Phorbol 12-Myristate 13-Acetate-dependent Protein Kinase CΔ-Tyr\(^{311}\) Phosphorylation in Cardiomyocyte Caveolae*

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Vitalyi O. Rybin, Jianfen Guo, Zoya Gertsberg, Steven J. Feinmark, and Susan F. Steinberg

From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Protein kinase Cδ (PKCδ) activation is generally attributed to lipid cofactor-dependent allosteric activation mechanisms at membranes. However, recent studies indicate that PKCδ also is dynamically regulated through tyrosine phosphorylation in H\(_2\)O\(_2\)- and phorbol 12-myristate 13-acetate (PMA)-treated cardiomyocytes. H\(_2\)O\(_2\) activates Src and related Src-family kinases (SFKs), which function as dual PKCδ-Tyr\(^{311}\) and -Tyr\(^{332}\) kinases in vitro and contribute to H\(_2\)O\(_2\)-dependent PKCδ-Tyr\(^{311}\)/Tyr\(^{332}\) phosphorylation in cardiomyocytes and in mouse embryo fibroblasts. H\(_2\)O\(_2\)-dependent PKCδ-Tyr\(^{311}\)/Tyr\(^{332}\) phosphorylation is defective in SYF cells (deficient in SFKs) and restored by Src re-expression. PMA also promotes PKCδ-Tyr\(^{311}\) phosphorylation, but this is not associated with SFK activation or PKCδ-Tyr\(^{332}\) phosphorylation. Rather, PMA increases PKCδ-Tyr\(^{311}\) phosphorylation by delivering PKCδ to SFK-enriched caveolae. Cyclodextrin treatment disrupts caveolae and blocks PMA-dependent PKCδ-Tyr\(^{311}\) phosphorylation, without blocking H\(_2\)O\(_2\)-dependent PKCδ-Tyr\(^{311}\) phosphorylation. The enzyme that acts as a PKCδ-Tyr\(^{311}\) kinase without increasing PKCδ phosphorylation at Tyr\(^{311}\) in PMA-treated cardiomyocytes is uncertain. Although in vitro kinase assays implicate c-Abl as a selective PKCδ-Tyr\(^{311}\) kinase, PMA-dependent PKCδ-Tyr\(^{311}\) phosphorylation persists in cardiomyocytes treated with the c-Abl inhibitor ST1571 and c-Abl is not detected in caveolae; these results effectively exclude a c-Abl-dependent process. Finally, we show that 1,2-dioleoyl-sn-glycerol mimics the effect of PMA to drive PKCδ to caveolae and increase PKCδ-Tyr\(^{311}\) phosphorylation, whereas G protein-coupled receptor agonists such as norepinephrine and endothelin-1 do not. These results suggest that norepinephrine and endothelin-1 increase 1,2-dioleoyl-sn-glycerol accumulation and activate PKCδ exclusively in non-caveolae membranes. Collectively, these results identify stimulus-specific PKCδ localization and tyrosine phosphorylation mechanisms that could be targeted for therapeutic advantage.

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1 To whom correspondence should be addressed: 630 West 168 St., New York, NY 10032. Tel.: 212-305-4297; Fax: 212-305-8780; E-mail: sfs1@columbia.edu.

2 The abbreviations used are: PKC, protein kinase C; PM, phorbol 12-myristate 13-acetate; SFK, Src-family kinases; DAG, 1,2-dioleoyl-sn-glycerol; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; dIC\(_8\), 1,2-diacylantolyl-sn-glycerol; MES, 4-morpholineethanesulfonic acid; PSSA, phospho-site specific antibodies; PS, phosphatidylserine; RP, reverse phase; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; AR, adrenergic receptor; NE, norepinephrine; ET-1, endothelin-1.
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partment (9), this study examines the role of caveolae as platforms for cross-talk between PKCα and SFKs in cardiomyocytes. Insofar as PKCα-Tyr311 phosphorylation is predicted to be associated with a coordinate increase in PKCα phosphorylation at Tyr332 (a site that also is a target for in vitro Src-dependent phosphorylation (7)), and PKCα-Tyr332 phosphorylation may impart functionally important properties (as a consensus binding sequence for the SH2 domain of the adapter protein Shc (10) or to influence PKCα proteolytic cleavage, which contributes to the induction of apoptosis (11)), the mechanisms that regulate PKCα-Tyr332 phosphorylation also were examined.

EXPERIMENTAL PROCEDURES

Materials—Antibodies were from the following sources: PKCα-Thr(P)\(^{305}\), PKCα-Tyr(P)\(^{311}\), Src-Tyr(P)\(^{416}\), ERK-Thr(P)\(^{202}/\)
Tyr(P)\(^{204}\), Abl, and FAK, Cell Signaling Technology; PKCε and Caveolin-3 (BD Transduction Laboratories); PKCα and PKCδ-Tyr(P)\(^{332}\), Santa Cruz Biotechnology; Src, Oncogene; Lyn, Fyn, and Yes, Santa Cruz Biotechnology; anti-Tyr(P), Clone 4G10, BD Transduction Laboratories; and FAK-Tyr(P)\(^{397}\), BIO-
SOURCE. Recombinant human PKCα (rPKCα) was from Sigma; active Src kinase was from Panvera; Lyn, Fyn, Yes, PDGFR β, FAK, JAK2, and c-Abl were from Upstate Biotechnolo-
gies. PMA and platelet-derived growth factor (PDGF) were from Sigma. 1,2-Dioleoyl-sn-glycerol (DAG) and 1,2-di-
octanoyl-sn-glycerol (diC8) were from Avanti Polar Lipids, Inc. All other chemicals were reagent grade.

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 cardiomyo-
cytes followed by irradiation as described previously (2, 12, 13). A differential attachment procedure to enrich for cardiomyo-
cytes of 2-day-old Wistar rats by a trypsin dispersion procedure using solubilized in detergent-containing lysis buffer (50 mM HEPES, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1.5 mM MgCl₂, 10% glycer-
erol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM phenyl-
methanesulfonyl fluoride, 1 mM sodium vanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), sonicated, and centrifuged at 14,000 × g for 15 min. The supernatant was saved as the soluble fraction and the detergent-insoluble frac-
tion was solubilized in SDS-PAGE sample buffer.

Preparation of Caveolae Membranes—Fractions enriched in the muscle-specific caveolin-3 isoform were prepared according to a detergent-free purification scheme described previously (9, 14). All steps were carried out at 4 °C. Briefly, cells from five 100-mm diameter dishes were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 ml sodium carbonate, pH 11.0 (0.5 ml/dish). Cells from five dishes were combined (total volume, 2.5 ml) for each preparation. The extract was sequentially disrupted by homogenization with a loose-fitting Dounce homogenizer (10 strokes), a Polytron tis-
sue grinder (three 10-s bursts), and a tip sonicator (three 20-s bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in MES-buff-
ered saline (25 mM MES, pH 6.5, and 0.15 mM NaCl), placed on the bottom of an ultracentrifuge tube, overlaid with a 5–30% discontinuous sucrose gradient (3 ml of 5% sucrose and 4 ml of 35% sucrose, both in MES-buffered saline containing 0.25 mM sodium carbonate), and centrifuged at 260,000 × g for 16 to 18 h in a SW40 rotor (Beckman Coulter, Palo Alto, CA). After centrifugation, 13 1-ml fractions were collected. A pooled caveola fraction (fractions 4–5, containing all of the buoyant caveolin-3 immunoreactivity and 0.5–1% total starting cell pro-
tein), a pooled fraction 8–13 (F8–13, which contains the bulk of the cellular material including the cytosol and most of the particulate membrane fraction), and the insoluble pellet (P, which is solubilized in SDS-PAGE sample buffer) were sub-
jected to SDS-PAGE and immunoblotting. The caveolin-3-en-
riched membrane fraction isolated according to this method is biochemically distinct from the surrounding phospholipid bilayer and is operationally defined as caveolae in this study. However, it is important to note that this buoyant membrane fraction undoubtedly contains both true caveolae (specialized lipid raft membranes that contain caveolin and form invagina-
tions at, or vesicles close to, the surface membrane) and mor-
phologically featureless lipid rafts (that coexist and may even associate with caveolae (15)). Biochemical methods to separate these distinct membrane subdomains and experiments to resolve their discrete cellular functions are beyond the scope of this study.

Immunoprecipitation and Immunoblot Analysis—Immunopre-
claretion on lystate or immunoprecipitated PKCα was according to methods described previously or the manufacturer’s instruc-
tions (2, 7, 9). All anti-PKC antibodies (including the phospho-
site specific antibodies (PSSAs) that specifically recognize PKCα phosphorylation at Thr\(^{305}\), Tyr\(^{311}\), and Tyr\(^{332}\) have been validated (2). Of note, the PKCα-Tyr(P)\(^{311}\) antibody is highly specific reagent that can be used to track PKCα phosphoryla-
tion (at endogenous levels of enzyme expression) in experi-
ments on cell lysates. The anti-PKCα-Tyr(P)\(^{332}\) antibody is less specific and requires immunoprecipitation for studies of endogenous PKCα phosphorylation. In each figure, each panel
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In Vitro PKCδ Phosphorylation by Src and Other Tyrosine Kinases—0.03 μg of recombinant human PKCδ (rPKCδ) was preincubated in the absence or presence of Src kinase (0.18 units) in 110 μl of a reaction buffer containing 43 mM Tris-Cl, pH 7.5, 5.45 mM MgCl₂, 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-32P]ATP (13 μCi, 83 μM). Incubations were carried out for 30 min at 30 °C in the absence or presence of 91 μM HCl, and 10 μM ammonium bicarbonate, 17 μM HCl, and 10 μg of sequencing grade trypsin. Digested peptides were eluted from the membrane by sonication, lyophilized, and the residue was reconstituted in 0.1% trifluoroacetic acid and fractionated by RP-HPLC on a Vydac semimicro C₁₈ column (2.1 × 250 mm). Peptides were eluted with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 140 min at a flow rate of 1 ml/min. The eluant was monitored at 220 nm and fractions were collected every 30 s for Cherenkov counting. Radioactive peptides of interest were submitted to the Howard Hughes Medical Institute/Columbia University Protein Chemistry Core Facility for sequencing by MALDI-MS.

RESULTS

Distinct Agonist-dependent PKCδ Phosphorylation Patterns in Cardiomyocytes—We previously reported that treatment with PMA, the α₁-adrenergic receptor (α₁-AR) agonist norepinephrine (NE), and H₂O₂ results in distinct PKCδ phosphorylation patterns in cardiomyocytes. Fig. 1 extends these studies to examine PKCδ regulation by endothelin-1 (ET-1) and PDGF. Fig. 1A shows that PKCδ is recovered from resting cardiomyocytes with little-to-no Thr⁵⁰⁵ or Tyr⁳¹¹ phosphorylation. NE and ET-1 increase PKCδ phosphorylation at Thr⁵⁰⁵. In each case, PKCδ-Thr⁵⁰⁵ phosphorylation is maximal at 5 min (the first time point sampled in the experiments) and sustained for at least another 10 min of continuous stimulation (Fig. 1B). NE- and ET-1-dependent increases in PKCδ-Thr⁵⁰⁵ phosphorylation are similar in magnitude, and comparable with the stimulatory effect of PMA (Fig. 1, A and C). We previously reported that PMA induces a rapid and sustained increase in PKCδ-Thr⁵⁰⁵ phosphorylation that falls only as PKCδ phosphorylation at Tyr³¹¹, whereas PKCδ-Thr⁵⁰⁵ phosphorylation at Thr⁵⁰⁵ decreases due to down-regulation during chronic stimulation (2). Fig. 1B shows that the NE-dependent increase in PKCδ-Thr⁵⁰⁵ phosphorylation also is sustained for at least 60 min, whereas the ET-1 response wanes with stimulation intervals greater than 30 min. PDGF also increases PKCδ-Thr⁵⁰⁵ phosphorylation, but this response is quite modest in magnitude when compared with the considerably more robust increases in PKCδ-Thr⁵⁰⁵ phosphorylation induced by NE, ET-1, or PMA. PDGF-dependent PKCδ-Thr⁵⁰⁵ phosphorylation is detected at 5 min and wanes as the stimulation interval is prolonged to >30 min (Fig. 1, B and C).

PDGF increases PKCδ phosphorylation at Tyr³¹¹, whereas NE and ET-1 treatments for 5 min (Fig. 1A) or selected time points up to 60 min of incubation (Ref. 8 and data not shown) do not lead to a detectable increase in PKCδ-Tyr³¹¹ phosphorylation. The magnitude of the PDGF-dependent increase in

FIGURE 1. Agonist-specific differences in PKCδ localization and phosphorylation mechanisms in cardiomyocytes. Immunoblot analysis of cell extracts (panels A and B) or soluble and particulate fractions (panel D) from cells treated with NE (1 μM), ET-1 (100 nM), PDGF (50 ng/ml), H₂O₂ (5 mM), or PMA (300 nM). Stimulations were for 5 min unless indicated otherwise. All samples in panel 8 are from a single experiment. Agonist-dependent increases in PKCδ-Thr⁵⁰⁵ and -Tyr⁳¹¹ phosphorylation at 5 (n = 7) and 30 min (n = 3) are quantified in panel C (mean ± S.E., *p < 0.05 compared with basal; †, p < 0.05 compared with H₂O₂).
PKCδ-Tyr\(^{311}\) phosphorylation is comparable with the stimulatory effect of PMA (Fig. 1, A and C). However, PDGF and PMA responses are not necessarily mediated by the identical signaling mechanism. Using an anti-Src-Tyr(P)\(^{416}\) PSSA that selectively recognizes the activation loop phosphorylated/activated forms of Src and related SFKs, Fig. 1B shows that PDGF induces a modest increase in Src activity. In contrast, PMA promotes PKCδ-Tyr\(^{311}\) phosphorylation without increasing Src-Tyr\(^{416}\) phosphorylation (Fig. 1B and Ref. 8). Finally, Fig. 1B shows that H\(_2\)O\(_2\) induces a robust increase in PKCδ-Tyr\(^{311}\) phosphorylation (to a level that exceeds PKCδ-Tyr\(^{311}\) phosphorylation in PDGF- or PMA-treated cardiomyocytes) and that the H\(_2\)O\(_2\) response is associated with a massive increase in Src-Tyr\(^{416}\) phosphorylation.

PKCδ partitions between the soluble and particulate fractions of resting cardiomyocytes (Fig. 1D). PMA, NE, and ET-1 translocate PKCδ protein from the soluble to the particulate fraction and increase PKCδ-Thr\(^{505}\) phosphorylation exclusively in the particulate fraction (Fig. 1D and data not shown). The PMA-dependent increase in PKCδ-Tyr\(^{311}\) phosphorylation also is detected exclusively in the particulate fraction. In contrast, treatment with H\(_2\)O\(_2\) (which does not translocate PKCδ to the particulate fraction, but rather releases PKCδ from the particulate fraction) leads to an increase in PKCδ-Tyr\(^{311}\) phosphorylation in both the soluble and particulate fractions. Fig. 1D also shows that H\(_2\)O\(_2\) does not increase PKCδ-Thr\(^{505}\) phosphorylation and NE does not increase PKCδ-Tyr\(^{311}\) phosphorylation.

Tyro332 has been identified as another major phosphorylation site on heterologously overexpressed PKCδ in H\(_2\)O\(_2\)-treated COS-7 cells (16). Because preliminary studies demonstrated that the anti-PKCδ-Tyr(P)\(^{332}\) antibody used in our studies is not sufficiently sensitive or specific to be used directly in immunoblotting studies on cell lysates, PKCδ was immunoprecipitated from resting and agonist-treated cardiomyocyte cultures followed by immunoblotting analysis with anti-PKCδ-Tyr(P)\(^{332}\), anti-PKCδ-Tyr(P)\(^{311}\), a general anti-Tyr(P) antibody (to track total PKCδ tyrosine phosphorylation), and an anti-PKCδ protein antibody (to validate equal protein recovery and loading). Fig. 2A shows that high concentrations of H\(_2\)O\(_2\) promote PKCδ-Tyr\(^{311}\) and -Tyr\(^{332}\) phosphorylation in association with an increase in Src-Tyr(P)\(^{416}\) immunoreactivity (used as a surrogate strategy to track SFK activity). In contrast, PMA increases PKCδ-Tyr\(^{311}\) phosphorylation without detectably activating SFKs or increasing PKCδ-Tyr\(^{332}\) phosphorylation; NE does not increase Src phosphorylation at Tyr\(^{416}\) or PKCδ phosphorylation at either tyrosine residue.

We previously used an immunoprecipitation strategy with antibodies that discriminate individual SFKs (Src, Lyn, and Fyn) followed by immunoblotting with the anti-Src-Tyr(P)\(^{416}\) PSSA to compare the time course for Src activation and PKCδ-Tyr\(^{311}\) phosphorylation. These previous studies established that Src activation precedes PKCδ-Tyr\(^{311}\) phosphorylation; H\(_2\)O\(_2\) induces a similar rapid and robust increase in Src, Lyn, and Fyn activity that is maximal by 2 min, whereas the H\(_2\)O\(_2\)-dependent increase in PKCδ-Tyr\(^{311}\) phosphorylation is detectable at 2 min and increases further as the incubation interval is prolonged to 5–30 min (8). Fig. 2B uses a similar strategy to compare the H\(_2\)O\(_2\) requirements for SFK activation and PKCδ-Tyr\(^{311}/\)Tyr\(^{332}\) phosphorylation. These studies show that high H\(_2\)O\(_2\) concentrations (5 mM, a level of oxidative stress that typically leads to cellular necrosis) activate Src, Lyn, and Fyn, whereas lower H\(_2\)O\(_2\) concentrations (0.1–1 mM) do not detectably increase Src, Lyn, or Fyn activity (although control experiments show that 0.1–1 mM H\(_2\)O\(_2\) activates ERK and previous literature links low H\(_2\)O\(_2\) concentrations to changes in gene expression, cardioprotection, and at least some features of the cardiac hypertrophic response (17, 18)). The steep concentration-response curves for H\(_2\)O\(_2\)-dependent SFK activation identified in
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A.

B.

C.

the observation that SYF cells support a low level of H2O2-dependent PKCδ-Tyr311 phosphorylation was surprising. Further studies suggest that this might be attributable to Lyn, which has been implicated as a PKCδ kinase (10) and is detected at similar low levels (exclusively in a detergent-insoluble fraction) of SYF and Src− cells (Fig. 3C). Lyn also is detected, as multiple molecular species with different electrophoretic mobilities, in both the soluble and insoluble fractions of neonatal rat cardiomyocytes. Lyn immunoreactivity in SYF and Src− cells co-migrates with the slowest migrating form of Lyn in the insoluble fraction of cardiomyocytes. Studies with the anti-Src-Tyr416 PSSA show a similar amount of active enzyme (co-migrating with Lyn protein) in the insoluble fractions of H2O2-treated SYF and Src− cells. The increase in anti-Src-Tyr416 immunoreactivity due to Src expression in Src− cells is detected exclusively in the soluble fraction. Collectively, these studies indicate that SYF cells contain some Lyn protein/activity (i.e. they are not completely devoid of SFK activity), introducing a note of caution regarding the use of SYF cells to resolve the cellular actions of SFKs. Nevertheless, whereas SYF cells support a low level of PKCδ-Tyr311 phosphorylation, studies in Src− cells suggest that Src itself is the major cellular PKCδ-Tyr311 kinase. The results also establish that PKCδ tyrosine phosphorylation (identified with PKCδ-pY311 and -pY332 antibodies in PKCδ extracts with the indicated antibodies. Panel C, immunoblotting to compare Lyn protein and SFK activity (Src-Tyr(P)416 immunoreactivity) in soluble and detergent-insoluble fractions from SYF cells, Src− cells, and cardiomyocytes cultures (CM). Results were replicated in three separate experiments on separate culture preparations.

FIGURE 3. H2O2- and PMA increase PKCδ tyrosine phosphorylation in Src+ cells, but not in SYF cells that lack Src, Yes, and Fyn expression. Panel A, SYF and Src+ cultures were treated with vehicle or H2O2 (alone or following a pretreatment with 10 μM PP1) followed by immunoprecipitation (IP) of PKCδ and immunoblotting (IB) for PKCδ-Tyr(P)332 and overall tyrosine phosphorylation. Panel B, SYF and Src− cultures were treated with vehicle, and the indicated concentrations of H2O2 or PMA (300 nM). Immunoblotting was on cell extracts with the indicated antibodies. Panel C, immunoblotting to compare Lyn protein and SFK activity (Src-Tyr(P)416 immunoreactivity) in soluble and detergent-insoluble fractions from SYF cells, Src− cells, and cardiomyocytes cultures (CM). Results were replicated in three separate experiments on separate culture preparations.

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phosphorylation is a general response to oxidative stress; this response is not unique to cardiomyocytes. PMA Translocates PKC to Caveolae and Increases PKC-Thr505/Tyr311 Phosphorylation in the Caveolae Fraction; H$_2$O$_2$ Increases PKC-Thr$_{505}$/Tyr$_{311}$ Phosphorylation in Both Caveolae and Non-caveolae Fractions—We previously demonstrated that PMA delivers phorbol ester-sensitive PKC isoforms to cardiomyocyte caveolae, specialized cholesterol-/glycosphingolipid-enriched buoyant membranes (9). Because caveolae function as platforms to facilitate cross-talk between signaling molecules (and SFKs partition to caveolae membranes in non-cardiomyocyte models (22)), we tested the hypothesis that PMA-dependent PKC$_{\delta}$-Tyr$_{311}$ phosphorylation requires PKC$_{\delta}$ translocation to SFK-enriched caveolae membranes. Fig. 4A shows that PKC$_{\delta}$ is detected at low levels, and PKC$_{\varepsilon}$ is largely excluded, from resting caveolae (tracked by immunoblotting for the muscle-specific caveolin-3 isoform that is used as a recovery marker in these experiments). The bulk of the PKC isoform immunoreactivity is recovered in the heavy gradient fractions (F8–13 fraction). PMA translocates PKC$_{\delta}$ and PKC$_{\varepsilon}$ to the caveolae fraction. Our previous estimates place ~15–25% of total PKC isoform immunoreactivity (for both PKC$_{\delta}$ and PKC$_{\varepsilon}$) in the caveolae fraction of PMA-treated cardiomyocytes (9). Because the bulk of the PKC protein immunoreactivity remains in the F8–13 fraction of PMA-treated cardiomyocytes, a reciprocal PMA-dependent decrease in PKC protein immunoreactivity in the F8–13 fraction is not resolved for technical reasons.

Initial studies using a generic anti-Tyr(P) antibody showed that PMA treatment leads to the appearance of a single band in caveolae (but not F8–13 or pellet fractions) that co-migrates with PKC$_{\delta}$ (Fig. 4A). Studies with the more specific anti-PKC$_{\delta}$-Tyr(P)311 PSSA show that a Tyr311-phosphorylated form of PKC$_{\delta}$ is detected in resting cardiomyocytes and is confined to the heavy F8–13 gradient fractions and that PMA increases PKC$_{\delta}$-Tyr$_{311}$ phosphorylation exclusively in the caveolae fraction. The PMA-dependent increment in PKC$_{\delta}$-Tyr$_{311}$ immunoreactivity is blocked by PP1, but not GF109203X (a general inhibitor of all PKC isoforms) or Go¨6976 (a compound that inhibits conventional PKC isoforms or PKD, but does not block the catalytic activity of PKC$_{\delta}$ or other novel PKC isoforms, Fig. 4B). PP1, GF102903X, and Go¨6976 do not interfere with PMA-dependent PKC$_{\delta}$ translocation to caveolae.

Fig. 4A shows that caveolae contain considerable amounts of (albeit not all) Src immunoreactivity and virtually all of the cellular Fyn, Lyn, and Yes proteins. These SFK subcellular local-

FIGURE 4. PMA-dependent PKC$_{\delta}$-Tyr$_{311}$ phosphorylation is confined to caveolae (and is disrupted by treatment with cyclodextrin); H$_2$O$_2$ does not drive PKC$_{\delta}$ to caveolae; H$_2$O$_2$ increases PKC$_{\delta}$-Thr$_{505}$/Tyr$_{311}$ phosphorylation in both caveolae and F8–13 fractions. Panels A–D, cardiomyocyte cultures were treated for 20 min with 300 nM PMA without or with a 45-min pretreatment with GF109203X (GFX, 5 μM), PP1 (10 μM), or Go6976 (5 μM) or with 5 mM H$_2$O$_2$ as indicated. Caveolae membranes (Cav) were separated from heavy gradient fractions (F8–13) and the insoluble pellet (P) and samples were subjected to immunoblotting with the indicated antibodies as described under “Experimental Procedures.” Some variability in the detection of PKC$_{\delta}$-Tyr(P)311 immunoactivity in the F8–13 fractions between experiments (compare panels A and B) is attributable to differences in protein loading and gel exposure time. Panel E, immunoblotting on cell extracts from cardiomyocyte cultures incubated for 1 h at 37°C in SFM containing vehicle, 2% cyclodextrin, or 2% cyclodextrin complexed with 1.3 mM cholesterol and then challenged with PMA or H$_2$O$_2$ as indicated. For each panel, the results were replicated in three to six separate experiments on separate culture preparations.
PKCδ abundance. PMA translocates PKCδ to caveolae (i.e. increases PKCδ abundance in caveolae), whereas \( \text{H}_2\text{O}_2 \) does not. Hence, the \( \text{H}_2\text{O}_2 \)-dependent increase in PKCδ-Tyr\(^{311} \) phosphorylation is largely in non-caveolae fractions.

We previously demonstrated that cycloexdrin (a membrane-impermeable cholesterol-binding drug) can be used to extract approximately two-thirds of total cellular cholesterol and redistribute caveolin-3 (and other resident caveolae proteins) from buoyant membranes to heavy sucrose fractions, without inducing any gross morphological toxicity or major changes in spontaneous automaticity (14). Fig. 4E shows that cycloexdrin treatment also leads to a defect in PMA-dependent PKCδ-Tyr\(^{311} \) phosphorylation, whereas PMA-dependent PKCδ-Thr\(^{505} \) phosphorylation and \( \text{H}_2\text{O}_2 \)-dependent PKCδ-Thr\(^{505} \) phosphorylation (which are detected in both caveolae and F8–13 fractions) persist in cycloexdrin-treated cardiomyocytes. Control experiments show that this effect requires cholesterol depletion, as it is not observed when treatment is with cycloexdrin-cholesterol complexes (that do not deplete cellular cholesterol, excluding a nonspecific effect of the cycloexdrin treatment).

Collectively, these results identify a critical role for cholesterol[SFK-enriched caveolae in the PMA-dependent PKCδ-Tyr\(^{311} \) phosphorylation pathway.

In Vitro PKCδ Tyrosine Phosphorylation—Cell-based studies show that PMA selectively increases PKCδ phosphorylation at Tyr\(^{311} \) (but not Tyr\(^{332} \)) in caveolae that contains multiple SFKs; pharmacologic studies implicate Src or a related PP1-sensitive enzyme as the PMA-dependent PKCδ-Tyr\(^{311} \) kinase. Because previous \textit{in vitro} kinase assays identified an effect of Src to phosphoprotein PKCδ at both Tyr\(^{311} \) and Tyr\(^{332} \) (7), and PP1 is an equipotent inhibitor of Src, related SFKs, and PDGFRs (23), we performed \textit{in vitro} kinase assays to determine whether other SFKs or the PDGFR might promote PKCδ-Tyr\(^{311} \) phosphorylation without inducing a coordinate increase in PKCδ phosphorylation at Tyr\(^{332} \). Fig. 5A shows that Lyn, Fyn, and Yes mimic the effect of Src to increase PKCδ phosphorylation at both Tyr\(^{311} \) and Tyr\(^{332} \) (\textit{i.e. in vitro} PKCδ tyrosine phosphorylation by individual SFKs cannot be distinguished). In contrast, an active fragment of recombinant human PDGFRβ that undergoes robust \textit{in vitro} autophosphorylation triggers only a very modest increase in PKCδ phosphorylation at Tyr\(^{311} \). Studies with the anti-PKCδ-Tyr\(^{311} \) PSSA suggest that this is not associated with a coordinate increase in PKCδ-Tyr\(^{332} \) phosphorylation. However, conclusions regarding the role of PDGFRs as a PKCδ-Tyr\(^{332} \) kinase are somewhat tenuous, because these immunoblotting experiments are undermined to some degree by the imperfect specificity of the anti-PKCδ-Tyr\(^{311} \) PSSA; this PSSA recognizes the autophosphorylated PDGFR. Although this should not entirely preclude the identification of imme-

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a PDGFR-dependent increase in PKCδ-Tyr332 phosphorylation (because the PDGFR and PKCδ have somewhat different mobilities and are resolved in these studies), this negative conclusion is considered more tentative. Nevertheless, the physiologic significance of a very modest PDGFR-dependent increase in PKCδ-Tyr311 phosphorylation (in the context of the very high levels of PDGFR autocatalytic activity) is uncertain.

FAK and JAK2 also have been identified as enzymes that cooperate with Src and localize to caveolae in other cell types (24). Fig. 5B shows that a pool of cellular FAK constitutively localizes to the cardiomyocyte caveolae with little-to-no phosphorylation at Tyr397 (a target for an autophosphorylation reaction that accompanies FAK activation (25)). Oxidative stress increases FAK abundance and FAK-Tyr397 phosphorylation in caveolae; FAK-Tyr397 phosphorylation also is detected at lower levels in the heavy F8–13 fraction. Of note, H2O2-dependent PKCδ-Tyr311 increases FAK abundance and FAK-Tyr397 phosphorylation in cardiomyocyte caveolae. This is presumed to reflect a docking interaction between the Src SH2 domain and Tyr397-phosphorylated FAK, which has been implicated in FAK activation by certain stimuli (i.e. integrin engagement but not VEGF or G protein-coupled receptor activation (25–28)). The observation that FAK localizes to (and is activated in) caveolae via a PP1-sensitive mechanism provided the rationale to consider a role for FAK as a putative PKCδ-Tyr311 kinase. Fig. 5C shows that FAK and JAK2 (another non-receptor tyrosine kinase that localizes to caveolae in other cell types) undergo robust autophosphorylation reactions but neither enzyme displays PKCδ-Tyr311 or -Tyr332 kinase activity.

Finally, we considered a role for c-Abl activity in the detergent-insoluble lipid raft fraction in certain cell types, is moderately sensitive to the direct inhibitory effects of PP1, and also can be inhibited by PP1 indirectly (because it is downstream from Src in certain signaling pathways). A recent study implicated c-Abl activity in a cellular PKCδ-Tyr311 phosphorylation pathway (29), but the direct in vitro effects of c-Abl (and a role for c-Abl in PKCδ-Tyr332 phosphorylation) were not considered. Fig. 6A shows that c-Abl mimics the effect of Src to phosphorylate PKCδ at Tyr311, but c-Abl does not act as a PKCδ-Tyr332 kinase. Of note, our previous studies showed that Src-dependent PKCδ-Tyr311 phosphorylation is via a PMA-dependent mechanism (presumably reflecting a role for lipid cofactors to induce a conformational change that renders PKCδ a better substrate for phosphorylation by Src). Fig. 6 extends the analysis by showing that c-Abl also phosphorylates PKCδ in a lipid cofactor-dependent manner and that PKCδ tyrosine phosphorylation (either by Src or c-Abl) is similar in assays with PS/PMA, PS/DAG, or PS/diC8 (a membrane-permeant DAG analogue typically used in cell based studies); PS alone does not support Src- or c-Abl-dependent PKCδ tyrosine phosphorylation. Collectively, these results indicate that: 1) c-Abl mediates PKCδ phosphorylation at Tyr311, but not Tyr332 and 2) that DAG analogues can substitute for PMA and render PKCδ a substrate for tyrosine phosphorylation by either Src or c-Abl.

Because the immunoblotting studies are biased by the availability of PSSAs, and previous reports in the literature using a mutagenesis strategy mapped c-Abl-dependent PKCδ phosphorylation to Tyr312 (a highly conserved kinase domain tyrosine residue corresponding to Tyr204 in PKA, a residue that participates in intramolecular interactions that radiate throughout the PKA structure and are critical for the transition from inactive/apo to active/closed conformations of the enzyme (30, 31)), we also subjected c-Abl- and Src-phosphorylated PKCδ to peptide mapping analysis. Radiolabeled PKCδ was purified by SDS-PAGE, blotted to nitrocellulose, excised from the membrane, digested with trypsin, and peptide fragments were separated by RP-HPLC (Fig. 6B). The four peaks, containing radioactive peptide fragments detected in assays...
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PKCδ from cells. Finally, peak 4 was specific for RP-HPLC chromatograms from in vitro kinase assays with PKCδ and either Src or c-Abl (and was not detected when in vitro kinase assays were performed with PKCδ alone). These peaks contained the oxidized and reduced forms of a phospho-Tyr311 fragment (note, differences in sequence, and importantly never recovered Tyr512-bearing peptide fragments, a role for c-Abl as a possible PKCδ-Tyr311 phosphorylation is via a c-Abl-independent mechanism.

Although the activity profile of c-Abl conforms to the prediction for a PMA-activated PKCδ-Tyr311 kinase, a role for c-Abl is excluded by additional studies showing that: 1) c-Abl immuno-reactivity is readily detected in the heavy F8-13 gradient fractions, but c-Abl is not detected in cardiomyocyte caveolae (Fig. 6C) and 2) ST1571 (also known as Gleevec, a potent c-Abl inhibitor that also inhibits c-kit and PDGFR activity, but does not inhibit Src (33)) attenuates the H2O2-activated PKCδ-Tyr311 phosphorylation pathway, but ST1571 does not block PMA-dependent PKCδ-Tyr311 phosphorylation (Fig. 6D). Studies of PDGFR activation (which is directly inhibited by ST1571) validate the efficacy of the ST1571 treatment protocol; PDGFR-dependent PKCδ-Tyr311 phosphorylation is blocked by ST1571, PP1, and AG1295 (a quinoxaline compound that acts as a selective inhibitor of PDGFRs, but does not inhibit Src (33)). These results indicate that PDGF promotes PKCδ-Tyr311 phosphorylation via a pathway that requires PDGFR activity and perhaps also Src (which is activated downstream of the PDGFR signaling pathway and is the better in vitro PKCδ-Tyr311 kinase). Collectively, these results indicate that c-Abl and SFKs cooperate to increase PKCδ tyrosine phosphorylation during oxidative stress, but PMA-dependent PKCδ-Tyr311 phosphorylation is via a c-Abl-independent mechanism.

**NE and ET-1 Do Not Promote PKCδ Translocation to Caveolae; NE and ET-1 Selectively Increase PKCδ-Thr505 (but Not Tyr311) Phosphorylation in Non-caveolae Membranes—**Experiments depicted in Fig. 1 show that NE treatment leads to the translocation of PKCδ from the cytosol to the particulate fraction and increased PKCδ-Thr505 phosphorylation in the particulate fraction. However, experiments depicted in Fig. 7A provide surprising evidence that NE and ET-1 do not mimic the effect of PMA to drive PKCδ or PKCe to caveolae membranes. NE and ET-1 also do not increase PKCδ-Thr505 phosphorylation in the caveolae fraction. NE- and ET-1-dependent increases in PKCδ-Thr505 phosphorylation are detected exclusively in the F8–13 fraction. Importantly, the effect of NE to promote PKCδ-Thr505 phosphorylation is blocked when DAG accumulation is prevented by the phospholipase C inhibitor U73123; control experiments show that U73123 does not block PMA-dependent PKCδ-Thr505 or -Tyr311 phosphorylation, excluding a nonspecific effect of U73123. These results indicate that NE activates PKCδ through a phospholipase C-dependent mechanism involving the hydrolysis of membrane phosphoinositides and the generation of DAG. The observation that NE and ET-1 do not drive PKCδ to caveolae suggests either: 1) GPCR-dependent lipid cofactor generation is defective in caveolae (i.e. α1-adrenergic and ET-1 receptors increase DAG exclusively in non-caveolae membranes) or 2) NE and ET-1 increase DAG in caveolae but a local increase in DAG is not sufficient to anchor PKCδ and PKCe to caveolae membranes. The notion that DAG might not be sufficient to anchor PKCδ to caveolae membranes is based upon recent evidence that the molecular determinants for PKCδ activation by PMA and DAG differ. PMA binds with high affinity to the PKCδ-C1A domain, whereas DAG anchors full-length PKCδ to membranes via an interaction with the PKCδ-C1A domain (34). Therefore, the
Tyrosine phosphorylation (in both caveolae and heavy fractions). The PKC\(\beta\) subfamily is predicated on the assumption that PKCs are regulated by lipid cofactors (that induce a conformational change that renders PKC\(\beta\) catalytically active conformation to their target substrates in membranes. However, PKC\(\beta\) binding to the C1A domain is sufficient to anchor full-length PKC\(\beta\) to the particulate fraction and to caveolae membranes. Fig. 8A shows that diC8 mimics the effects of PMA to translocate PKC\(\delta\) and PKC\(\epsilon\) from the soluble to the particulate fraction; this effect is detected largely as a PMA-/diC8-dependent decrease in soluble enzyme; the already high levels of PKC\(\beta\) decrease in soluble enzyme; the already high levels of PKC\(\beta\) abundance in this fraction. diC8 also mimics the effects of PMA to increase PKC\(\delta\)-Thr\(^{505}\) and -Tyr\(^{311}\) phosphorylation in the particulate fraction. Fig. 8B shows that diC8 translocates PKC\(\delta\) and PKC\(\epsilon\) to caveolae; diC8 also increases PKC\(\delta\)-Tyr\(^{311}\) phosphorylation (exclusively in caveolae) and PKC\(\delta\)-Thr\(^{505}\) phosphorylation (in both caveolae and heavy fractions). The observation that DAG analogues localize PKC\(\delta\) (and support PKC\(\delta\)-Thr\(^{505}\) phosphorylation) in caveolae suggests that the defect in NE-/ET-1-dependent PKC\(\delta\) and PKC\(\epsilon\) localization to caveolae (and PKC\(\delta\)-Tyr\(^{311}\) phosphorylation in caveolae) is due to a defect in agonist-dependent DAG accumulation in this membrane fraction.

**DISCUSSION**

The conventional model of PKC activation by lipid cofactors is predicated on the assumption that PKCs are regulated entirely by translocation events that deliver the enzyme in an active conformation to their target substrates in membranes. This model, which assumes that PKC catalytic activity is an inherent and immutable property of the enzyme that is not altered by the activation process, has recently been challenged by studies showing that PKC\(\delta\) undergoes a series of highly regulated phosphorylation events at both an activation loop threonine and on tyrosine residues that contribute to the regulation of PKC\(\delta\) catalytic function (and particularly PKC\(\delta\) substrate specificity (5, 6)). These recent findings provide a rationale for studies that examine the mechanisms that regulate PKC\(\delta\) phosphorylation in a cellular context. This study identifies stimulus-specific differences in PKC\(\delta\) localization and phosphorylation that are likely to have physiologic relevance.

The PKC\(\delta\) sequence contains at least nine highly conserved tyrosine residues that have been implicated as sites for regulatory phosphorylation in various cellular contexts. Although the mechanisms and consequences of overall PKC\(\delta\) tyrosine phosphorylation have been scrutinized in considerable detail, there is still relatively limited information on the mechanisms that specifically regulate PKC\(\delta\) phosphorylation at Tyr\(^{311}\) and/or Tyr\(^{332}\) (sites that are the major targets for PKC\(\delta\) phosphorylation during oxidative stress and are critical for PKC\(\delta\) signaling to certain proapoptotic pathways (35)). We previously demonstrated that H\(_2\)O\(_2\) and PMA promote PKC\(\delta\)-Tyr\(^{311}\) phosphorylation via a PP1-sensitive pathway in cardiomyocytes (8). These results were interpreted as tentative evidence that agonist-dependent PKC\(\delta\)-Tyr\(^{311}\) phosphorylation is via Src or a related SFK. This conclusion gains further support from studies reported herein showing that Src expression is required for H\(_2\)O\(_2\)-dependent PKC\(\delta\)-Tyr\(^{311}\)/-Tyr\(^{332}\) (and PMA-dependent PKC\(\delta\)-Tyr\(^{311}\) phosphorylation in the SYF/Src\(^+\) cell lines and that Src, Lyn, Fyn, and Yes act as in vitro PKC\(\delta\)-Tyr\(^{311}\) and -Tyr\(^{332}\) kinases. However, our studies also identify stimulus-specific differences in PKC\(\delta\) tyrosine phosphorylation mechanisms in H\(_2\)O\(_2\)- and PMA-treated cardiomyocytes. H\(_2\)O\(_2\) activates SFKs and markedly increases PKC\(\delta\)-Tyr\(^{311}/\)Tyr\(^{332}\) phosphorylation in both soluble and particulate subcellular compartments. Although the H\(_2\)O\(_2\)-dependent increase in PKC\(\delta\)-Tyr\(^{311}\) phosphorylation is blocked by PP1, it also is attenuated by the c-Abl inhibitor ST1571, suggesting that c-Abl contributes to PKC\(\delta\)-Tyr\(^{311}\) phosphorylation in H\(_2\)O\(_2\)-treated cardiomyocytes (similar to its role as an in vivo H\(_2\)O\(_2\)-activated PKC\(\delta\)-Tyr\(^{311}\) kinase in glioma cells (29)). In contrast, PMA translocates PKC\(\delta\) to caveolae and leads to a highly localized increase in PKC\(\delta\)-Tyr\(^{311}\) phosphorylation in this subcellular compartment. Although PMA treatment does not activate SFKs, PMA-dependent PKC\(\delta\)-Tyr\(^{311}\) phosphorylation also is blocked by PP1; it is not blocked by ST1571 (effectively excluding a role for c-Abl, which is not detected in the caveolae fraction). On the basis of further studies showing that Src acts as an in vitro PKC\(\delta\)-Tyr\(^{311}\) kinase only in assays containing lipid cofactors (that induce a conformational change that renders PKC\(\delta\) a better substrate for SFKs) and that PMA-dependent PKC\(\delta\)-Tyr\(^{311}\) phosphorylation is blocked by cyclodextrin, which disrupts caveolae, our studies suggest that PMA promotes PKC\(\delta\)-Tyr\(^{311}\) phosphorylation by delivering PKC\(\delta\) in its active conformation to caveolae, in close proximity to SFKs. These studies also expose distinct PKC\(\delta\) phosphorylation patterns in caveolae and non-caveolae membranes. PKC\(\delta\) accumulates as a dually Thr\(^{505}/\)Tyr\(^{311}\)-phosphorylated enzyme in the caveolae fraction of PMA-treated cardiomyocytes, whereas PKC\(\delta\) is selectively phosphorylated at Thr\(^{505}\) in other membrane fractions. These results are consistent with recent findings from Markou et al. (36) showing that fast protein liquid chromatography can be used to resolve PKC\(\delta\) from agonist-treated cardiomyocytes into pools of enzyme with distinct phosphorylation patterns and activities. Our study extends the analysis by identifying the unique subcellular compartment of individual PKC\(\delta\) subspecies.

This study provides novel evidence that H\(_2\)O\(_2\) promotes PKC\(\delta\) phosphorylation at both Tyr\(^{311}\) and Tyr\(^{332}\), but PMA

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stimulation leads to a selective increase in PKCδ phosphorylation at Tyr311 (and not Tyr325). Because the pharmacologic studies implicate a PP1-sensitive kinase in the PMA-dependent PKCδ-Tyr311 phosphorylation pathway, and in vitro kinase assays implicate Src as a dual PKCδ-Tyr311/Tyr332 kinase, we performed additional in vitro kinase assays to identify an enzyme that selectively phosphorylates PKCδ at Tyr311, but not Tyr332. These studies identified a unique role for c-Abl as a selective Tyr311 kinase; other tyrosine kinases induced either a coordinate increase in PKCδ phosphorylation at both Tyr311 and Tyr332 (other SFKs such as Lyn, Fyn, and Yes) or exhibited little to no PKCδ-Tyr311/Tyr332 kinase activity (PDGFR, FAK, and JAK2). Although our results suggest that c-Abl may contribute to PKCδ-Tyr311 phosphorylation during oxidative stress (and there is ample evidence that c-Abl-dependent PKCδ phosphorylation at Tyr311, and perhaps other sites such as Tyr512, may contribute to the catalytic or pro-apoptotic pathways of this enzyme (31, 37)), results pertaining to c-Abl do not resolve the dilemma regarding the identity of the PMA activation/PP1-sensitive PKCδ-Tyr311 kinase in cardiomyocyte caveolae. PMA-dependent PKCδ-Tyr311 phosphorylation is not blocked by ST1571 and c-Abl is not detected in cardiomyocyte caveolae fractions. Rather, several other mechanisms should be considered. First, whereas the literature has focused primarily on the kinases that promote PKCδ tyrosine phosphorylation, phosphatases that control PKCδ dephosphorylation may also regulate this process. Second, PKC phosphorylation may be controlled through protein-protein interactions. Lee et al. (38) recently identified heat shock protein 25 (HSP25) as a PKCδ binding partner that inhibits PKCδ tyrosine phosphorylation and PKCδ-dependent cellular responses. Studies to date suggest that HSP25 inhibits PKCδ by interacting with the V5 domain, an accessible binding surface that engages in long range intramolecular interactions that influence C1 domain interactions with lipid cofactors, PKC interactions with membranes, and the conformation of the catalytic pocket (regulating catalysis). The PKCδ hinge region represents another relatively exposed surface that also might participate in protein-protein interactions that regulate enzyme function and also should be considered in future studies. Finally, it is worth noting that in vitro kinase assays examined Src-dependent phosphorylation of human PKCδ, whereas the cell-based studies were performed in rat cardiomyocytes. Although the residues immediately surrounding Tyr332 are conserved across species, the remainder of the V3 region is considerably more variable. Recent studies also implicate alternative splicing as a mechanism that further increases PKCδ V3 domain structural diversity by introducing inserts into the caspase-3 cleavage site of both rodent (DIL ↓ DNNQTY332 (39)) and human (DMQ ↓ DNSQTY334 (40)) V3 domains. These inserts disrupt the caspase cleavage site, prevent proteolytic PKCδ activation, and protect cells from proapoptotic stimuli. An additional role for V3 domain inserts (which are in close proximity to V3 domain phosphorylation sites) to influence Src-dependent PKCδ tyrosine phosphorylation is possible and will be considered in future studies.

This study exposes a mechanism for differential activation of PKCδ by PMA and G protein-coupled receptor agonists such as NE and ET-1. We and others previously reported that NE and ET-1 translocate PKCδ to membranes and promote PKCδ-Thr505 phosphorylation. This study shows that NE-dependent PKCδ-Thr505 phosphorylation requires PLC activity (presumably reflecting a role for DAG to stabilize PKCδ at membranes). However, NE and ET-1 do not deliver PKCδ to Src-enriched caveolae membranes or increase PKCδ-Tyr311 phosphorylation. These agonist-specific differences in PKCδ regulation were surprising. The observation that DAG analogues mimic the effect of PMA to recruit PKCδ to caveolae and increase PKCδ-Tyr311 (and Thr505) phosphorylation in vivo in cardiomyocytes, and that DAG analogues effectively substitute for PMA in vitro in kinase assays, suggests that α1-ARs do not increase PKCδ tyrosine phosphorylation because they do not promote DAG accumulation in caveolae membranes. This could suggest that the density of α1-ARs or their downstream signaling partners is limiting in cardiomyocytes caveolae. In this regard, it is worth noting that caveolae were originally implicated as the source of hormone-sensitive phosphatidylinositol bisphosphate pools, but this conclusion has recently been challenged (41, 42). The role of caveolae as signaling domains for ET-1 and α1-adrenergic receptors also remains uncertain. Although ET-1 receptors are reported to co-localize with caveolin-1 in a variety of non-cardiomyocyte models (43, 44), ET-1 receptors apparently do not colocalize with caveolin-3 in cardiomyocytes (45). Similarly, there is evidence that α1-ARs are recovered in phosphatidylinositol bisphosphate-enriched caveolae membranes (and regulate phosphatidylinositol bisphosphate levels) in rat heart, but the consensus of most recent studies is that α1-ARs localize primarily to intracellular membranes (46–49). These inconsistencies in the literature could reflect differences in the localization patterns for native and heterologously over-expressed G protein-coupled receptors or cell specific differences in G protein-coupled receptor localization (that may or may not be attributable to differences in receptor interactions with caveolin-1 and the muscle-specific caveolin-3 isoform) and will require further studies. Finally, an α1-AR-dependent increase in DAG accumulation that is offset by the simultaneous activation of a diacylglycerol kinase (an enzyme that catalyzes the conversion of DAG to phosphatidic acid and locally depletes DAG) also might limit PKCδ activation in caveolae. In fact, there is recent evidence that NE activates diacylglycerol kinase-θ leading to feedback inhibition of PKC in the caveolae/raft fraction of rat mesenteric small arteries (48). The relevance of this finding to PKC regulation in cardiomyocytes, where a different diacylglycerol kinase isoform (namely diacylglycerol kinase-ζ) negatively regulates hypertrophic signaling responses to G protein-coupled receptor agonists requires further study (50, 51).

In summary, this study identifies distinct PKCδ phosphorylation/localization mechanisms in cardiomyocytes treated with PMA, NE, and H2O2. Our studies suggest that models of PKCδ function must be revised to allow for stimulus-specific PKCδ signaling “modes” that trigger functionally distinct signaling responses in cells. In this manner, growth factors (that promote DAG generation in membranes) can trigger PKCδ-mediated events that are membrane-delimited and distinct from events induced by the Tyr311/Tyr332-phosphorylated form of PKCδ.
that accumulates during oxidative stress. According to this model, PKCδ acts both as an enzyme with highly regulated catalytic function (that is calibrated by phosphorylations at the activation loop and on tyrosine residues) and perhaps also by protein–protein interactions that regulate the efficiency/fidelity of signal transduction in cells. PKCδ signaling modes that differ, according to the inciting stimulus, would provide an attractive explanation for the diverse (and at times contradictory) cellular responses that have been attributed to PKCδ in the heart, where PKCδ inhibitors have emerged as clinically important cardioprotective agents but PKCδ activation also is reported to afford cardioprotection during ischemic preconditioning. These findings are not necessarily contradictory if the molecular forms of the enzyme (with different phosphorylation patterns) in specific subcellular localizations. This model would suggest that stimulus-specific differences in PKCδ activation loop and tyrosine phosphorylation, that lead to dynamic changes in the enzymology and signaling function of PKCδ in cells, might be targeted for therapeutic advantage.

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