Stimulus-specific Differences in Protein Kinase Cδ Localization and Activation Mechanisms in Cardiomyocytes*

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Protein kinase C (PKC) isoforms play key roles in the regulation of cardiac contraction, ischemic preconditioning, and hypertrophy/failure. Models of PKC activation generally focus on lipid cofactor-induced PKC translocation to membranes. This study identifies tyrosine phosphorylation as an additional mechanism that regulates PKCδ actions in cardiomyocytes. Using immunoblot analysis with antibodies to total PKCδ and PKCδ-pY311, we demonstrate that PKCδ partitions between soluble and particulate fractions (with little Tyr311 phosphorylation) in resting cardiomyocytes. Phorbol 12-myristate 13-acetate (PMA) promotes PKCδ phosphorylation in resting cardiomyocytes. Phorbol 12-myristate and soluble and particulate fractions (with little Tyr311 phosphorylation) of H2O2-treated cardiomyocytes as a tyrosine-phosphorylation by precomplexed Src kinases (without Src activation) and increased Src-PKCδ/H9254 kinase activity. PKCδ is recovered from the soluble fraction of H2O2-treated cardiomyocytes as a tyrosine-phosphorylated, lipid-independent enzyme with altered substrate specificity. In vitro PKCδ phosphorylation by Src also increases lipid-independent kinase activity. The magnitude of this effect varies, depending upon the substrate, suggesting that tyrosine phosphorylation fine-tunes PKCδ substrate specificity. The stimulus-specific modes for PKCδ signaling identified in this study allow for distinct PKCδ-mediated phosphorylation events and responses during growth factor stimulation and oxidant stress in cardiomyocytes.

Protein kinase C (PKC)1 comprises a multigene family of at least 10 structurally distinct phospholipid-dependent serine/threonine kinases that regulate cardiac contraction, play a role in ischemic preconditioning, and contribute to the pathogenesis of cardiac hypertrophy and heart failure (1, 2). PKC isoforms are single polypeptide chains with structurally homologous C-terminal catalytic domains and more variable N-terminal regulatory domains. This diverse group of enzymes is subdivided into three distinct subfamilies based upon structural differences in their N-terminal regulatory domain that confer distinct patterns of cofactor activation. Conventional PKC isoforms (cPKCs; α, β, βII, γ) contain an autoinhibitory pseudosubstrate domain followed by membrane-targeting C1 and C2 domains that are regulated by diacylglycerol (DAG) and calcium, respectively. Novel PKCs (nPKCs; δ, ε, η, and θ) lack a calcium-binding C2 domain and are maximally activated by DAG and phorbol ester, in the absence of calcium. Atypical PKCs (aPKCs; ζ and ι/λ) are regulated by lipids, but are not activated by second messengers such as calcium and DAG. Current models of PKC isoform activation in the heart have focused largely on the conformational changes induced by cofactor interactions with N-terminal membrane-targeting modules that anchor the enzyme to membranes, expel the autoinhibitory pseudosubstrate domain from the substrate-binding pocket, and thereby relieve autoinhibition. According to this model, individual PKC isoforms elicit distinct (and occasionally functionally opposing) cellular responses as a result of cofactor-induced compartmentation to distinct membrane subdomains, in close proximity to their unique sets of target protein substrates (1).

Recent studies identify an additional mechanism for PKC regulation via sequential phosphorylations on a conserved threonine in the activation loop and two conserved serine/threonines in turn and hydrophobic motifs in the C terminus (3). For cPKCs, these phosphorylation events are completed during enzyme maturation (and are critical for the generation of an enzymatically active and conformationally stable protein in the cytosol). In contrast, our recent studies identify dynamically regulated nPKC phosphorylation events that accompany agonist-induced nPKC activation/translocation (and contribute to the regulation of nPKC function) in cardiomyocytes (4). Agonist-induced phosphorylation of PKCδ at Thr505 in the activation loop regulates its kinase activity, whereas agonist-induced phosphorylation of PKCε at its C-terminal hydrophobic motif influences the kinetics of trafficking/down-regulation. PKC isoforms (in particular PKCδ) also serve as targets for regulatory phosphorylations on tyrosine residues in cells transformed with Src and Ras or acutely stimulated with PMA, epidermal growth factor, platelet-derived growth factor, and...
H$_2$O$_2$ (5–9). However, the precise biological consequences of PKCδ tyrosine phosphorylation have been difficult to decipher, at least in part because of the presence of multiple sites for independently regulated tyrosine phosphorylations in PKCδ regulatory domain (Tyr$^{457}$, Tyr$^{455}$, and Tyr$^{447}$), catalytic domain (Tyr$^{432}$ and Tyr$^{456}$), and hinge region (Tyr$^{411}$ and Tyr$^{436}$). Current literature suggests that there is no uniform pattern or consequence of PKCδ tyrosine phosphorylation. Rather, the precise configuration of tyrosine residues phosphorylated on PKCδ depends upon the nature of the activating stimulus and dictates the functional properties of the enzyme in cells. Most studies have relied on in vitro kinase assays to resolve tyrosine phosphorylation-dependent changes in PKCδ function and variably describe the catalytic activity of tyrosine phosphorylated PKCδ as decreased, increased or even altered its substrate specificity and cofactor requirements (5–9). However, a singular focus on phosphorylation-driven effects for PKCδ may be myopic (or even misplaced), given recent evidence that phosphotyrosines on PKCδ can also serve as docking sites for other signaling proteins (10, 11) and that the PKCδ role as a signal-regulated scaffold (rather than as an active serine/threonine kinase) may underlie certain of its effector functions (12).

Oxidative stress is a common feature of many cardiovascular risks (including hypertension, diabetes, and smoking) and contributes to the pathogenesis of heart failure syndromes (13). There is ample evidence that intracellularly generated reactive oxygen species (ROS) and exogenously administered hydrogen peroxide (H$_2$O$_2$) activate both mitogenic and apoptotic signaling pathways in cardiomyocytes (14, 15). PKCδ is one of several signaling molecules typically activated in this context. However, the precise mechanisms whereby oxidant stress activates PKCδ and the role of PKCδ in ROS triggered structural and functional remodeling of the ventricle has not been examined. This study demonstrates that H$_2$O$_2$ treatment releases PKCδ from membranes and generates a tyrosine phosphorylated form of PKCδ that exhibits lipid-independent catalytic function (and is poised to phosphorylate distinct PKCδ target proteins throughout the cell, not just on lipid membranes). These results suggest that there are two independent signaling modes for PKCδ, with PKCδ actions/phosphorylations resulting from GPCR activation and the generation of DAG in membranes functionally distinct from the events induced by the tyrosine-phosphorylated form of PKCδ in the soluble fraction of cells exposed to oxidant stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies were from the following sources: PKCδ-pY$^{111}$ (BioSource); PKCδ-pT$^{505}$ and Src-pT$^{416}$ (Cell Signaling Technology); PKCα and PKCε (Invitrogen, Life Technologies, Inc.); PKCδ (Santa Cruz Biotechnology); Src (Oncogene), Fyn (Santa Cruz Biotechnology and BD Transduction Laboratories); Lyn (Santa Cruz Biotechnology), Yes (Santa Cruz Biotechnology and BD Transduction Laboratories), Phosphotyrosine (Clone 4G10, UBI). Recombinant human PKCδ (rPKCδ) and histone III-S were from Sigma. Active Src kinase was from Panvera. The PKCδ substrate peptide ($\beta$-peptide) and the PKCδ substrate peptide (e-peptide) were purchased from Calbiochem and UBI, respectively. PMA was from Sigma. All other chemicals were reagent grade.

**Cardiomyocyte Culture and Transfection**—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 previously (4). The yield of cardiomyocytes typically is 2.5–3 × 10$^6$ cells per neonatal ventricle. Cells were plated on protamine sulfate-coated culture dishes at a density of 5 × 10$^4$ cells/100-mm dish. Experiments were performed on cultures grown for 5 days in MEM (Invitrogen, Life Technologies, Inc.) supplemented with 10% fetal calf serum and then serum-deprived for the subsequent 24 h.

**Immunoblot Analysis**—Immunoblot analysis was performed on whole cell extracts or soluble and particulate fractions prepared according to methods described previously (16, 17). Briefly, cells were washed with 20 mM HEPES-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 β-mercaptoethanol, 50 μg/ml aprotinin, 48 μg/ml leupeptin, 5 μm pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium vanadate, and 50 mM NaF). Cells were lysed by sonication, centrifuged at 100,000 × g for 1 h, and the supernatant was saved as the soluble fraction. PKC isoforms were extracted from the pellets by incubation on ice for 10 min in homogenization buffer containing 1% Triton X-100 followed by centrifugation at 100,000 × g for 30 min at 4 °C. Electrophoretic separation was performed using 8% SDS-polyacrylamide gels, followed by transfer to nitrocellulose for immunoblotting with a panel of antibodies that recognize total PKCs, PKCδ, or another kinase expression (previously identified as the oxidant-sensitive PKC isoforms expressed by cardiomyocyte cultures (16, 18)). PKCδ phosphorylated at the activation loop (anti-PKCδ-pT$^{505}$), or PKCδ tyrosine-phosphorylated at the hinge region (anti-PKCδ-pY$^{111}$). The specificity of the anti-PKCδ antibodies was established previously (4, 19); the specificity of all anti-phospho-PKC antibodies was validated in control experiments showing that anti-phospho-PKC immunoreactivity is stripped by treatment of samples with alkaline phosphatase (data not shown) and is down-regulated along with PMA-dependent down-regulation of the cognate protein (4). Bands were detected by enhanced chemiluminescence, with each panel in each figure from a single gel exposed for a uniform duration.

**Immunoprecipitation and Immunocomplex PKCδ Kinase Assay**—Following incubation with compounds indicated in the figures, cells were lysed in homogenization buffer (20 mM TrisCl, pH7.5, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 1 μg/ml aprotinin, 5.5 μg/ml leupeptin, 0.25 μg phenylmethylsulfonyl fluoride, 1 μg pepstatin A, 0.1 mM sodium orthovanadate, and 0.2% Triton X-100). Lysates were centrifuged at 4 °C for 15 min at 1,500 × g. For immunoprecipitation, the supernatant from five 100-mm dishes was incubated with 5 μg of anti-PKCδ antibody for 1 h at 4 °C, followed by addition of 200 μl of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) and incubation for 3 h at 4 °C. The immunoprecipitates were washed two times with washing buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT and 0.5% NaCl) and suspended in 1.15 ml of storage buffer (18 mM Tris-Cl, pH 7.5, 1.8 mM EDTA, 1.8 mM EGTA, 0.45 mM DTT, 225 mM NaCl, and 10% glycerol).

Immunoprecipitates (equivalent to the amount of protein extracted from 0.3 × 100-mm dish per assay) were used in immunocomplex kinase assays carried out in 200 μl of a reaction mixture containing buffer (26 mM Tris, pH 7.5, 5 mM MgCl$_2$, 0.6 mM EGTA, 0.6 mM EDTA, 0.5 μM PKI, 10 μM PIP1, and 0.25 mM DTT) in the absence and presence of 80 μg/ml phosphatidylserine (PS) plus 160 ng/ml PMA with histone III-S (0.5 mg/ml), PKC-ε-peptide (50 μM) or PKC-δ-β-peptide (50 μM) as substrate. PKC-ε-peptide is a synthetic peptide that corresponds to the pseudosubstrate domain of PKCε, with a phosphorylatable serine for the pseudosubstrate substitution (ERMRPKRGQPSGRRR). PKCδ-β-peptide serves as a good substrate for all PKCs except PKCδ (20). PKC-δ-β-peptide (which corresponds to amino acids 422–443 of marine eEF-1α, RFAVDRMRQTVAVGVYKAVKK) is a relatively specific substrate for PKCδ (and not other PKC isoforms, Ref. 20). Of note, we previously established that the phosphorylation of β- and ε-peptide (which do not contain tyrosines) or histone is mediated by PKCδ (rather than another co-immunoprecipitating kinase), all phosphorylations are completely inhibited by the addition of 5 μM GF109203X to the in vitro kinase assay buffer (4). Reactions were initiated by the addition [γ$^{32}$P]ATP (13 μCi, 66 μM) and were performed in quadruplicate at 30 °C for 16 min. Assays were terminated by placing samples on ice followed by centrifugation at 15,000 × g for 10 min at 4 °C. Aliquots of 40 μl of each supernatant were spotted onto phosphocellulose filter papers (P-81). Each P-81 disc was immediately dropped into water, washed (five times for 5 min), and counted for radioactivity. Pellets were subjected to SDS-PAGE and immunoblotting for PKCδ, to normalize for minor differences in the amount of immunoprecipitated enzyme.

**In Vitro Phosphorylation of PKCδ by Src—**To 0.1 μg of recombinant human PKCδ (rPKCδ) was preincubated in the absence or presence of Src kinase (0.66 units) in 160 μl of a reaction buffer containing 43 mM Tris-Cl, pH 7.5, 6.25 mM MgCl$_2$, 10 mM MnCl$_2$, 0.75 mM EDTA, 0.77 mM EGTA, 0.3 mM DTT, 125 mM NaCl, 5% glycerol, 0.06% Brij-35, 0.04 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, and [γ$^{32}$P]ATP (83 μM). Incubations were for 15 min at 30 °C. Reaction mixtures were terminated by placing samples on ice. PKC substrates were then added, and kinase assays were carried out (in a final volume of 200 μl) in the absence or presence of PS/PMA as described above. Triplicate 10-μl aliquots of each sample were spotted onto phosphocellulose filter paper (P-81),

*Stimulus-specific Differences in PKCδ Signaling in the Heart*
RESULTS

Distinct Effects of H2O2, PMA, and Norepinephrine on PKCδ Translocation and Phosphorylation in Cardiomyocytes—Immunoprecipitation of PKCδ followed by immunoblot analysis with anti-YP was used as a screen for PKCδ tyrosine phosphorylation by the α1-adrenergic receptor agonist norepinephrine (NE), PMA, and H2O2. Fig. 1A shows that there is a low level of PKCδ tyrosine phosphorylation in quiescent cultures and that PKCδ tyrosine phosphorylation is increased in cultures treated with NE, PMA, and H2O2. The magnitude of the NE-dependent increase in PKCδ tyrosine phosphorylation is relatively modest. In contrast, PMA and H2O2 induce substantial increases in PKCδ tyrosine phosphorylation, with the effect of H2O2 considerably greater than the effect of PMA. To begin to resolve an agonist-dependent difference in the level of PKCδ tyrosine phosphorylation at Tyr311, we focused on this tyrosine (in the hinge region, between the cysteine-rich and kinase domains) because it has been identified as a major site for tyrosine phosphorylation in cells treated with H2O2, it is flanked by sequence that conforms to an optimal Src substrate (and is reported to be a target for Src-dependent phosphorylation), and it represents a modification that reportedly alters PKCδ kinase activity and accelerates its down-regulation kinetics (6, 22, 23). Fig. 1A shows that PKCδ-Y311 phosphorylation is increased by PMA and (to a somewhat greater extent) by H2O2, but not by NE.

Cardiomyocyte cultures were partitioned into soluble and particulate fractions prior to immunoblot analysis to determine whether PKC isoforms (including the Tyr311-phosphorylated form of PKCδ) reside in distinct subcellular locations following stimulation with PMA and H2O2. Fig. 1B shows that PKCδ protein partitions between the soluble and particulate fractions of resting cardiomyocytes. Stimulation with PMA leads to the translocation of PKCδ to the particulate fraction and a decline in its mobility in SDS-PAGE, characteristic of a phosphorylation-induced change in electrophoretic mobility. In contrast, H2O2 promotes a marked redistribution of PKCδ from the particulate to the soluble fraction without changing its electrophoretic mobility in SDS-PAGE. PKCδ also partitions between the soluble and particulate fractions of resting cardiomyocytes and translocates to the particulate fraction upon activation with PMA. Whereas H2O2 promotes the redistribution of PKCδ to the soluble fraction, the magnitude of this effect is relatively modest compared with the H2O2 effect on PKCδ. In contrast, PKCα resides mainly in the soluble fraction of resting cardiomyocytes and is recovered in the particulate fraction of PMA-treated cardiomyocytes; PKCα partitioning between soluble and particulate fractions is not appreciably altered by H2O2.

PKCδ-Y311 phosphorylation is detected at very low levels (and only in the particulate fraction) in resting cardiomyocytes (Fig. 1B). Stimulation with PMA leads to a large increase in particulate PKCδ-pY311 (in association with the PMA-induced...
translocation of PKCδ to membranes). PKCδ-Y311 phosphorylation also is markedly increased in cells treated with H2O2. However, under this condition PKCδ-pY311 is detected in both soluble and particulate fractions. This and subsequent experiments in this study report the cellular response to 5 mM H2O2; increased amounts of Tyr311-phosphorylated PKCδ also are recovered from cardiomyocytes subjected to lower levels of oxidant stress (100–500 μM, data not shown). Interestingly, both H2O2 and PMA promote PKCδ-Y311 phosphorylation, but only PMA slows the mobility of PKCδ in SDS-PAGE (Fig. 1B; also see Figs. 2 and 4). This result suggests that the electrophoretic mobility of PKCδ is not influenced by phosphorylation at Tyr311; rather the PMA-induced mobility shift must derive from a phosphorylation at a site distinct from Tyr311. We recently demonstrated that PKCδ is phosphorylated at the turn motif (Ser643) and hydrophobic motif (Ser662) sites, but not at the activation loop (Thr505) site, in resting cultures and that phosphorylation at Thr505 (but not Ser643 or Ser662) increases during stimulation with PMA (4). Similarly, Fig. 1B shows that PKCδ is not detectably phosphorylated at Thr505 in resting cultures. PMA (which slows PKCδ mobility in SDS-PAGE) promotes PKCδ phosphorylation at Thr505, whereas H2O2 (which does not alter the mobility of PKCδ) does not. H2O2 also does not induce any change in PKCδ phosphorylation at Ser643 or Ser662 in the C terminus (data not shown). These results suggest that PKCδ phosphorylation at Thr505 (or another site that becomes phosphorylated in parallel in cells treated with PMA, but not H2O2) slows the mobility of PKCδ in SDS-PAGE; the mobility of PKCδ is not appreciably altered by tyrosine phosphorylation. Finally, Fig. 1C shows that NE does not promote PKCδ-Y311 phosphorylation at any time point, under conditions where an effect of NE to induce PKCδ-T505 phosphorylation is readily detected. Collectively, these results identify distinct phosphorylation profiles for PKCδ in cardiomyocytes stimulated with PMA, H2O2, and NE. All three agonists promote PKCδ tyrosine phosphorylation, but only PMA and H2O2 promote tyrosine phosphorylation at Tyr311. PKCδ is localized to the particulate fraction, and phosphorylated at both Tyr311 and Thr505, in cardiomyocytes treated with PMA. In contrast, PKCδ distributes between the soluble and particulate fractions and is phosphorylated at Tyr311 (but not Thr505) in cardiomyocytes treated with H2O2. Whereas immunoreactivity for PKCδ-pY311 is slightly greater in H2O2-treated cardiomyocyte cultures, than in the PMA-treated cultures studied in parallel, this difference can not account for the considerably higher overall level of PKCδ tyrosine phosphorylation in H2O2-treated cultures. This result suggests that PKCδ phosphorylation in H2O2-treated cultures is at Tyr311 as well as at other tyrosine residues.

To better understand agonist-induced compartmentation of PKCδ, we studied the kinetics of agonist-induced PKCδ translocation and Tyr311 phosphorylation in greater detail (Fig. 2). The H2O2-induced release of PKCδ from membranes (and translocation to the soluble fraction) is quite rapid (detectable within the first min, and maximal by 2 min); PKCδ recovery in the soluble fractions remains increased for at least 30 min of H2O2 stimulation. PKCδ-Y311 phosphorylation accompanies the translocation to the soluble fraction. PKCδ-Y311 phosphorylation is detected within the first 1–2 min and increases progressively (in both the soluble and particulate fractions) during the first 30 min of H2O2 treatment. Of note, PKCδ-Y311 phosphorylation increases progressively in the particulate fraction, despite a dwindling amount of PKCδ protein recovery. PMA-induced PKCδ-Y311 phosphorylation also follows rapid kinetics (Fig. 2B), with both PKCδ translocation to the particulate fraction and PKCδ-Y311 phosphorylation being maximal at 5 min (the first time point sampled). PMA-induced PKCδ-Y311 phosphorylation is sustained for up to 5 h, declining in parallel with the PMA-induced down-regulation of the protein.

Most cardiac disorders are characterized by the simultaneous activation of hormone/neurotransmitter receptors coupled to lipid signaling (and the generation of cofactors that anchor PKC to membranes) as well as variable amounts of oxidant stress. To determine whether the H2O2-dependent release of PKCδ from membranes competes with the PMA-dependent translocation of PKCδ to membranes, PKCδ localization and phosphorylation were compared in cardiomyocytes exposed to H2O2 or PMA alone, versus PMA in the presence of H2O2. Fig. 3 shows that H2O2 alone promotes the release of PKCδ from membranes, whereas PMA alone effectively clears PKCδ from the soluble fraction. However, PMA-dependent translocation of PKCδ to the particulate fraction is attenuated by H2O2, illus-
stimulating the dynamic convergence of these signals. Although PMA-dependent phosphorylation of PKCα at Thr505 proceeds unhindered in the membrane fraction, no PMA-dependent PKCα-Thr505 phosphorylation is detected in the pool of PKCα that remains in the soluble fraction of PMA + H₂O₂-treated cardiomyocytes. This result is consistent with the notion that PKCα-Thr505 phosphorylation requires PKCα localization in an open conformation, to expose the activation loop Thr505 in membranes. The effects of H₂O₂ to modify PMA (and presumably receptor-dependent) activation of PKCα appear to be specific (as opposed to a nonspecific effect of H₂O₂ to inactivate PMA), because PMA-dependent translocation of PKCα is not influenced by H₂O₂.

PMA- and H₂O₂-dependent Tyrosine Phosphorylation of PKCα Requires Src Kinase Activity—Fig. 4A shows that H₂O₂- and PMA-dependent PKCα-Y311 phosphorylations are markedly inhibited by the relatively specific Src family kinase inhibitor PP1. In contrast, H₂O₂- and PMA-dependent PKCα-Y311 phosphorylations are not blocked by AG1478 (an inhibitor of the EGF receptor tyrosine kinase), GF109203X (a general inhibitor of cPKCs, PKCδ, and PKCe, under conditions that were previously validated to completely inhibit cardiomyocyte PKC isoforms (4)) or PP3 (a structurally similar, but functionally inactive, PP1 analogue that serves as a negative control; Fig. 4B and data not shown). Neither PP1 nor GF109203X influence the PMA-dependent translocation of PKCδ to the particulate fraction or the H₂O₂-dependent release of PKCδ from membranes. Because H₂O₂-dependent tyrosine phosphorylation of PKCδ is not necessarily limited to Tyr311, and there is evidence that PKCδ phosphorylation at certain regulatory domain tyrosines need not be limited to the actions of Src family kinases (24), the effect of PP1 to more generally inhibit H₂O₂-dependent PKCδ tyrosine phosphorylation was considered. PKCδ was immunoprecipitated from cardiomyocytes exposed to vehicle or H₂O₂ (in the presence or absence of PP1) and analyzed using an anti-YP antibody. In this context, H₂O₂-dependent PKCδ tyrosine phosphorylation (at all potential sites recognized by the anti-YP antibody) is completely blocked by PP1 (Fig. 4C). The observation that PP1 prevents H₂O₂-dependent PKCδ tyrosine phosphorylation but it does not prevent the H₂O₂-dependent release of PKCδ from membranes suggests that H₂O₂-dependent release of PKCδ from membranes is via a mechanism that does not involve PKCδ tyrosine phosphorylation.

Src Kinase Activation and Complex Formation with PKCδ—Neonatal rat cardiomyocyte cultures express multiple Src family kinases that might mediate PKCδ tyrosine phosphorylation (25). To begin to explore the mechanism(s) for PKCδ tyrosine phosphorylation in cardiomyocytes treated with H₂O₂ or PMA, we examined Src family kinase expression, partitioning between soluble and particulate fractions, and activity in resting and activated cardiomyocytes. Fig. 5A shows that Src, Fyn, Yes, and Lyn are readily detected, and recovered largely in the particulate fraction, in resting cardiomyocytes. Whereas the partitioning of each of these proteins between soluble and particulate fractions is not altered by PMA, treatment with H₂O₂ consistently increased the recovery of Lyn (and more variably Src) in the soluble fraction. Yes and Fyn partitioning between soluble and particulate fractions were not influenced by H₂O₂.

Immunoblot analysis with anti-phospho-Src-Y416 was used as a screen to detect H₂O₂- or PMA-dependent changes in Src kinase activity. This antibody specifically recognizes the activated (activation loop phosphorylated) form of Src; anti-phospho-Src-Y416 also cross-reacts with activated forms of Fyn, Lyn, and Yes (which are phosphorylated at equivalent activation loop residues). Fig. 5B shows that anti-Src-pY416 immunoreactivity is at the limits of detection in resting cultures. Anti-Src-
pY416 immunoreactivity increases rapidly in the particulate fraction (maximal activation detected at 2 min, the first time point assayed) when cardiomyocytes are treated with H2O2. Src-pY416 immunoreactivity also increases in the soluble fraction, but with a slight delay relative to the increase in membranes. Of note, Src-pY416 immunoreactivity is detected in both particulate and soluble fractions as multiple bands with mobilities corresponding to Src [60-kDa], Fyn [59-kDa], Yes [62-kDa], and Lyn [a 53- and 56-kDa doublet]. This suggests that multiple Src family kinases are activated in parallel during stimulation with H2O2. In the particulate fraction, the increase in Src-pY416 immunoreactivity denotes activation of resident Src kinases. However, the increase in Src-pY416 immunoreactivity in the soluble fraction is the combined result of increased Lyn protein recovery (an event that follows a time course that closely tracks the increase in Src-pY416 immunoreactivity) as well as Src, Fyn, and/or Lyn activation. In striking contrast to the stimulatory actions of H2O2, PMA does not increase Src-pY416 immunoreactivity.

To determine whether there are potential differences in the activation kinetics for individual Src kinase family members, Src family kinases were immunoprecipitated individually from vehicle- or H2O2-treated cardiomyocyte cultures and Src-pY416 immunoreactivity was monitored as a measure of their activity. These experiments used whole cell lysates and track Src activation loop phosphorylation throughout the cell (without resolving potential differences in soluble and particulate fractions). Fig. 5C shows that H2O2 markedly increases Src, Lyn, and Fyn activation loop phosphorylation. In each case, the H2O2-dependent response is maximal by 2 min and sustained for an additional 20 min. In contrast, Src family kinase activation loop phosphorylation is not increased by PMA. Similar results were obtained in separate experiments that used immune complex kinase assays with enolase as substrate (data not shown).

Interactions between PKCδ and several Src family kinases have been reported. To determine whether Src kinases interact (either in a constitutive or regulated fashion) with PKCδ in
cardiomyocytes, lysates from cardiomyocyte cultures treated with vehicle, PMA, or H2O2 were subjected to immunoprecipitation with antibodies to Src, Fyn, Yes, or Lyn followed by immunoblot analysis to probe for PKCδ. Fig. 6 shows that Src, and to a lesser extent other Src family kinase members, are recovered constitutively in complexes with PKCδ. H2O2 induces a dramatic increase in complex formation between PKCδ and Fyn, Yes, and Lyn. PKCδ complexes with Src, Fyn, and Yes are not influenced by PMA. Of note, PMA consistently decreased complex formation between Lyn and PKCδ. A similar effect of PMA to disrupt PKCδ-Lyn complexes was recently identified in mast cells (where it was attributed to PKC-dependent phosphorylation of Lyn, (11)). Src family kinase interactions with PKCδ are isoform specific; PKCe or PKCμ were not detected in these complexes.

The marked H2O2-dependent increase in Src-pY416 immunoreactivity (and Src kinase family member complex formation with PKCδ) provides a plausible mechanism to explain H2O2-dependent tyrosine phosphorylation of PKCδ. However, a mechanism for PKCδ-Y311 phosphorylation in cells treated with PMA (where Src kinases are not detectably activated) is less obvious. To explore the mechanism for PKCδ-dependent phosphorylation, we performed reciprocal immunoprecipitation/immunoblotting experiments. PKCδ was immunoprecipitated from quiescent cultures and subjected to kinase assays with [γ-32P]ATP in the absence or presence of sonicated vesicles containing the PKC-activating lipid cofactors phosphatidylserine [PS] plus PMA (PS/PMA). This protocol allows for PKCδ autophosphorylation as well as PKCδ phosphorylation in trans by associated kinases that co-purify during immuno precipitation. Samples were subjected to SDS-PAGE followed by autoradiography (as a general measure of phosphorylation at serine, threonine, or tyrosine residues) as well as immunoblot analysis with anti-YP and anti-PKCδ-pY311 antibodies (to resolve total and Tyr311-specific tyrosine phosphorylation).

Fig. 7 shows that PKCδ is recovered from quiescent cardiomyocytes with only a low level of Tyr311 phosphorylation (including at Tyr311). Fyn kinase assays without added lipid resulted in a low level of 32P incorporation into PKCδ (detected by autoradiography) and PKCδ tyrosine phosphorylation (detected by the anti-YP antibody). PKCδ radio labeling was enhanced further by added PS/PMA. Of note, the PS/PMA-dependent increase in 32P incorporation into PKCδ was blocked by GF109203X (but not PP1); this result indicates that 32P incorporation largely provides a measure of PKC-dependent autophosphorylation. In contrast, PS/PMA increases PKCδ tyrosine phosphorylation (including at Tyr311); the PS/PMA-induced tyrosine phosphorylation of PKCδ is blocked by PP1 (but not GF109203X). Collectively, these results identify distinct mechanisms for in vitro PKCδ phosphorylation at serine/threonine versus tyrosine residues. The phosphorylation of PKCδ at Tyr311 (and perhaps other tyrosine residues) during the in vitro kinase assays validates the conclusion that PKCδ is phosphorylated by one (or more) Src family kinases(s) that constitutively associates (and co-precipitates) with PKCδ from resting cardiomyocyte cultures. In fact, both Src and Lyn were identified by immunoblot analyses in these anti-PKCδ immune complexes (data not shown). Because PS/PMA allosterically activates PKC (but does not activate Src kinases), the mechanism for the PS/PMA-induced increase in PKCδ tyrosine phosphorylation is presumed to involve a PS/PMA-induced conformational change that renders PKCδ a better substrate for phosphorylation by one or more precomplexed Src kinase.
from H_2O_2-activated cardiomyocytes is quite low (<10% of the PS/PP1-stimulated histone kinase activity of PKCδ recovered from quiescent cultures).

Fig. 9B shows that H_2O_2 increases PKCδ kinase activity in both soluble and particulate fractions. H_2O_2-dependent changes in PKCδ kinase activity are illustrated in this figure, without correcting for the H_2O_2-dependent translocation of PKCδ from the particulate to the soluble fraction. This serves to underestimate the H_2O_2-dependent increase in particulate PKCδ kinase activity. However, the H_2O_2-dependent increase in PKCδ kinase activity in the soluble fraction could result from an increase in PKCδ recovery in the soluble fraction, activation of the soluble pool of PKCδ, or (more likely) both, because the 3.5- to 2.8-fold increment in δ- and ε-peptide kinase activity in the soluble fraction exceeds the ~2-fold increase in soluble PKCδ protein recovery.

H_2O_2 can modulate PKCδ kinase activity indirectly by stimulating Src kinase-dependent tyrosine phosphorylation or directly through oxidative modification of cysteines in the regulatory and catalytic domains. Oxidation of reactive cysteines in the zinc fingers of the regulatory C1 domain induces a conformational change that relieves autoinhibition and leads to a constitutively active enzyme. Oxidation of critical cysteines in the catalytic domain has the opposite effect and disrupts catalytic activity (26). Fig. 9C shows that H_2O_2, directly added to the in vitro assay buffer according to conditions used for the intact cell experiments (in an attempt to mimic the amount of oxidant stress achieved in vivo in cardiomyocytes), induces a significant increase in both lipid-independent and PS/PP1-dependent PKCδ kinase activity; under these in vitro assay conditions, the stimulatory effects of PMA and H_2O_2 are additive. This result is surprising, because activating oxidative modifications in the regulatory domain of PKCδ are predicted to produce a form of PKCδ that no longer binds phorbol esters (i.e. is not further activated by PMA). These results suggest that additional types of redox modifications may exist for PKCδ and that oxidative modifications may contribute to PKCδ activation in H_2O_2-treated cardiomyocytes.

**Fig. 8.** The time course for PMA-dependent PKCδ down-regulation is not influenced by Tyr³¹¹ phosphorylation. Cardiomyocytes were pretreated with vehicle or PP1 (10 μM for 45 min) followed by PMA for the indicated intervals. Immunoblot analysis of whole cell lysates (35 μg) was with antibodies for PKCδ and PKCα. Similar results were obtained in two separate experiments on separate culture preparations.

Tyrosine Phosphorylation Regulates the Kinase Activity (but not the PMA-dependent Down-regulation Kinetics) for PKCδ—PKCδ-pY³¹¹ has been viewed as a modification that destabilizes the enzyme and promotes down-regulation (23). However, Fig. 8 shows similar kinetics for PKCδ down-regulation in cardiomyocytes exposed to PMA alone and PMA plus PP1 (to prevent Tyr³¹¹ phosphorylation); the down-regulation kinetics for PKCδ (examined in parallel) also are not influenced by PP1. These results argue that Src-dependent PKCδ phosphorylation at Tyr³¹¹ does not appreciably influence PMA-dependent PKC trafficking/down-regulation mechanism(s) in cardiomyocytes.

Tyrosine phosphorylation also has been implicated as a mechanism to regulate PKC kinase activity. Therefore, we compared the catalytic activity of PKCδ recovered from quiescent cultures and cultures treated with PMA or H_2O_2: assays were performed under basal conditions and with lipid (PS/PMA) added to the in vitro incubations. To determine whether PKCδ activation/phosphorylation leads to a change in substrate specificity, kinase assays measured the incorporation of ³²P from [γ-³²P]ATP into δ-peptide and ε-peptide (model substrates for PKCδ and PKCε, respectively) and histone (generally considered to be a better substrate for cPKC isoforms, than for nPKC isoforms). Fig. 9A shows that PKCδ immunopurified from quiescent cardiomyocyte cultures exhibits little-to-no lipid-independent kinase activity toward any substrate; histone-, δ-peptide-, and ε-peptide-kinase activities increase markedly upon addition of PS/PMA to the in vitro kinase assays (although as recently reported, ³²P incorporation into δ-peptide is surprisingly modest, compared with the activity measured with ε-peptide or histone as substrates (4)). PKCδ is recovered from PMA- and H_2O_2-treated cultures with markedly different cofactor requirements, substrate specificity, and activity. Here, lipid-independent kinase activity is markedly increased. With δ-peptide or ε-peptide as substrates, the lipid-independent kinase activity of the in vivo stimulated enzyme (recovered from PMA- or H_2O_2-stimulated cardiomyocytes) approaches the in vitro PS/PMA-stimulated (maximally activated) PKCδ kinase activity recovered from resting cardiomyocytes. Although lipid-independent histone kinase activity also is significantly increased by in vivo treatment with PMA or H_2O_2, addition of lipid cofactors leads to little further increase in histone phosphorylation. Consequently, the absolute level of histone kinase activity (even in the presence of PS/PMA) for PKCδ recovered...
histone is detected (Fig. 10A, right). $^{32}$P incorporation into PKCδ also largely provides a measure of autophosphorylation, because it is detected in incubations without Src. However, immunoblot analysis with anti-YP and anti-PKCδ-pY$^{311}$ shows that Src also promotes PKCδ-tyrosine phosphorylation, including at Tyr$^{311}$.

Fig. 10B shows that Src-dependent tyrosine phosphorylation influences PKCδ catalytic activity (with the magnitude of the effect varying for different substrates). Tyrosine phosphorylation leads to a ~50% increase in lipid-independent catalytic activity toward ε-peptide and histone. Maximal PS/PMA-stimulated PKCδ kinase activity toward histone also is increased, whereas maximal PS/PMA-dependent phosphorylation of ε-peptide is not. The robust PMA-dependent histone kinase activity displayed by Src-phosphorylated rPKCδ contrasts with the very low level of histone phosphorylation (even in the presence of lipid cofactors) by PKCδ recovered from H$_2$O$_2$-treated cardiomyocytes (Fig. 9A). These results suggest that PKCδ-pY$^{311}$ phosphorylation alone is not sufficient to impair histone kinase activity, and that treatment with H$_2$O$_2$ leads to

Fig. 9. In vitro PKCδ kinase assays; PKCδ is recovered from PMA- and H$_2$O$_2$-activated cardiomyocytes with altered activity, cofactor requirements, and substrate-specificity. Panel A, cultures were treated with vehicle, PMA (300 nM) or H$_2$O$_2$ (5 mM, each for 10 min); whole cell lysates were subjected to immunoprecipitation and immune complex PKCδ kinase assays with histone, δ-peptide, or ε-peptide as substrate. Assays were in the absence or presence of PS/PMA as described under “Experimental Procedures.” Panel B, cultures were treated for 10 min with vehicle or H$_2$O$_2$. Lysates were partitioned into soluble and particulate fractions and subjected to immunoprecipitation and immune complex PKCδ kinase assays (without added PS/PMA) with δ-peptide or ε-peptide as substrate. Samples also were subjected to immunoblot analysis for total PKCδ and PKCδ-pY$^{311}$. Panel C, cultures were treated for 10 min with vehicle or 5 mM H$_2$O$_2$. Whole cell lysates were subjected to immunoprecipitation and immune complex PKCδ kinase assays were performed in the absence or presence of PS/PMA or 1 mM H$_2$O$_2$, with δ-peptide as substrate; similar results were obtained in separate assays that used ε-peptide as substrate, (data not shown). Results for kinase assays are mean ± S.E. of quadruplicate measurements from a representative experiment, with similar result obtained in three (panels A and B) or two (panel C) separate experiments on separate culture preparations.
defective histone phosphorylation via a different modification on PKCδ (that could include tyrosine phosphorylation of PKCδ at other tyrosine residues, perhaps by other non-receptor tyrosine kinases). Finally, Fig. 10B shows that tyrosine phosphorylation of PKCδ markedly augments lipid-independent and PS/PMA-dependent PKCδ kinase activity toward δ-peptide (34- and 5-fold, respectively). Collectively, these results indicate that PKCδ is phosphorylated at Tyr311 by Src, that Src-dependent phosphorylation of PKCδ at Tyr311 (and potentially other tyrosine residues) leads to an increase in the catalytic activity of PKCδ, and that the magnitude of the increment is skewed by the choice of substrate used in the assay; the effect is most striking in assays with a substrate that conforms to an optimal PKCδ phosphorylation motif.

DISCUSSION

PKC regulates a wide range of functions in cardiomyocytes. Signaling specificity for PKC generally has been attributed to the co-expression of multiple isoforms with distinct modes of activation, subcellular localizations, and/or target substrates. However, this study suggests that (at least for PKCδ) this model must be broadened to consider distinct modes of activation and functions for a single PKC isoform. In addition to the traditional mode of allosteric activation by lipid cofactors, PKCδ is regulated by oxidative stress and tyrosine phosphorylation. This mechanism holds particular interest for cardiomyocyte physiology, where the traditional model of receptor-driven lipid cofactor-dependent PKCδ activation readily accounts for substrate phosphorylation in lipid membranes, but it does not adequately explain the well known effects of PKC to modulate contractile function by phosphorylating proteins in the sarcomere (27). This study provides a viable solution to this dilemma by identifying conditions that allow for lipid-independent PKCδ kinase activity in the soluble fraction of cardiomyocytes.

Three distinct stimulus-specific patterns for PKCδ phosphorylation in cardiomyocytes treated with NE, PMA, and H2O2 were identified. All three stimuli increase PKCδ tyrosine phosphorylation, but only PMA and H2O2 promote PKCδ-pY311 phosphorylation. Because NE does not increase PKCδ-pY311, it must promote a tyrosine phosphorylation event elsewhere in the protein. Similarly, H2O2 and PMA promote quantitatively similar increases in PKCδ-pY311 phosphorylation, but overall PKCδ tyrosine phosphorylation (detected with anti-YP) is substantially higher in cardiomyocytes treated with H2O2. This

Fig. 10. Src-dependent phosphorylation increases the kinase activity of recombinant PKCδ. Recombinant PKCδ was incubated with vehicle (lanes 1 and 2) or active Src (lanes 3 and 4) and then used in kinase assays with histone, δ-peptide, or ε-peptide as substrate in the absence (lanes 1 and 3) or presence of PS/PMA (lanes 2 and 4) as described under “Experimental Procedures.” Panel A (left), autoradiogram showing 32P incorporation into PKCδ, Src, and histone. Ponceau staining to show the position of the major and minor bands corresponding to histone is depicted in lane 5. Panel A (right), immunoblot analysis with anti-PY and anti-PKCδ-pY311. Panel B, results for kinase assays (mean ± S.E. of triplicate measurements from a representative experiment). Similar results were obtained in two separate experiments.
The effects of H$_2$O$_2$ and PMA to promote PKC$_\delta$-Y$_{311}$ phosphorylation are mediated by a Src (or related) nonreceptor tyrosine kinase, but via distinct mechanisms. The H$_2$O$_2$-induced increase in PKC$_\delta$-Y$_{311}$ phosphorylation is associated with Src family kinase activation; PKC$_\delta$-Y$_{311}$ phosphorylation presumably is by Src itself (which phosphorylates PKC$_\delta$ at this site in vitro) and/or other Src family kinases. In contrast, the PMA-dependent increase in PKC$_\delta$-Y$_{311}$ phosphorylation is not accompanied by Src kinase activation; it must be via a different mechanism. Immunoprecipitation/in vitro immunocomplex kinase assays provide insight into this mechanism by demonstrating that PKC$_\delta$ constitutively interacts with multiple Src family kinases and that PKC$_\delta$-Y$_{311}$ phosphorylation is increased by PS/PMA. These results suggest that PKC$_\delta$-Y$_{311}$ phosphorylation can result from a conformational change that accompanies PKC$_\delta$ activation, exposes Tyr$_{311}$, and renders PKC$_\delta$ a better substrate for phosphorylation by precomplexed Src kinases. In this regard, our previous studies demonstrating that PMA promotes PKC$_\delta$ translocation to caveolin-3-enriched lipid raft membranes may be pertinent, because certain Src family kinases retain basal catalytic activity in these specialized membranes subdomains (28, 29). Finally, this study has focused on PKC$_\delta$ phosphorylation by one or more Src family kinases, but we have not excluded a potential additional role for c-Abl (which also would be inhibited by the PP1 concentrations used in this study). c-Abl is reported to physically interact with and phosphorylate PKC$_\delta$; c-Abl also can cooperate with PKC$_\delta$ in the cellular response to oxidative stress (30–32).

This study identifies considerably more elaborate regulation of PKC$_\delta$ enzyme activity than previously appreciated. The results suggest that the conventional notion that PKC$_\delta$ acts as a generic kinase, with phosphorylation events regulated by translocation to distinct microenvironments (i.e., access to substrate), must be broadened to include additional factors that dramatically influence the cofactor requirements and substrate specificity of the enzyme. For example, we demonstrate that histone (generally considered a poor substrate for nPKC isoforms) is effectively phosphorylated by the form of PKC$_\delta$ recovered from resting cardiomyocytes. Only the phosphorylated/activated form of PKC$_\delta$ (recovered from PMA-/H$_2$O$_2$-stimulated cardiomyocytes) is a poor histone kinase. This reflects an activation-dependent change in PKC$_\delta$ substrate specificity, rather than an increase in the enzyme’s in vitro thermal lability, because the phosphorylation of model PKC peptide substrates persists in this context. The PMA- and H$_2$O$_2$-induced changes in substrate specificity are accompanied by equally striking changes in cofactor requirements, because phosphorylated/activated PKC$_\delta$ acts as a lipid-independent kinase. Multiple regulatory mechanisms are likely to contribute to this process. We recently reported that PMA enhances PKC$_\delta$ catalytic activity by promoting activation loop PKC$_\delta$-T$_{505}$ phosphorylation (4). This mechanism is specific for PKC$_\delta$ regulation by PMA, because H$_2$O$_2$ does not promote PKC$_\delta$-T$_{505}$ phosphorylation. H$_2$O$_2$ (through oxidative modification of susceptible regulatory domain cysteines) and PMA (by binding to the regulatory domain) are reported to increase lipid-independent PKC kinase activity by releasing tetrahedrically coordinated zinc ions from the zinc finger motifs (33). Finally, in vitro kinase assays with rPKC$_\delta$ implicate tyrosine phosphorylation as a mechanism that alters the cofactor requirements and substrate specificity of PKC$_\delta$. Of note, tyrosine phosphorylation appears to fine-tune PKC$_\delta$ substrate specificity, by preferentially enhancing the phosphorylation of sequences that conform to an optimal PKC$_\delta$ substrate. This aspect of PKC$_\delta$ regulatory control may be pertinent to the identification of physiologically relevant PKC$_\delta$ substrates in cardiomyocytes.

There is still only limited information on the biological consequences of PKC$_\delta$-Y$_{311}$ phosphorylation. A single previous study from Blake et al. (23) concluded that PKC$_\delta$-Y$_{311}$ phosphorylation accelerates the kinetics of PKC$_\delta$ trafficking/down-regulation on the basis of the observations that Src transformed NIH3T3 cells exhibit reduced levels of PKC$_\delta$ protein expression, pronounced PKC$_\delta$ tyrosine phosphorylation, and accelerated PKC$_\delta$ degradation (relatively to parental NIH3T3 cells). The biochemical experiments in this previous study suggested that ‘Tyr$_{311}$ is not the only (or perhaps even major) site for Src-dependent PKC$_\delta$ tyrosine phosphorylation. However, a single Tyr $\rightarrow$ Phe mutation at Tyr$_{311}$ was sufficient to completely abrogate Src-dependent PKC$_\delta$ tyrosine phosphorylation and effectively prevent the accelerated PKC$_\delta$ degradation kinetics. These results were interpreted as evidence that PKC$_\delta$ undergoes a highly ordered sequence of tyrosine phosphorylation initiated at Tyr$_{311}$ and that Src-dependent PKC$_\delta$-Y$_{311}$ phosphorylation (itself, or in conjunction with a subsequent modification) accelerates the kinetics of PKC$_\delta$ down-regulation. However, additional experiments from Blake et al and others (23, 24) show that Tyr $\rightarrow$ Phe mutations at Tyr$_{311}$ (as well as Tyr$_{52}$, Tyr$_{64}$, Tyr$_{155}$, and Tyr$_{187}$) do not grossly influence the kinetics of PMA-dependent PKC$_\delta$ down-regulation. These published results are consistent with our finding that PP1 inhibits PKC$_\delta$-Y$_{311}$ phosphorylation but it does not influence the kinetics of PMA-dependent PKC$_\delta$ down-regulation in cardiomyocytes. Collectively, these results are at odds with the conclusion that PKC$_\delta$ tyrosine phosphorylation (including at Tyr$_{311}$) plays a major role to modulate physiologic PKC$_\delta$ trafficking/down-regulation events (although an effect of PKC$_\delta$-Y$_{311}$ phosphorylation to influence a distinct PKC$_\delta$ trafficking/down-regulation mechanism that gains prominence in Src-dependent cells remains possible).

The H$_2$O$_2$-dependent release of PKC$_\delta$ from membranes identified in this study is reminiscent of the hypoxia-induced PKC$_\delta$ release from membranes identified previously (35). The mechanism for PKC$_\delta$ translocation to the soluble fraction remains uncertain. The observation that PKC$_\delta$ translocates to the soluble fraction of cardiomyocytes treated with H$_2$O$_2$ plus PP1 (where PKC$_\delta$ tyrosine phosphorylation is prevented) effectively excludes a role for tyrosine phosphorylation in this process. A more likely trigger is ceramide, which is reported to accumulate in cardiomyocytes exposed to hypoxia/reoxygenation or H$_2$O$_2$ (36, 37). Recent studies identify a complex reciprocal relationship between ceramide formation (which is increased by PKC$_\delta$) and PKC$_\delta$ release from membranes and translocation to mitochondria (which is induced by ceramide). The importance of this amplification loop in the mitochondrial apoptosis pathway in cardiomyocyte subjected to oxidant stress deserves further study (38).

PKC$_\delta$ is implicated in signaling pathways leading to apoptosis in many cell types (1). PKC$_\delta$ overexpression activates JNK and p38-MAPK cascades and induces apoptosis in cardiomyocyte cultures; PKC$_\delta$ signaling leads to enhanced ischemic injury in the intact heart (21, 39, 40). In the absence of detailed information on the cellular targets that mediate the deleterious actions of PKC$_\delta$ in the heart, current strategies to prevent PKC$_\delta$ cardiac actions rely largely on peptide inhibitors that interfere with PKC$_\delta$ interactions with its RACK (receptor for activated C-Kinase), a protein that anchors activated PKC$_\delta$ at membranes, in close proximity with lipid co-factors and its target substrates. However, the current study identifies cir-
Phosphorylations that occur in the soluble fraction of H$_2$O$_2$-treated branes (and free it of its lipid requirement). Substrate phosphorylations during growth factor signaling (and its consequences, which are predicted to differ from the actions of PKC during growth factor signaling) in future efforts to design and evaluate PKC-targeted therapeutics.

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Stimulus-specific Differences in Protein Kinase Cδ Localization and Activation Mechanisms in Cardiomyocytes
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