Protein kinase Cε (PKCε) and Src Control PKCδ activation
loop phosphorylation in cardiomyocytes*

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Protein kinase Cδ (PKCδ) is unusual among AGC kinases in that it does not require activation loop (Thr505) phosphorylation for catalytic competence. Nevertheless, Thr505 phosphorylation has been implicated as a mechanism that influences PKCδ activity. This study examines the controls of PKCδ-Thr505 phosphorylation in cardiomyocytes. We implicate phosphoinositide-dependent kinase-1 and PKCδ autophosphorylation in the "priming" maturational PKCδ-Thr505 phosphorylation that accompanies de novo enzyme synthesis. In contrast, we show that PKCδ-Thr505 phosphorylation dynamically increases in cardiomyocytes treated with phorbol 12-myristate 13-acetate or the α1-adrenergic receptor agonist norepinephrine via a mechanism that requires novel PKC isoform activity and not phosphoinositide-dependent kinase-1.

We used a PKCδ overexpression strategy as an initial approach to discriminate two possible novel PKC mechanisms, namely PKCδ-Thr505 autophosphorylation and PKCδ-Thr505 phosphorylation in trans by PKCε. Our studies show that adenovirus-mediated PKCε overexpression leads to an increase in PKCδ-Thr505 phosphorylation. However, this cannot be attributed to an effect of PKCε to function as a direct PKCδ-Thr505 kinase, since the PKCε-dependent increase in PKCδ-Thr505 phosphorylation is accompanied by (and dependent upon) increased PKCδ phosphorylation at Tyr311 and Tyr332. Further studies implicate Src in this mechanism, showing that 1) PKCε overexpression increases PKCδ-Thr505 phosphorylation in cardiomyocytes and Src+ cells but not in SYF cells (that lack Src, Yes, and Fyn and exhibit a defect in PKCδ-Tyr311/Tyr332 phosphorylation), and 2) in vitro PKCδ-Thr505 autophosphorylation is augmented in assays performed with Src (which promotes PKCδ-Tyr311/Tyr332 phosphorylation). Collectively, these results identify a novel PKCδ-Thr505 autophosphorylation mechanism that is triggered by PKCδ overexpression and involves Src-dependent PKCδ-Thr311/Tyr332 phosphorylation.

Traditional models of PKCδ activation have focused on lipid cofactor binding to determinants in the N-terminal regulatory domain that anchor PKCδ to membranes and promote a conformational change that expels the autoinhibitory pseudosubstrate domain from the substrate-binding pocket. This effectively relieves autoinhibition and enables PKCδ-dependent phosphorylation of target substrates. However, we and others recently demonstrated that PKCδ also is dynamically regulated through phosphorylation at a conserved threonine residue in the activation loop (Thr505) (1, 2). Other PKCs require activation loop phosphorylation as a “priming” event to generate a catalytically competent enzyme. In contrast, PKCδ is catalytically active even without activation loop phosphorylation. Rather, activation loop phosphorylation plays a distinctive role to regulate the enzymology (activity, substrate specificity) of membrane-associated allosterically activated PKCδ (1–5). The precise controls and consequences of the coordinate events that govern PKCδ phosphorylation and translocation in highly differentiated cells, such as cardiomyocytes, remain uncertain.

PKCδ activation loop phosphorylation has been attributed to phosphoinositide-dependent kinase-1 (PDK-1, a general AGC activation loop kinase) on the basis of studies examining in vitro phosphorylation events on heterologously overexpressed PKCδ in undifferentiated cell types (3). Our recent studies suggest that this model is not sufficient to describe the control of PKCδ-Thr505 phosphorylation in the heart, where the α1-adrenergic receptor agonist norepinephrine (NE) and PMA increase PKCδ-Thr505 phosphorylation via a mechanism that is blocked by GF109203X (a relatively nonselective inhibitor of most PKC isoforms), and not by Go6976 (an inhibitor that preferentially blocks calcium-sensitive PKC isoforms (1)). These results provided tentative evidence that the dynamic stimulus-dependent increase in PKCδ-Thr505 phosphorylation is mediated by an nPKC isoform and not PDK-1 (which is a GF109203X-insensitive enzyme). Since this conclusion runs counter to the general consensus that PKC activation loop phosphorylations are via a PDK-1-dependent mechanism, the relative roles of PDK-1 and nPKC isoforms as PKCδ-Thr505 kinases are examined in greater detail in this study.

**EXPERIMENTAL PROCEDURES**

**Materials**—All antibodies were from Cell Signaling Technology with the following exceptions: anti-PKCδ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Tyr(P), anti-PKCα, and anti-AKT (Upstate Biotechnology); anti-PKCε (BD Transduction); and anti-Src (Oncogene).

**Cell Culture**—Cardiomyocytes were isolated from the hearts of 2-day-old Wistar rats by a trypsin dispersion procedure using a differential attachment procedure to enrich for cardiomyocytes followed by irradiation as described in previous publica-
The Control of PKC-Thr\textsuperscript{505} Phosphorylation in Cardiomyocytes

![Image](https://via.placeholder.com/150)

**A.** NE- and PMA-dependent PKC-Thr\textsuperscript{505} phosphorylation mechanisms in cardiomyocytes. Immunoblots of cell extracts from cardiomyocyte cultures pretreated for 45 min with vehicle, GF109203X (GFX; 5 \textmu M in A and B or the indicated concentrations in C), Go6976 (5 \textmu M) (A), LY294002 (LY; 10 \textmu M) (A), or UCN-01 (0.1 \textmu M in B, the indicated concentrations in C), followed by stimulations for 10 min with NE (10 \textmu M), PMA (300 \textmu M), or H\textsubscript{2}O\textsubscript{2} (5 mM) as indicated. pT, phosphothreonine; pS, phosphoserine.

**B.** AKT-Thr\textsuperscript{308} and PKC-Thr\textsuperscript{505} phosphorylation increases dynamically in NE- and PMA-dependent activation loops. Samples were subjected to SDS-PAGE, autoradiography, and immunoblotting with the indicated antibodies.

**C.** PKC-Thr\textsuperscript{505} and PKD-Thr\textsuperscript{744/748} phosphorylation increases dynamically in NE- and PMA-dependent activation loops. Samples were subjected to SDS-PAGE, autoradiography, and immunoblotting with the indicated antibodies.



The yield of cardiomyocytes typically is 2.5–3 \times 10^6 cells/neonatal ventricle. Cells were plated on protramate sulfate-coated culture dishes at a density of 5 \times 10^6 cells/100-mm dish. Experiments were performed on cultures grown for 5 days in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum and then serum-deprived for the subsequent 24 h. Primary cardiac fibroblast cultures were obtained from the cells adherent to the culture dishes during the preplating step, as described previously (6).

PKCe\textsuperscript{-/-} mouse embryonic fibroblast cell lines (MEFs) were described previously and generously provided by Dr. Peter Parker (7). PKCe\textsuperscript{-/-} MEFs were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 100 \mu g/ml hygromycin at 37 °C in a 5% CO\textsubscript{2} atmosphere.

Adenoviral Infections—Cell infections with adenoviral vectors that drive expression of wild-type (WT) or kinase-dead (KD) PKC\delta, WT-PKCe, KD-PKCe, or \beta-galactosidase (as a control) were according to protocols published previously (8). Protein extracts were prepared 44–48 h following infections.

Immunoprecipitation and Immunoblot Analysis—Immunoblotting on lysates or immunoprecipitated PKC\delta was according to methods described previously or the manufacturer’s instructions (6). In each figure, each panel represents the results from a single gel (exposed for a uniform duration); detection was with enhanced chemiluminescence. All results were replicated in at least four experiments on separate culture preparations.

Preparation of Soluble and Particulate Fractions—Cells were washed with phosphate-buffered saline and then immediately transferred to ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 6 mM \beta-mercaptoethanol, 50 \mu g/ml aprotinin, 48 \mu g/ml leupeptin, 5 \mu M pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, and 50 mM NaF), lysed by sonication, and centrifuged at 100,000 \times g for 1 h. The supernatant was saved as the soluble fraction, and the particulate fraction was solubilized in SDS-PAGE sample buffer.

In Vitro Phosphorylation of PKC\delta by Src—0.1 \mu g of recombinant human PKC\delta was preincubated for 15 min at 30 °C in the absence or presence of Src kinase (0.66 units) in 160 \mu l of a reaction buffer containing 43 mM Tris-Cl, pH 7.5, 6.25 mM MgCl\textsubscript{2}, 10 mM MnCl\textsubscript{2}, 0.75 mM EDTA, 0.77 mM EGTA, 0.3 mM dithiothreitol, 125 mM NaCl, 5% glycerol, 0.006% Brij-35, 0.04 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, and [γ\textsuperscript{32}P]ATP (13 \mu Ci, 83 \mu M) in the absence or presence of phosphatidylinerine/PA. Samples were subjected to SDS-PAGE, autoradiography, and immunoblotting with the indicated antibodies.

**RESULTS**

PMA Promotes PKC-\textsuperscript{Thr}505 Phosphorylation via a Mechanism That Requires nPKC Activity and Not PDK-1—Our initial experiments used a pharmacologic approach to identify the PKC-\textsuperscript{Thr}505 phosphorylation mechanism in NE- and PMA-treated cardiomyocytes. Fig. 1A shows that PKC\delta retains a low level of Thr\textsuperscript{505} phosphorylation in resting cardiomyocytes; PKC-\textsuperscript{Thr}505 phosphorylation increases dynamically in response to treatment with either NE or PMA. These stimulus-induced increases in PKC-\textsuperscript{Thr}505 phosphorylation are blocked by GF109203X (a general PKC isoform inhibitor) but not by Go6976 (which selectively blocks calcium-sensitive PKC isoforms, the PKC effector protein kinase D (PKD), and JAK2 (8, 9)). Since some PDK-1-dependent phosphorylation mechanisms require the generation of 3'-phosphoinositides that colocalize PDK-1 with substrates at the plasma membrane, the effect of LY294002 (a phosphatidylinositol 3-kinase inhibitor) also was examined. Fig. 1A shows that LY294002 does not block agonist-dependent PKC-\textsuperscript{Thr}505 phosphorylation.

These pharmacologic studies implicate a nPKC activity in agonist-dependent PKC-\textsuperscript{Thr}505 phosphorylation. Since they run counter to the prevailing notion that PKC-\textsuperscript{Thr}505 phosphorylation is mediated by PDK-1 (a GF109203X-insensitive enzyme), we performed a more detailed analysis of the relative roles of PDK-1 and PKC isoforms as in vivo PKC-\textsuperscript{Thr}505 kinases. These studies took advantage of the distinct inhibitory profiles of GF109203X and UCN-01 (a 7-hydroxyxysterosporine derivative that was first identified as a PKC inhibitor and subsequently characterized as an even better inhibitor of PDK-1 (10)) (Fig. 1, B and C). Stimulus-dependent activation loop phosphorylation events on PKC\delta (Thr\textsuperscript{505}), AKT (Thr\textsuperscript{308}), and PKD (PKD-\textsuperscript{Ser}744/748) were examined in parallel. AKT activation loop phosphorylation was tracked as a control for PDK-1 inhibition by UCN-01, since AKT is a bona fide PDK-1 target. Similarly, PKD activation loop phosphorylation was included to control for PKC inhibition by GF109203X, since PKD activa-
tion loop phosphorylation is mediated by a nPKC (PKCδ or PKCe, depending upon the specific stimulus and cell type (11)); PKD phosphorylation via PDK-1-dependent mechanisms has not been reported.

Fig. 1B shows that H₂O₂ increases AKT-Thr³⁰⁸ phosphorylation via a PDK-1-dependent mechanism that is fully abrogated by a very low concentration of UCN-01 (<0.1 μM). PMA does not increase AKT-Thr³⁰⁸ phosphorylation, and H₂O₂-dependent AKT-Thr³⁰⁸ phosphorylation is not blocked by GF109203X. These results identify distinct inhibitory profiles for UCN-01 and GF109203X and implicate PDK-1 (and effectively exclude PKC isoforms) as the AKT-T³⁰⁸ kinase.

Fig. 1C shows that PMA and NE increase PKCδ-Thr⁵⁰⁵ and PKD-Ser⁷⁴⁴/⁷⁴⁸ phosphorylation via a mechanism that is blocked by 1–3 μM GF109203X. Although UCN-01 also inhibits the PMA- and NE-dependent increases in PKCδ-Thr⁵⁰⁵ and PKD-Ser⁷⁴⁴/⁷⁴⁸ phosphorylation, these inhibitory actions of UCN-01 are detected only at high concentrations (>10-fold higher that the UCN-01 concentrations required to abrogate H₂O₂-dependent AKT-Thr³⁰⁸ phosphorylation). This presents a promiscuous action of high UCN-01 concentrations to inhibit PKC isoforms. Collectively, these results indicate that 1) inhibitor studies with UCN-01 and GF109203X can be used to distinguish PDK-1-dependent AKT-Thr³⁰⁸ phosphorylation (which is blocked by low UCN-01 concentrations but not by GF109203X) from PKC-dependent phosphorylation of PKD-Ser⁷⁴⁴/⁷⁴⁸ and PKCδ-Thr⁵⁰⁵ (which are blocked by GF109203X and only high UCN-01 concentrations), and 2) the dynamic cycling of PKCδ between a fully active (Thr⁵⁰⁵-phosphorylated) and a less active (unphosphorylated) form in response to PMA or NE is mediated by a GF109203X-sensitive kinase with properties resembling a nPKC isoform. This could involve either a PKCδ-Thr⁵⁰⁵ autophosphorylation reaction or PKCδ-Thr⁵⁰⁵ phosphorylation in trans by PKCe.

PDK-1 Contributes to Activation Loop Phosphorylation during de Novo PKCδ Synthesis—The evidence that PKCδ-Thr⁵⁰⁵ phosphorylation is dynamically controlled through an nPKC-dependent mechanism is at odds with the prevailing model that attributes activation loop phosphorylation (for PKCδ and other AGC kinases) to PDK-1. However, this discrepancy might be reconciled if PKCδ-Thr⁵⁰⁵ phosphorylation is controlled through dual mechanisms, with an nPKC activity contributing to the dynamic regulation of PKCδ-Thr⁵⁰⁵ phosphorylation in response to receptor activation and PDK-1 functioning to phosphorylate the activation loop site of newly synthesized PKCδ. Therefore, we used an adenovirus-mediated gene transfer strategy to examine activation loop phosphorylation on heterologously overexpressed WT- and KD-PKCδ enzymes. We previously showed that WT-PKCδ is expressed at levels ~7–8 times higher than endogenous PKCδ under these conditions. Fig. 2 shows that WT-PKCδ and KD-PKCδ are both constitutively Thr⁵⁰⁵ phosphorylated in resting cardiomyocytes, indicating that PKCδ activity is not absolutely required for activation loop phosphorylation. However, at similar MOIs, KD-PKCδ expression is consistently ~3–4 times lower than WT-PKCδ expression. Moreover, Thr⁵⁰⁵ phosphorylation of KD-PKCδ is reduced relative to WT-PKCδ, when protein loading is normalized for differences in protein expression. These results suggest that an autophosphorylation mechanism contributes to Thr⁵⁰⁵ phosphorylation on newly synthesized PKCδ in cells. This phosphorylation defect presumably limits KD-PKCδ expression, since priming phosphorylations play a role to stabilize the phosphatase-/protease-resistant conformation of the enzyme (12).

Fig. 2 also shows that UCN-01 treatment (to inhibit PDK-1) results in a modest decrease in WT-PKCδ-Thr⁵⁰⁵ phosphorylation; UCN-01 completely abrogates KD-PKCδ-Thr⁵⁰⁵ phosphorylation. Collectively, these results indicate that the activation loop site of newly synthesized PKCδ is phosphorylated via a dual mechanism involving both an autophosphorylation reaction and PDK-1-dependent phosphorylation in trans.

PKCe Overexpression Increases PKCδ-Thr⁵⁰⁵ Phosphorylation—Our pharmacologic studies implicate an nPKC activity (either PKCδ-Thr⁵⁰⁵ autophosphorylation or PKCδ-Thr⁵⁰⁵ phosphorylation in trans by PKCe) in PMA-dependent PKCδ-Thr⁵⁰⁵ phosphorylation. We used an adenovirus-mediated gene transfer strategy to overexpress PKCe and test the hypothesis that PKCe acts as a PKCδ-Thr⁵⁰⁵ kinase. Fig. 3A shows that WT-PKCe overexpression (MOI of 100 pfu/cell) increases PKCδ-Thr⁵⁰⁵ phosphorylation. This is not associated with a change in PKCδ protein abundance. It is specific to catalytically active PKCe; KD-PKCe and WT-PKCα do not increase PKCδ-Thr⁵⁰⁵ phosphorylation (Fig. 3 and data not shown).

Although these results could suggest that PKCe overexpression increases PKCδ phosphorylation by acting as a direct PKCδ-Thr⁵⁰⁵ kinase, other mechanisms are possible and were considered. Fig. 3 shows that Ad-WT-PKCe overexpression does not lead to any detectable changes in PDK-1 protein expression, PDK-1-Ser²⁴¹ (activation loop) phosphorylation (panel A); Ad-wt-PKCe overexpression also does not increase PDK-1 activity, measured as basal or agonist-dependent AKT phosphorylation.
phosphorylation (panel B). These results effectively exclude an indirect effect of PKCe overexpression to regulate PKCδ via PDK-1.

Our previous studies showed that the PMA-dependent increase in PKCδ-Thr505 phosphorylation is confined to the pool of enzyme recovered in the particulate fraction. Therefore, we considered an alternative indirect mechanism for nPKC isoform cross-regulation involving an effect of PKCe to regulate a lipid-modifying enzyme (such as a phospholipase C or diacylglycerol kinase (13, 14)), leading to increased DAG levels and the stabilization of PKCδ at membranes. To address this alternative mechanism for PKCe-dependent PKCδ-Thr505 phosphorylation, we compared the subcellular distributions of PKCα and PKCδ in resting and PMA-treated Ad-β-galactosidase and Ad-PKCe cultures. Fig. 4 shows that PKCα is recovered largely in the soluble fraction, whereas PKCδ partitions between the soluble and particulate fractions of resting Ad-β-galactosidase and Ad-PKCe cultures. These results argue that the effect of PKCe overexpression to increase PKCδ-Thr505 phosphorylation cannot readily be attributed to a gross change in PKCδ targeting to membranes. Rather, Fig. 4 shows that PKCe overexpression leads to dysregulated PKCδ-Thr505 phosphorylation. PKCδ-Thr505 immunoreactivity is confined to the pool of PKCδ that localizes to the particulate fraction following PMA treatment in Ad-β-galactosidase cultures. In contrast, PKCδ-Thr505 immunoreactivity is detected in both the soluble and particulate fractions of resting Ad-PKCe cultures. Fig. 4 also shows that PMA treatment for 24 h leads to the complete loss of PKC immunoreactivity in Ad-β-galactosidase cultures, whereas the Thr505-phosphorylated form of PKCδ (and lesser amounts of PKCe) accumulates in the particulate fraction of Ad-PKCe cultures under these conditions. These results indicate that Ad-PKCe overexpression leads to a defect in PKC down-regulation.

PKCe Overexpression Increases PKCδ-Tyr311/Tyr332 Phosphorylation—The studies thus far identify PKCδ as a downstream target of the PKCe signaling pathway but neither implicate nor refute the role of PKCe as a direct PKCδ-Thr505 kinase. Therefore, additional mechanisms for nPKC cross-talk were considered. In particular, PKCδ is a well known target for regulated tyrosine phosphorylation. We previously demonstrated that H2O2 increases PKCδ phosphorylation at Tyr311 (8). Other studies identify PKCδ phosphorylation at Tyr332 in cells subjected to oxidative stress (15). Although PKCδ-Thr505 and tyrosine phosphorylations are generally viewed as independently regulated events (and there was no a priori reason to anticipate that PKCe overexpression would lead to PKCδ tyrosine phosphorylation), Fig. 5A provides surprising evidence that WT-PKCe (but not KD-PKCe) markedly increases basal and H2O2-dependent PKCδ tyrosine phosphorylation. The PKCe-dependent increase in PKCδ tyrosine phosphorylation is detected with an anti-phospho-PKCδ-Tyr311 antibody (that can be used directly on cell extracts) as well as with anti-Phospho-Tyr and anti-phospho-PKCδ-Tyr332 antibodies (that require immunoprecipitation; the anti-phospho-PKCδ-Tyr332...
phosphorylation site-specific antibodies detects too many non-specific bands to be informative in studies on cell extracts).

We previously reported that H$_2$O$_2$ increases PKC$_\delta$ tyrosine phosphorylation via an Src-dependent mechanism in cardiomyocytes (8). Fig. 5 shows that the Ad-PKCe-dependent increases in PKC$_\delta$-Tyr$^{311}$ and -Tyr$^{332}$ phosphorylation are not accompanied by a detectable increase in Src protein or Src activity (tracked by an antibody that recognizes Src activation loop phosphorylation, a useful surrogate for Src activity). However, the Ad-PKCe-dependent increment in PKC$_\delta$-Thr$^{505}$ phosphorylation requires Src activity, since PKC$_\delta$-Thr$^{505}$ phosphorylation is not increased in Ad-PKCe cultures treated with PP1 (which inhibits Src activity and PKC$_\delta$ tyrosine phosphorylation). These studies provide novel evidence that PKC$_\delta$ tyrosine and Thr$^{505}$ phosphorylation are interdependent events. Our results indicate that PKCe overexpression leads to Src-dependent PKC$_\delta$ tyrosine phosphorylation and that PKC$_\delta$ tyrosine phosphorylation facilitates further PKC$_\delta$ phosphorylation at Thr$^{505}$ (although these results still do not discriminate a PKC$_\delta$ autophosphorylation reaction from PKC$_\delta$ phosphorylation in trans by PKCe).

Previous studies in genetically engineered mouse models have suggested that PKCe exerts an inhibitory control on PKC$_\delta$ protein expression and/or phosphorylation, which is lost in the PKCe$^{-/-}$ mouse (i.e. PKC protein and/or phosphorylation is already increased in PKCe$^{-/-}$ cells) (16, 17). However, Fig. 6 shows that Ad-PKCe overexpression (at increasing MOIs) leads to a dose-dependent increase in PKC$_\delta$-Thr$^{505}$ and -Tyr$^{311}$ phosphorylation in PKCe$^{-/-}$ MEFs and primary cardiac fibroblast cultures. In these cells (which exhibit robust PKCe overexpression, even at relatively low MOIs), PKC$_\delta$-Thr$^{505}$/PKC$_\delta$-Tyr$^{311}$ phosphorylation increases without an associated change in PKC$_\delta$ abundance at low MOI (2 pfu/cell), whereas PKC$_\delta$ protein also accumulates as PKCe overexpression levels increase (Fig. 6) (data not shown). These results emphasize that PKC$_\delta$ is not necessarily constitutively activated in PKC$^{-/-}$ cells and that PKCe overexpression leads to a general increase in PKC$_\delta$ phosphorylation in many cell types, not just cardiomyocytes.

\*In vitro Kinase Assays Show that Src Phosphorylates PKC$_\delta$ at Tyr$^{311}$/Tyr$^{332}$, Leading to Enhanced PKC$_\delta$ Autophosphorylation at Thr$^{505}$—In vitro kinase assays with recombinant PKC$_\delta$ and active Src provided novel evidence that PKC$_\delta$ undergoes a Src-regulated Thr$^{505}$ autophosphorylation reaction. Fig. 7 shows that PKC$_\delta$ autophosphorylation at a very low rate in the absence of lipids; PKC$_\delta$ autophosphorylation at Thr$^{505}$ is increased by the addition of lipid micelles containing phosphatidylserine/PMA. Src induces only a trivial increase in PKC$_\delta$ tyrosine phosphorylation without lipid cofactors. However, Src induces a prominent increase in PKC$_\delta$-Tyr$^{311}$ and -Tyr$^{332}$ phosphorylation when incubations are performed in the presence of PMA (which does not alter Src activity but rather induces a conformational change that renders PKC$_\delta$ a better substrate for Src). Importantly, Fig. 7 provides unanticipated evidence that the Src-dependent increase in PKC$_\delta$-

\*The Control of PKC$_\delta$-Thr$^{505}$ Phosphorylation in Cardiomyocytes

**FIGURE 6.** Ad-PKCe increases PKC$_\delta$-Thr$^{505}$ (T$^{505}$) and -Tyr$^{311}$ (T$^{311}$) phosphorylation in PKCe$^{-/-}$ MEFs and cardiac fibroblasts. Adenovirus-mediated gene transfer was used to overexpress WT-PKCe (at increasing MOI) in PKCe$^{-/-}$ MEFs and primary cardiac fibroblast cultures. Immunoblotting on lysates prepared 48 h following infection was with the indicated antibodies.

**FIGURE 7.** Src enhances in vitro PKC$_\delta$ autophosphorylation at Thr$^{505}$. In vitro kinase assays were performed with PKC$_\delta$ and active Src as described under "Experimental Procedures." Proteins were separated by SDS-PAGE and subjected to autoradiography and immunoblotting for PKC$_\delta$ protein and phosphorylation. pT, phosphothreonine; pY, phosphotyrosine.
The Control of PKCδ-Thr<sup>505</sup> Phosphorylation in Cardiomyocytes

**DISCUSSION**

PKCδ was originally characterized as an allosterically activated enzyme that transduces signals from stimuli that trigger the hydrolysis of membrane phosphoinositides. However, recent studies identify additional dynamic regulatory controls through activation loop phosphorylation. PKCδ-Thr<sup>505</sup> phosphorylation was originally attributed to PDK-1, based upon an early study showing that PDK-1 complexes with and phosphorylates PKCδ (as well as the extensive literature implicating PDK-1 as a general activation loop kinase for a diverse array of AGC kinases (3, 19)). However, we recently implicated a novel PKC activity in the dynamic agonist-dependent increase in PKCδ-Thr<sup>505</sup> phosphorylation in cardiomyocytes. Results reported herein extend these findings by identifying PKCδ-Thr<sup>505</sup> phosphorylation as an elaborately controlled mechanism that is regulated by PDK-1, PKCδ autophosphorylation, PKCe, and Src, depending upon cell context.

We previously demonstrated that endogenous PKCδ is recovered from resting cardiomyocytes with little to no activation loop phosphorylation. In contrast, PKCδ retains high levels of activation loop phosphorylation when overexpressed (even at relatively modest levels) in cardiomyocyte cultures. We exploited this feature of the overexpressed enzyme to delineate the mechanisms that set basal PKCδ-Thr<sup>505</sup> phosphorylation in cardiac cultures. Our studies show that WT-PKCδ and KD-PKCδ are both recovered with some level of activation loop phosphorylation, indicating that PKCδ activity is not absolutely required for PKCδ-Thr<sup>505</sup> phosphorylation. However, KD-PKCδ exhibits a relatively low level of Thr<sup>505</sup> phosphorylation, even when corrected for the reduced levels of KD-PKCδ protein expression. This residual KD-PKCδ-Thr<sup>505</sup> phosphorylation is completely abrogated by a low concentration of UCN-01 (that selectively inhibits PDK-1). Collectively, these results indicate that PDK-1 cooperates with PKCδ to generate the fully phosphorylated form of PKCδ during de novo enzyme synthesis. Although PKCδ-Thr<sup>505</sup> phosphorylation is generally attributed to PDK-1 (and a role for PKCδ autophosphorylation is not generally considered), it is worth noting that current models implicating PDK-1 as a PKCδ-Thr<sup>505</sup> kinase are based largely upon an early study that used a bacterially expressed PKCδ preparation that retained only very limited catalytic activity (3). In fact, there is ample evidence that related AGC kinases, such as PKA, can be processed to an active form via an autocatalytic mechanism in certain in vivo environments and that PDK1 is not necessarily rate-limiting for PKA activation loop phosphorylation (since PKA activation loop phosphorylation and enzyme activity are similar in PDK1<sup>−/−</sup> and PDK1<sup>+/−</sup> ES cells (20, 21)). Of note, PKCδ protein is detected in PDK1<sup>−/−</sup> ES cells, although PKCδ expression is reduced (presumably as a result of a relative activation loop phosphorylation defect and the associated C-terminal autophosphorylation defect that destabilizes the nascent enzyme (22)). In contrast, PKCe is completely dependent upon PDK-1 for activation loop phosphorylation; PKCe protein is not detectable in PDK1<sup>−/−</sup> ES cells (22).

Although PDK-1 cooperates with PKCδ to generate the fully phosphorylated form of PKCδ during de novo enzyme synthesis, our pharmacologic studies indicate that PDK-1 does not participate in the PMA- or α<sub>1</sub>-adrenergic receptor-dependent mechanism that dynamically increases PKCδ-Thr<sup>505</sup> phosphorylation in cells. Here, PKCδ-Thr<sup>505</sup> phosphorylation is attributable to an nPKC activity, either an autophosphorylation reaction or a trans phosphorylation by PKCe. We used an adenovirus-mediated overexpression strategy as an initial strat-
ergy to determine whether PKCe can act as a direct PKCd-Thr505 kinase. Our studies show that PKCe overexpression leads to a robust increase in PKCd-Thr505 phosphorylation. Had we stopped at this level of analysis, we might have concluded that PKCe acts as a direct in vivo PKCd-Thr505 kinase (and in fact, we have no direct evidence to exclude a role for this mechanism under certain circumstances). However, further studies provided compelling evidence that PKCe increases PKCd-Thr505 phosphorylation indirectly via a mechanism involving Src and Src-dependent PKCd tyrosine phosphorylation. Specifically, we found that PKCe increases PKCd-Thr505 phosphorylation in association with an increase in PKCd phosphor- ylation at Tyr311 and Tyr332. We also found that the PKCe-dependent increase in PKCd-Thr505 phosphorylation requires Src activity (and PKCd tyrosine phosphorylation), since 1) PKCe does not increase PKCd-Thr505 phosphorylation in cardiomyocytes treated with PP1, and 2) the PKCe-dependent increase in PKCd-Thr505 phosphorylation is not detected in SYF cells (that lack Src activity), and it is restored by Src reex- pression (in the Src+ cell line). In vitro studies exposed the underlying mechanism, showing that PKCd undergoes a Thr505 autophosphorylation reaction that is facilitated when assays are performed in the presence of active Src (under conditions lead- ing to PKCd-Tyr311/Tyr332 phosphorylation). Collectively, these results add a new dimension to models of PKCd signaling, showing that Src (and PKCd tyrosine phosphorylation) con- trols PKCd-Thr505 autophosphorylation in cells. These studies indicate that PKCd is uniquely positioned to sense signaling inputs from both Src and PKCe pathways in cells. These studies also indicate that tyrosine phosphorylation plays a fundamental role in the control of PKCd activity. Although the full functional implications of PKCd-Thr505 phosphorylation are not fully resolved, recent studies provide intriguing evidence that PKCd-Thr505 phosphorylation “fine tunes” the enzymology of PKCd by altering its substrate specificity (5). Hence, our studies suggest that activation loop phosphorylation might repre- sent a final common mechanism to control the catalytic function of PKCd in a variety of contexts, including in the context of oxidative stress and Src-dependent PKCd tyrosine phosphorylation.

Many laboratories have come to rely on an adenovirus-me- diated overexpression strategies to resolve PKCe and PKCd actions in cells, at least in part to avoid using pharmacologic inhibitors, such as chelerythrine and rottlerin, that exert toxic/ PKC-independent actions. PKCe overexpression protocols are generally validated by experiments showing that PKCe overex- pression does not lead to compensatory changes in the abun- dance of other PKC isoforms (23). Our studies emphasize that measurements of nPKC isoform protein expression are inade- quate, since even relatively low levels of PKCe overexpression lead to coordinate increases in PKCd phosphorylation at Thr505, Tyr311, and Tyr332. These results identify a serious limi- tation associated with the use of an adenovirus-mediated PKCe overexpression strategy to resolve the cellular actions of PKCe and PKCd in cells (perhaps explaining some of the ambiguities identified in previous studies that have used this experimental strategy (24)).

Previous studies in genetically engineered mouse models have offered hints that PKCd might be a downstream target of PKCe. Klein et al. (17) reported that PKCd protein expression and Thr505 phosphorylation are increased in PKCeH9280 (but not normal) hearts subjected to pressure overload. Although Klein et al. did not identify changes in base-line PKCd protein or phosphorylation in the absence of a hypertrophic stimulus, Gray et al. (16) identified increased PKCd expression and PKCd localization to perinuclear structures (a sign of chronic PKCd activation) in resting cardiomyocytes isolated from PKCeH9280 mice. These previous studies in genetically engineered mouse models were interpreted as evidence that PKCe functions to inhibit PKCd (and that this inhibitory effect of PKCe is lost in the PKCe−/− mouse). Based upon this literature, the effect of PKCe overexpression to increase PKCd phosphorylation was surprising (and serves to emphasize that despite years of research, we still have attained only a rudimentary understanding of mechanisms that control nPKC isoform cross-talk in highly differentiated tissues). The effect of PKCe overexpres- sion to induce a generalized increase PKCd phosphorylation in many cell types (including cardiomyocytes, primary cardiac fibroblasts, Src− cells, and PKCe−/− MEFs) suggests that the elevated levels of PKCd protein and/or phosphorylation observed in PKCe−/− mice may reflect a compensatory response to total body PKCe knock-out from embryonic life onward and have little relevance to the physiologic control of PKCd in cells. These results serve to underscore the potential pitfalls inherent in extrapolations based upon data obtained in genetic models in mice, where some aspects of the phenotype may not necessarily be physiologically relevant.

Our studies suggest that PKCd is uniquely positioned within cellular signaling networks to integrate input from PKCe and Src signaling pathways. The precise molecular determinants that link PKCe overexpression to an increase in PKCd-Tyr311/ Tyr332 and Thr505 phosphorylation (that is not associated with any gross changes in Src family kinase or PDK-1 activity and may involve a PKCd-targeted phosphatase) are the focus of ongoing studies. However, to the best of our knowledge, the Src-dependent mechanism that controls activation loop phos- phorylation identified in this study is unique to PKCd; similar Src-dependent mechanisms that control activation loop phos- phorylation have not been described for other AGC kinases. Insofar as PKCd-Thr505 phosphorylation has emerged as an important determinant of PKCd specificity toward heterolo- gous substrates (5), an intervention that prevents Src-dependent PKCd phosphorylation might constitute a novel therapeutic strategy to selectively regulate only a subset of PKCd actions in cells.

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The Control of PKC-α-Thr505 Phosphorylation in Cardiomyocytes

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