrate inhibition at 10 \(\mu\)M TnI (the highest concentration tested) was noted for PKC-\(\alpha\) but not for other isozymes. These findings were consistent with those from the studies using the Tn complex mentioned above (Figs. 1 and 2), where PKC-\(\zeta\) when compared with other isozymes phosphorylated TnI poorly, and TnI phosphorylation by PKC-\(\alpha\) was highly susceptible to substrate inhibition. PKA preferentially phosphorylated TnI-(17–30) compared with the other two peptides, in line with the findings that PKA selectively phosphorylated Ser-23/Ser-24 in spot 5 shown above (Fig. 3). We found that PKC-\(\delta\), unlike other isozymes but more like PKA, favorably phosphorylated TnI-(17–30), which is in agreement with its ability to effectively incorporate \(^{32}\)P into spot 5 in TnI shown above (Fig. 3). Similar kinetic studies were also carried out for TnT and its peptides (Fig. 7). The representative peptides used were TnT-(179–198), TnT-(191–209), and TnT-(268–284), which contain phosphorylation sites Thr-190/Thr-194, Thr-194/Thr-199, and Thr-280, respectively. As judged by the initial phosphorylation rates, TnT was a poorer substrate than TnI was for PKC-\(\alpha\), -\(\delta\), and -\(\epsilon\). In contrast, PKC-\(\zeta\) phosphorylated both TnI and TnT at comparable, although low, rates. These findings were roughly in agreement with the relative rates (and extents) of phosphorylation of TnI and TnT subunits in the Tn complex by PKC isozymes shown earlier in Figs. 1 and 2, with the exception that PKC-\(\zeta\) phosphorylated TnT much better than TnI when these proteins were in the Tn complex compared with when they were the free subunits. The synthetic peptides were generally poorer substrates than the intact TnT. Although a correlation between phosphorylation of synthetic peptides and phosphopeptide maps of TnT (Fig. 4) was not uncovered, the following observations deserved comment. The relative phosphorylation efficacies of peptides TnT-(179–198), TnT-(191–209), and TnT-
PKC- to identify the site in spot 2 preferentially phosphorylated by the poorest substrate for the two isozymes. It would be of interest to determine whether PKC- (15, 16) or pan PKC (16) reduced the Ca\(^{2+}\) activity and that phosphorylation of Ser-23/Ser-24 by PKA and Ser-43/Ser-45 by pan PKC decreased the maximal activity, whereas that by PKA exclusively caused a pronounced decrease in the Ca\(^{2+}\) sensitivity without affecting the activity of MgATPase (Fig. 9). Because phosphorylation of TnT by PKC- yielded marked decreases in both Ca\(^{2+}\) sensitivity and activity of MgATPase, in line with earlier studies with pan PKC (19, 20, 21), it was somewhat surprising that phosphorylation by PKC- was unique in its ability to selectively phosphorylate unknown sites in TnT. Because phosphorylation of TnI by PKC- yielded similar sites (therefore likely having similar effects) that were distinct from those phosphorylated by PKC-, we chose only PKC- and - for the functional study (Fig. 9).Because phosphorylation of TnT by PKC- yielded marked decreases in both Ca\(^{2+}\) sensitivity and activity of MgATPase, in line with earlier studies with pan PKC (19, 20, 21), it was somewhat surprising that phosphorylation by PKC- was unique in its ability to selectively phosphorylate unknown sites in TnT. It was also noted that phosphorylation of TnI by PKC- produced effects on MgATPase that were more pronounced than the effects of phosphorylation of TnI by PKC- or PKA.

**DISCUSSION**

We have presented evidence in the present study showing that PKC isoforms displayed distinct specificities in phosphorylating two physiological substrates in the myocardium (i.e. TnI and TnT), various sites within these proteins, and synthetic peptides containing the respective phosphorylation sites. It seems particularly worth noting that (a) PKC- was unique among all isoforms in its ability to mimic PKA in phosphorylating Ser-23/Ser-24 of TnI, resulting in a decreased Ca\(^{2+}\) sensitivity of actomyosin S-1 MgATPase, and (b) PKC- was unique in its ability to selectively phosphorylate unknown sites in TnT, leading to a slight increase in the Ca\(^{2+}\) sensitivity without affecting the activity of MgATPase. The crucial issues needed to be addressed are whether the in vitro phosphorylation of TnI and TnT is physiologically relevant and which PKC isoforms are functionally involved in cardiomyocytes. As reported earlier (20), the in vitro TnI phosphorylation sites for the isoforms have been collectively con-
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The values were calculated from the data given in Figs. 8 and 9 and are shown as the means ± S.E. of three experiments. The maximal enzyme activity is expressed as s⁻¹ (mol of ATP hydrolized per mol of S-1 per s). The numbers in parentheses are percentages of values obtained for phosphorylated TnI or TnT compared with respective unphosphorylated controls, which are taken as 100%.

| Phosphorylation | Maximal Ca²⁺-stimulated MgATPase activity | EC₅₀ for Ca²⁺ μM | Hill coefficient |
|-----------------|-------------------------------------------|-----------------|-----------------|
| TnI             |                                           |                 |                 |
| None (control)  | 0.46 ± 0.01 (100)                         | 1.4 ± 0.1       |                 |
| PKC-α           | 0.30 ± 0.01* (65)                         | 1.7 ± 0.2       |                 |
| PKC-δ           | 0.39 ± 0.02* (85)                         | 1.1 ± 0.1       |                 |
| PKA             | 0.45 ± 0.02 (98)                          | 1.0 ± 0.1       |                 |
| TnT             |                                           |                 |                 |
| None (control)  | 0.56 ± 0.02 (100)                         | 1.3 ± 0.1       |                 |
| PKC-α           | 0.18 ± 0.01* (32)                         | 1.0 ± 0.1       |                 |
| PKC-ζ           | 0.52 ± 0.06 (93)                          | 1.0 ± 0.2       |                 |

* Significantly different from the unphosphorylated control (p < 0.01-0.05, paired Student’s t test).

In conclusion, the present study has demonstrated distinct substrate preferences for PKC isoforms. More importantly, dramatic differences emerged upon examination of phosphorylation sites in TnI and TnT, which appear to correlate in some instances with distinct functional differences. This biochemical approach promises to be very useful in determining the functional specificities of PKC isoforms in cardiac myofibrils.

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