Regulation of Protein Kinase C δ by Phorbol Ester, Endothelin-1, and Platelet-derived Growth Factor in Cardiac Myocytes*

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Protein kinase C (PKC) δ is regulated allosterically by phosphatidylserine and diacylglycerol (which promote its translocation to the membrane) and by phosphorylation of Ser/Thr and Tyr residues. Although phosphorylation on Thr-505/Ser-643/Ser-662 may simply “prime” PKCδ for activation, it could be regulatory. We examined the regulation of PKCδ in cardiac myocytes by endothelin-1 (Eν, protein-coupled receptor agonist) and platelet-derived growth factor (receptor tyrosine kinase agonist) in comparison with phorbol 12-myristate 13-acetate (PMA). All increased phosphorylation of PKCδ(Thr-505/Ser-643) and of Tyr residues, although to differing extents. *De novo* phosphorylation occurred mainly after translocation of PKCδ to the particulate fraction, and phosphorylations of Thr-505/Ser-643 versusb vs Tyr residues were essentially independent events. Following chromatographic separation of the PKCδ subpopulations, species were correlated with immunoreactivity profiles of total and phosphorylated forms. In unstimulated cells, ~25% of PKCδ lacked phosphorylation of Thr-505/Ser-643 and displayed minimal activity (assayed in the presence of phosphatidylserine/ PMA following chromatography). Endothelin-1 or PMA (10 min) promoted Thr-505/Ser-643 phosphorylation of this pool, and this was associated with an increase in total recoverable PKCδ activity. Meanwhile, in cells exposed to endothelin-1 or PMA, the overall pool of PKCδ translocated rapidly (30 s) to the particulate fraction and was phosphorylated on Tyr residues. This was associated with an increase in lipid-independent activity (*i.e.* the phosphatidyserine/PMA requirement disappeared). For endothelin-1, Tyr phosphorylation of PKCδ and the increase in phosphorylase-dependent PKCδ/ PMA-independent activity persisted after PKCδ retrotranslocated to the soluble fraction. We concluded that, with this physiological agonist, PKCδ becomes activated in the particulate fraction but retains activity following its retrotranslocation, presumably to phosphorylate substrates elsewhere.

The structure and regulation of protein kinase C (PKC) family members (1–3) have been under investigation over many years. All family members have an N-terminal regulatory domain and a C-terminal catalytic domain joined by a flexible hinge region. The regulatory domain contains the pseudosubstrate sequence that folds into the active site to retain the kinase in an inactive form. Interaction of allosteric activators with the regulatory domain results in release of the pseudosubstrate sequence from the active site, producing an open, active conformation. The PKC family members are divided into three subfamilies on the basis of their regulatory domains and their activators. “Classical” cPKCs (α, β, and γ) contain a C1 domain with two Zn2+ fingers (C1A and C1B) that interact with diacylglycerol (DAG) and phosphatidylserine (PtdSer) and a C2 domain that binds Ca2++. DAG, PtdSer, and Ca2+ are all required for PKC activation. “Novel” nPKCs (δ, ε, θ, and η) have a modified C2 domain that does not bind to Ca2++ but contain C1A and C1B Zn2+ fingers. nPKCs are therefore Ca2+-independent but require DAG and PtdSer for activation. Atypical PKCs (ζ and ι/λ) require neither Ca2+ nor DAG, having a modified C1 domain with only a single Zn2+ finger, and the mechanisms of their activation are not fully understood. DAG is generated in cell membranes from the hydrolysis of phosphatidylinositol 4,5-bisphosphate by the action of phospholipase C enzymes (4). Because DAG is retained in the membrane, activation of c/nPKCs is often taken as synonymous with their translocation from the soluble to the particulate fractions of cells. Phorbol esters (e.g. phorbol 12-myristate 13-acetate (PMA)) become embedded in cell membranes and interact with the C1 domains of c/nPKCs to promote their activation (1, 2). Although such phorbol esters are regarded as DAG mimetics, they appear to interact preferentially with the C1B domain of PKCδ, whereas DAGs interact preferentially with the C1A domain (5, 6).

PKCs are also regulated by phosphorylation. Studies with cPKCs identified Ser/Thr phosphorylations in the activation loop, the turn motif, and a C-terminal hydrophobic domain as critical for activity (7). Although these phosphorylations do not appear to influence PKC activity directly, they are required for the kinase to adopt a mature form that can be activated by Ca2+/ DAG. Phosphorylation of the activation loop is mediated by 3-phosphoinositide-dependent kinase-1 (PDK1) downstream of phosphoinositide 3-kinase (PI3K), and this leads to phosphorylation (potentially autophosphorylation) of the turn and hydrophobic motifs. PKCδ also exhibits phosphorylation of the activation loop (Thr-505), turn motif (Ser-663), and hydrophobic motif (Ser-662) (8). Although some reports indicate that, in contrast to cPKCs, phosphorylation of PKCδ activation loop (Thr-505) is not essential for activity (9), other groups suggest that the activity of PKCδ is nonetheless considerably reduced in the absence of this phosphorylation (10). PKCδ is also subject to phosphorylation on multiple Tyr residues in cells exposed to stimuli, including H2O2, PMA, and platelet-derived growth factor (PDGF).

PMAD, phorbol 12-myristate 13-acetate; PP2, 4-amino-5-(4-chlorophenyl)7-(3-butyryl)pyrazol-3,4-dipyrimidine; PtdSer, phosphatidylserine.

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3 The abbreviations used are: PKC, protein kinase C; cPKC, classical PKC; nPKC, novel PKC; DAG, diacylglycerol; E64, trans-epoxy-succinyl]-leucylamido-(4-hydantoin)butane; ET-1, endothelin-1; FPLC, fast protein liquid chromatography; GγPCR, Gγ protein-coupled receptor; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate; PP2, 4-amino-5-(4-chlorophenyl)7-(3-butyryl)pyrazol-3,4-dipyrimidine; PtdSer, phosphatidylserine.
Regulation of PKCδ in Cardiac Myocytes

factor (PDGF) (11). The effects and significance of each of these phosphorylations are not fully understood.

Cardiac myocytes (the contractile cells of the heart) are terminally differentiated cells that undergo hypertrophic growth (i.e. increase in cell size in the absence of further cell division) in response to G protein–coupled receptor (GqPCR) agonists such as endothelin-1 (ET-1), which activate phospholipase C and stimulate PKCs (12). Indeed, PKCs are central to the hypertrophic response, although there is little agreement about the isoform(s) responsible (13). ET-1 and PMA both promote the translocation of PKCδ from the soluble to particulate fractions of cardiac myocytes, but although the response to PMA is sustained (14), translocation of PKCδ induced by ET-1 is transient (15). Recent studies indicate that PMA increases the phosphorylation of PKCδ(Thr-505) in cardiac myocytes and PKCδ(Ser-643) but not PKCε(Ser-643) (11). It is proposed that phosphorylation of PKCδ(Thr-505) is mediated by PKCε. The increase in phosphorylation is associated with an increase in lipid-independent activity (i.e. reduced requirement for allosteric activation by PtdSer and PMA/DAG) and altered substrate specificity. Although cardiac myocytes are particularly responsive to GqPCR agonists, they also respond to peptide growth factors acting through their receptor protein–tyrosine kinases (16). Of these, only PDGF (not, for example, epidermal growth factor) clearly regulates PKCδ in cardiac myocytes, but this is associated with Tyr phosphorylation of PKCδ rather than translocation to the particulate fraction of the cell (16).

Because of the paucity of information on the phosphorylation and regulation of PKCδ by physiological agonists and the probability that activation by PMA is not the same as by physiological agonists, we conducted a detailed investigation of the regulation of endogenous PKCδ in cardiac myocytes by a GqPCR agonist (ET-1) and a peptide growth factor (PDGF) for comparison with that of PMA. By using fast protein liquid chromatography (FPLC), we identified at least four PKCδ subspecies in cardiac myocytes with differing patterns of phosphorylation and activities. We demonstrate that phosphorylation of Thr-505/Ser-643 appears to be required for any significant activity, whereas the increase in phosphorylation of Tyr residues is associated with increased lipid-independent activity.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies to the C-terminal regions of PKCδ or PKCε and the monoclonal antibody to phospho-Tyr (PY99) were from Santa Cruz Biotechnology (catalog numbers sc-213, sc-1681 and sc-7020, respectively). The monoclonal antibody to PKCδ (residues 114–289) was from Pharmingen (catalog number 610398). Antibodies to phospho-PKCδ(Thr-505) and phospho-PKCδ(Ser-643) were from Cell Signaling Technology (catalog numbers 9374 and 9376, respectively). The antibody to phospho-PKCδ(Tyr-311) was from Oncogene Research Products (catalog number ST1019).

Primary Cultures of Neonatal Rat Cardiac Myocytes—Myocytes were dissociated from the ventricles of 1–3-day-old Sprague-Dawley rat hearts by an adaptation of the method of Iwaki et al. (17) as described previously (18). Cells were plated at a density of 1,400 cells/mm in 60-mm Primaria culture dishes (BD Biosciences) for 24 h in serum, and serum was withdrawn for the 24 h prior to experimentation. Myocytes were exposed to PMA (2 μM), ET-1 (100 nM), or PDGF (20 ng/ml) with or without pretreatment with inhibitors (10 min).

Preparation of Extracts—Following incubation with agonists, myocytes were washed in ice-cold phosphate-buffered saline. For whole cell extracts, myocytes were scraped into Buffer A (20 mM β-glycerophosphate, pH 7.5, 20 mM NaF, 2 mM EDTA, 0.2 mM Na3VO4, 10 mM benzamidine, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 2 μM microcystin LR, and 10 μM E64) and centrifuged (10,000 × g, 5 min at 4 °C). The supernatants were retained.

Preparation of FPLC Fractions—Myocytes were washed in ice-cold phosphate-buffered saline. For whole cell extracts, myocytes were scraped into Buffer A (20 mM β-glycerophosphate, pH 7.5, 20 mM NaF, 2 mM EDTA, 0.2 mM Na3VO4, 10 mM benzamidine, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 2 μM microcystin LR, and 10 μM E64) and centrifuged (10,000 × g, 5 min at 4 °C). The supernatants were retained. To prepare soluble and particulate fractions, myocytes were scraped into 150 μl dish Buffer B (12.5 mM Tris-HCl, pH 7.4, 2.5 mM EGTA, 1 mM EDTA, 50 mM NaF, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 2 μM microcystin LR, and 10 μM E64). Samples were centrifuged (10,000 × g, 5 min, 4 °C) and the soluble fractions were collected. The pellets were resuspended in 150 μl of Buffer B containing 2% (v/v) Triton X-100 and incubated on ice for 10 min, and the solubilized particulate fractions were collected following centrifugation (10,000 × g, 5 min, 4 °C).

Immunoblot Analysis—Samples were boiled with 0.33 volumes of SDS-PAGE sample buffer (10% (w/v) SDS, 13% (w/v) glycerol, 300 mM Tris-HCl, pH 6.8, 130 mM dithiothreitol, and 0.2% (w/v) bromphenol blue). Proteins were separated by SDS-PAGE using 8% resolving gels with 6% stacking gels and transferred to nitrocellulose membranes as described previously (15). Nonspecific binding sites were blocked (15 min at room temperature) with 5% (w/v) nonfat milk powder in TBST Buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (w/v) Tween 20). Membranes were incubated (overnight, 4 °C) with primary antibodies diluted in TBST Buffer containing 1% (w/v) bovine serum albumin and then washed in TBST Buffer (three times for 5 min at room temperature). Primary antibodies were used at the following dilutions: phospho-PKCδ (Thr-505), phospho-PKCε(Ser-643), and phospho-PKCζ(Ty-311), 1/1000; PKCδ (C-terminal region antibody), 1/500 but 1/250 for FPLC fractions; and PKCε, 1/250 for FPLC fractions. Membranes were incubated (60 min at room temperature) with horseradish peroxidase-conjugated secondary antibodies (1/5000, Dako) in TBST Buffer containing 1% (w/v) nonfat milk powder, and then washed in TBST Buffer (three times for 5 min at room temperature). Bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.), and blots were quantified by scanning densitometry using ImageMaster 1D calibrated with Kodak photographic step tablet number 2 (Amersham Biosciences). For FPLC fractions, the fractions were first immunoblotted in order across separate blots, and bands were quantified. Representative fractions from each of these blots were then immunoblotted together, and the quantification from this blot was used to normalize the densitometry across all fractions. The data are expressed for each fraction relative to the total immunoreactivity (following normalization) across all fractions from a specific sample (whole cell extract, or soluble + particulate extracts).

FPLC—Myocytes were extracted with Buffer A or Buffer B as appropriate. Supernatants from four dishes (whole cell extracts) or five dishes (soluble and particulate fractions) were applied to a Mono Q HR 5/5 column equilibrated with 50 mM HEPES, pH 7.5, 1 mM EDTA, 0.2 mM Na3VO4, 0.1% (v/v) Triton X-100, and 4 μg/ml leupeptin. Following a 5-ml isocratic wash, proteins were eluted using a linear NaCl gradient (20 ml, 0–0.4 M) at a flow rate of 1 ml/min with collection of 0.5-ml fractions. Samples from each fraction were assayed for PKC activity, used for immunoprecipitations, or were boiled with 0.33 volume of SDS-PAGE sample buffer for immunoblotting.

Immunodetection of Phospho-Tyr-containing PKCδ—Myocytes were scraped into Buffer C (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mM KCl, 5 mM NaF, 0.2 mM Na3VO4, 5 mM MgCl2, 0.05% (v/v) 2-mercaptoethanol, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 2 μM microcystin LR, and 10 μM E64) and centrifuged (10,000 × g, 5 min at 4 °C). The supernatants were incubated (1 h at 4 °C) with anti-phospho-

8322 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 281 • NUMBER 13 • MARCH 31, 2006
Tyr (PY99) antibodies (2 μg) prebound to Protein G-Sepharose. To assess tyrosine phosphorylation of PKCε purified by FPLC, column fractions were incubated overnight (4 °C) with anti-phospho-Tyr antibodies prebound to Protein G-Sepharose. Immunoprecipitates were washed in Buffer C, resuspended in 30 μl of Buffer C, and boiled with SDS-polyacrylamide gel sample buffer (30 μl). Following SDS-PAGE, PKCε was detected using the antibody to the C-terminal region.

Assay of PKC Activity—PKC activity in column fractions was assayed by the incorporation of [γ-32P]ATP into AKRKRKGSFFYGG peptide (19) substrate (Advanced Biotechnology Centre, Imperial College London) in the absence or presence of PtdSer (0.125 mg/ml in the assay) and PMA (6.5 μM in the assay), or in the presence of PtdSer, PMA, and 10 μM (in the assay) GF109203X (to inhibit PKC). Assays were performed for 30 min at 30 °C. Column fractions (15 μl) were added to 20 μl of Buffer D (100 mM HEPES, pH 7.5, 1 mM EDTA, 0.4% (v/v) Triton X-100, and 400 μM AKRKRKGSFFYGG, with PtdSer, PMA, and GF109203X as required). Reactions were initiated by addition of 5 μl of 1 mM ATP, 100 mM MgCl2 containing 0.5 μCi of [γ-32P]ATP. Assays were terminated by spotting 30 μl of the reaction mixtures onto Whatman P81 papers that were then washed in 75 mM H3PO4 (one time for 5 min and three times for 30 min). Radioactivity incorporated was determined by Cerenkov counting. The activity in the presence of GF109203X was subtracted from the activities in the absence or presence of PtdSer/PMA to give a measure of lipid-independent and total activities, respectively. The activity in each fraction is expressed relative to the total activity for the sample (whole cell extract, or soluble + particulate extracts).

Immunokinase Assays—Myocytes were scraped into Buffer C and centrifuged (10,000 × g, 5 min, 4 °C). The supernatants were incubated (4 h at 4 °C) with 2 μg of monoclonal anti-PKCε antibody (BD Biosciences) prebound to Protein G-Sepharose. Following two washes in Buffer C and one wash in Buffer D (diluted 1/1 with water), immunoprecipitates were resuspended in 20 μl of Buffer D and 15 μl of H2O. PKCε activity was assayed as described above.

RESULTS

Effects of PMA, ET-1, or PDGF on Phosphorylation of PKCε—PKCε can be phosphorylated on numerous residues, including Thr-505 and Ser-643 (8). By analogy with classical PKCs, phosphorylation of Thr-505 and Ser-643 may be maturational/facilitatory events required for PKCε to become “activable” by DAG/PtdSer (7). However, in cardiac myocytes, a previous
Regulation of PKCβ in Cardiac Myocytes

study by Rybin et al. (11) indicated that PMA promotes an increase in phosphorylation of these residues, suggesting that phosphorylation of Thr-505 and Ser-643 may directly regulate activity. Activation of PKCβ by physiological agonists via DAG may differ from activation by PMA. Using phospho-specific antibodies for immunoblotting, we first studied the phosphorylation of Thr-505 and Ser-643 in cardiac myocytes exposed to PMA (for comparison with the work of Rybin et al. (11)), ET-1 (a GqPCR agonist that stimulates PKCβ translocation (15)), or PDGF (a receptor protein-tyrosine kinase agonist that also activates PKCβ in these cells (16)). PKCβ was identified as a band of ~74 kDa. Consistent with the study of Rybin et al. (11), PMA (1 μM) promoted an increase in phosphorylation of PKCβ(Thr-505) in cardiac myocytes (Fig. 1A). In contrast to Rybin et al. (11), we also detected an increase in phosphorylation of PKCβ(Ser-643). Phosphorylation of both residues was maximal within 5 min and sustained over at least 15 min. ET-1 (100 nM) promoted a similar increase in phosphorylation of PKCβ(Ser-643) as PMA but had a lesser effect on PKCβ(Thr-505) (Fig. 1B). PDGF (20 ng/ml) increased phosphorylation of PKCβ(Thr-505) but had a minimal effect on phosphorylation of PKCβ(Ser-643) (Fig. 1C).

We have shown previously that ET-1 stimulates rapid (within 15–30 s) translocation of PKCβ to the particulate fraction of cardiac myocytes (15). This is transient and PKCβ returns to the soluble fraction from ~3 min. To investigate how the increase in phosphorylation of Thr-505 and Ser-643 of PKCβ relates to this, soluble and particulate fractions prepared from cardiac myocytes exposed to ET-1 were analyzed by immunoblotting with phospho-specific antibodies or antibodies to the C terminus of PKCβ (to assess the global pool). As demonstrated previously (15), ET-1 promoted rapid but transient translocation of total PKCβ from the soluble to the particulate fraction of cardiac myocytes (Fig. 1D). The immunoblots for phospho-PKCβ(Ser-643) indicated that maximal translocation occurred within 5 s (Fig. 1E). There was no increase in the amount of phospho-PKCβ(Ser-643) or phospho-PKCβ(Thr-505) prior to translocation to the particulate fraction, but the total amount of both phosphorylations was elevated in soluble and particulate fractions subsequent to translocation (Fig. 1, E and F). It is therefore probable that phosphorylation of both these sites occurred at about the time of translocation of the global pool of PKCβ to the particulate fraction.

PKCβ may be phosphorylated on numerous Tyr residues (8). To determine the effects of PMA or ET-1 on Tyr phosphorylation of PKCβ, phospho-Tyr-containing proteins were immunoprecipitated from cardiac myocyte extracts and immunoblotted for total PKCβ. PMA (Fig. 2A) or ET-1 (Fig. 2B) increased Tyr phosphorylation of PKCβ. For both stimuli, an increase in phosphorylation was not detected before 1 min, indicating that Tyr phosphorylation occurred subsequent to translocation to the particulate fraction (Fig. 1D). We have shown previously that PDGF increases Tyr phosphorylation of PKCβ to a much greater degree than PMA or ET-1 but also with maximal phosphorylation at 3–5 min (16). The Src family kinase inhibitor PP2 (5 μM) attenuated the increase in Tyr phosphorylation of PKCβ induced by PMA or ET-1 (Fig. 2C), but the PKC inhibitor GF109203X (10 μM) had no effect (Fig. 2D). In contrast, phosphorylation of PKCβ(Thr-505) or PKCβ(Ser-643) induced by PMA (Fig. 3, A, C, and D) or ET-1 (Fig. 3, B–D) was inhibited by GF109203X. Thus, the increase in Tyr phosphorylation of PKCβ induced by PMA or ET-1 is unrelated to the increase in phosphorylation of PKCβ(Thr-505) or PKCβ(Ser-643). The increase in phospho-PKCβ(Thr-505) induced by PMA or ET-1 was not inhibited by the PI3K inhibitor LY294002 (50 μM) (Fig. 3, A–C), consistent with other studies in which PKC activity rather than PI3K activity was shown to be required for the increase in phosphorylation of this site induced by PMA (11). It was also not inhibited by PP2, so this phosphorylation is independent of any of the Tyr phosphorylation events. The regulation of phospho-PKCβ(Ser-643) appeared more complex because GF109203X, LY294002, or PP2 each had some inhibitory effect (Fig. 3, A, B, and D).
Subfractionation of PKC$_\delta$ Species—The studies with pharmacological inhibitors indicated that phosphorylation of PKC$_\delta$ on Tyr residues, Thr-505, and Ser-643 (Figs. 2 and 3) are probably independent events, and differential phosphorylation may generate subspecies of the protein. Because phosphorylation of proteins increases their net negative charge, we used Mono Q FPLC of total myocyte extracts (prepared with 2% Triton X-100) to separate PKC$_\delta$ subspecies according to charge. Fractions were then immunoblotted with PKC$_\delta$ antibodies. The immunoreactivity (quantified by scanning densitometry) in each fraction was calculated relative to the total immunoreactivity for all fractions following normalization between immunoblots. In unstimulated myocytes, PKC$_\delta$ eluted across a broad NaCl range (0.13–0.35 M NaCl), but with two clearly separated peaks of immunoreactivity at ~0.14–0.17 M NaCl (fractions 14–17; peak I) and ~0.18–0.29 M NaCl (fractions 18–29; peak II) (Fig. 4, A and B; Table 1). A small proportion of PKC$_\delta$ eluted at ~0.30–0.35 M NaCl (fractions 30–35; peak III). Following stimulation with PMA (10 min), minimal PKC$_\delta$ immunoreactivity was detected in peak I with an increase in the proportion of immunoreactivity in peak II and also in peak III, although the increase in peak III was not statistically significant (Fig. 4, A and C; Table 1). In response to ET-1 (10 min), the proportion of PKC$_\delta$ immunoreactivity in peak I was substantially reduced (15.6% of control values) with an increase in peak II (Fig. 4, A and D; Table 1). Overall, there was no increase in the proportion of PKC$_\delta$ in peak III relative to that in unstimulated cells. PDGF (10 min) also reduced the proportion of PKC$_\delta$ immunoreactivity in peak I, although to a lesser degree than ET-1 (42.5% of control values), with an increase in peak II (Fig. 4, A and E; Table 1).

These data indicate that stimulation with PMA, ET-1, or PDGF increases the negative charge on PKC$_\delta$ subspecies, which therefore require higher concentrations of NaCl for elution from Mono Q FPLC. This is consistent with the increase in phosphorylation induced by these agonists (Figs. 1 and 2). Fractions from each of peaks I, II, and III from the FPLC elutions were immunoblotted with phospho-specific antibodies for PKC$_\delta$ (Thr-505), PKC$_\delta$(Ser-643), or PKC$_\delta$(Tyr311) (the only currently available antibody to the C terminus of PKC$_\delta$) for 10 min. Extracts were subfractionated by Mono Q FPLC, and fractions were immunoblotted for total PKC$_\delta$. Bands were quantified by scanning densitometry (see Fig. 4). The amounts of PKC$_\delta$ immunoreactivity in peaks I (fractions 14–17), II (fractions 18–29), and III (fractions 30–35) were determined for each experiment. Results are means ± S.E. for five (PMA) or six (ET-1) independent preparations of myocytes.

### TABLE 1

| Peptide | Peak I | Peak II | Peak III |
|---------|--------|---------|----------|
| Control | 16.7 ± 2.4 | 74.1 ± 4.1 | 3.2 ± 0.9 |
| PMA     | 0.1 ± 0.1$^*$ | 89.4 ± 4.1$^*$ | 8.3 ± 3.7 |
| ET-1    | 2.6 ± 0.7$^*$ | 93.8 ± 0.8$^*$ | 1.8 ± 0.5 |
| PDGF    | 7.1 ± 2.2 | 86.7 ± 4.5 | 3.9 ± 2.0 |

* $p < 0.001$ versus respective control (one-way analysis of variance with Tukey post-test).

$^*$ $p < 0.05$.

$^{+} p < 0.001$.
Regulation of PKCδ in Cardiac Myocytes

FIGURE 5. Phosphorylation status of PKCδ subspecies. Cardiac myocytes were unstimulated (Control, A, C, and G) or exposed to 1 μM PMA (B, D, and H), 100 nM ET-1 (E and I), or 20 ng/ml PDGF (F and J) for 10 min. Proteins in whole cell extracts were separated by Mono Q FPLC. A and B, fractions were immunoblotted with antibodies to phospho-PKCδ(Thr 505) (upper panels) or phospho-PKCδ(Ser-643) (lower panels). Images are representative of at least four independent preparations of myocytes. C–F, fractions were immunoblotted with antibodies to phospho-PKCδ(Thr 505) (upper panels), phospho-PKCδ(Ser-643) (upper center panels), phospho-PKCδ(Tyr 311) (lower center panels), or total PKCδ (lower panels). Images are representative of at least four independent preparations of myocytes. G–I, fractions 14–17 (peak I), 18–23 (peak II), 24–29 (peak III), and 31–37 (peak III) were pooled. Samples of each of the pooled fractions were immunoprecipitated with antibodies to total PKCδ (upper panels). Phospho-Tyr-containing proteins were immunoprecipitated from the remainder and immunoblotted with antibodies to total PKCδ (upper panels). The experiment was repeated with similar results.

FIGURE 6. Identification of PKCδ subspecies in cardiac myocytes and regulation by PMA or ET-1. Cardiac myocytes were unstimulated (Control, A (upper panels) and B) or exposed to 1 μM PMA (A (center panels) and C), or 100 nM ET-1 (A (lower panels) and D) for 10 min. A, proteins in whole cell extracts were separated by Mono Q FPLC, and fractions were immunoblotted with antibodies to PKCδ. Arrowheads indicate PKCδ, identified on the basis of relative molecular mass (~92 kDa). Images are representative of three independent preparations of myocytes. B–D, immunoreactive bands were quantified by scanning densitometry, and the densitometry for each experiment was normalized as described under “Experimental Procedures.” Results are means ± S.E. for three independent preparations of myocytes.

the control samples (Fig. 5C) is of higher relative molecular mass than PKCδ (compare with the total PKCδ immunoblot) and probably represents cross-reactivity with another protein. In contrast to phosphorylation of Thr-505 and Ser-643, phospho-PKCδ(Tyr 311) was detected in peaks I and II of control samples (Fig. 5C), in peaks II and III of samples from cells exposed to PMA (Fig. 5D) or ET-1 (Fig. 5E), and in all peaks of samples from cells exposed to PDGF (Fig. 5F). However, relative to the total amount of PKCδ, phospho-PKCδ(Tyr 311) was greatly enriched in peak III suggesting that Tyr phosphorylation of PKCδ is particularly prevalent in this peak. Consistent with this, for all conditions studied, Tyr-phosphorylated PKCδ was particularly enriched in peak III relative to the total amount of PKCδ as assessed by immunoprecipitation of phospho-Tyr-containing proteins followed by immunoblotting for total PKCδ (Fig. 5, G–J). Most interestingly, Tyr-phosphorylated PKCδ was also more abundant relative to total protein in the second part of peak II (ILB, fractions 24–29).

Subfractionation of PKCδ Species—Of the PKC isoforms expressed in cardiac myocytes, PKCδ is most closely related to PKCδ, so we also examined the Mono Q FPLC profile of PKCδ. Although PKCδ was detected in a number of fractions, in unstimulated cells the majority of immunoreactivity eluted at ~0.14–0.16 M NaCl (Fig. 6, A and B, fractions 14–16). Following stimulation with PMA or ET-1, the proportion of PKCδ immunoreactivity in this peak declined, and a second peak of immunoreactivity was eluted at ~0.19–0.22 M NaCl (Fig. 6, A, C, and D, fractions 19–22). This suggests that, like PKCδ, PKCδ is subject to modification following stimulation with PMA or ET-1, and the data are consistent with studies indicating that the phosphorylation status of PKCδ also increases in cardiac myocytes on stimulation with PMA (11). However, the elution profiles for PKCδ and PKCδ are clearly different.

Activities of PKCδ Subspecies—Clearly, stimulation of cardiac myocytes with PMA, ET-1, or PDGF increases phosphorylation of PKCδ. However, the relationship between phosphorylation and activity is ill-defined. There are few antibodies available that reliably immunoprecipitate PKCδ for activity measurements, probably because of the phosphorylations and conformational changes that are associated with its activation. Of the commercial antibodies available, we identified only one antibody (monoclonal), raised to an epitope spanning the Cys-rich region (residues 114–289) which could immunoprecipitate PKCδ in unstimulated cells. Following stimulation with PMA or ET-1 for 30 s, there was a small increase in total PKCδ activity, but most unexpectedly, total PKCδ activity was substantially reduced following stimulation for 10 min (Fig. 7A). However, at 10 min, the total amount of PKCδ recovered in the immunoprecipitate was similarly much reduced (Fig. 7B). Because the immunoprecipititating antibody was raised to a region encompassing two Tyr residues that are known to become phosphorylated in response to PMA stimulation (Tyr-155 and Tyr-187 [20–22]) and because Tyr phosphorylation of PKCδ is increased by PMA or ET-1 in cardiac myocytes by 10 min (Fig. 2), it seems probable that phosphorylation of one or both of these residues reduces the affinity of the antibody for PKCδ.
Because immunokinase assays for PKCδ proved problematical, we developed in vitro kinase assays using a peptide substrate corresponding to the optimal peptide substrate sequence predicted for PKCδ (19). Assays were performed in the absence or presence of lipid co-factors (PMA plus PtdSer) to determine lipid-independent and total activities, respectively. Assays were also performed in the absence or presence of the selective PKC inhibitor GF109203X (10 μM) to eliminate any contribution from other protein kinases. This assay should exclude activities of cPKCs because Ca2+ was not included, and EDTA was present. Although PKCδ and PKCμ/δ/protein kinase D are expressed in cardiac myocytes (15, 23, 24), the PKCδ peptide substrate is not efficiently phosphorylated by these isoforms (19). Furthermore, because the Mono Q FPLC elution profile for PKCδ (Fig. 6) did not correlate with the activity profile using the optimal PKCδ substrate (Fig. 8), we conclude that the assay essentially measures only the activities of PKCδ.

Total extracts were prepared from cells using 2% (v/v) Triton X-100, and proteins were separated by Mono Q FPLC. In extracts from unstimulated cells, there was no significant activity in fractions 12–17 (Fig. 8A) that encompassed peak I of PKCδ immunoreactivity (Fig. 4, A and B). Presumably, this pool of PKCδ, which lacks phosphorylation of Thr-505 or Ser-643 (Fig. 5C), has minimal activity, consistent with the original proposal that Thr-505 and/or Ser-643 are essentially maturation events required for PKCδ to become activable (7). It is also consistent with reports that bacterially expressed PKCδ has considerably reduced activity compared with PKCδ expressed in insect cells (10). PKCδ activity was detected in unstimulated cells principally in fractions 19–26 (Fig. 8, A and B), corresponding to peak II of PKCδ immunoreactivity (Fig. 4, A and B), and this activity was essentially lipid-dependent (Fig. 8, A, B, F, and G). PKCδ activity was also detected in fractions 31–37 (Fig. 8, A and B), corresponding to peak III of PKCδ immunoreactivity (Fig. 4, A and B), but this activity was lipid-independent (Fig. 8, A, B, F, and G).

Following stimulation with PMA (10 min), there was an increase in total PKCδ activity relative to unstimulated controls (132.7 ± 6.1% of controls, n = 4; p < 0.05, paired t test). This is consistent with an increase in activable PKCδ resulting from phosphorylation of Thr-505 and/or Ser-643 of peak I PKCδ and recruitment into peak II (Fig. 4, A and C; Fig. 5D). With PMA, PKCδ activity eluted principally in fractions 20–29 (peak II of PKCδ immunoreactivity; Fig. 4C) and 31–39 (peak III of PKCδ immunoreactivity; Fig. 4C) (Fig. 8C). The activity in peak III was lipid-independent and not significantly different from that of control cells. However, compared with unstimulated cells, a greater proportion of the activity in peak II eluted in fractions 24–29.

The majority of PKCδ activity in these fractions was lipid-independent, whereas PKCδ activity eluting in fractions 20–23 required exogenous lipids (Fig. 8, C, F, and G). It is notable that the lipid-independent activity in fractions 24–29 correlates with increased Tyr phosphorylation of...
PKC in these fractions (Fig. 5H), and it is possible that Tyr phosphorylation may cause the switch to lipid-independent activity.

ET-1 (10 min) also increased total PKC activity relative to unstimulated controls (125.3 ± 2.9% of controls, n = 4; p < 0.01, paired t test). This is also consistent with an increase in activable PKC resulting from phosphorylation of Thr-505 and/or Ser-643 of some peak I PKC with recruitment into peak II (Fig. 4, A and D; Fig. 5E). As with PMA, PKC activity eluted principally in fractions 21–29 with an increase in the proportion of activity in fractions 24–29 relative to controls (Fig. 8D). A large proportion of this activity did not require exogenous lipids, whereas the activity in fractions 21–23 was largely lipid-dependent (Fig. 8, D, F and G). As with PMA, the lipid-independent activity in fractions 24–29 correlates with increased Tyr phosphorylation of PKC (Fig. 5I), and such phosphorylation events could account for the increase in lipid-independent activity. There was no significant increase in total PKC activity in response to PDGF, potentially because of the much reduced effect on phosphorylation of Thr-505 and, particularly, Ser-643 (Fig. 1C). Despite this, there was an increase in lipid-independent PKC activity in fractions 25–29 (Fig. 8, E–G), coincident with the increased Tyr phosphorylation (Fig. 5J).

Partitioning of PKC Subspecies and PKC Activities between Soluble and Particulate Fractions—We examined the effects of ET-1 on the partitioning of the different PKC subspecies identified by Mono Q FPLC between the soluble and particulate fractions. In unstimulated cells, total PKC immunoreactivity was approximately equally distributed between soluble and particulate fractions (48.7 ± 8.2% in particulate fraction; mean ± S.E., n = 5). The majority of peak I of PKC immunoreactivity was detected in the soluble fraction, whereas PKC immunoreactivity in peak II was distributed between the soluble and particulate fractions (Fig. 9A). Following stimulation with ET-1 for 30 s,
the majority of PKCβ immunoreactivity was detected in the particulate fraction (90.8 ± 2.1%; mean ± S.D., n = 2), and this was principally present in peak II with a small increase in peak III (Fig. 9B). After 10 min of stimulation with ET-1, PKCβ immunoreactivity was again approximately equally distributed between soluble and particulate fractions (55.8 ± 8.6% in particulate fraction; mean ± S.D., n = 2) and, for both fractions, was detected almost entirely in peak II (Fig. 9C).

Although PKCβ immunoreactivity was approximately equally distributed between soluble and particulate fractions in unstimulated cells (Fig. 9A), a greater proportion of PKCβ activity was present in the soluble fraction (64.9 ± 5.0%; mean ± S.D., n = 2). Almost all of the detected PKCβ activity in either the soluble or particulate fraction was present in fractions 21–29 corresponding to peak II of PKCβ immunoreactivity and was lipid-dependent (Fig. 10, A and B). Following stimulation with ET-1 for 30 s, translocation of PKCβ activity to the particulate fraction was apparent, but the proportion in the soluble fraction (44.6 ± 4.7%; mean ± S.D., n = 2) was still greater than expected from the immunoreactivity profiles (Fig. 9B). As in unstimulated cells (Fig. 10, A and B), virtually all of the detected PKCβ activity eluted in fractions 21–29 (peak II of PKCβ immunoreactivity) and was lipid-dependent (Fig. 10, C and D). Stimulation of myocytes with ET-1 for 10 min resulted in some redistribution of PKCβ activity to the soluble fraction (54.5 ± 1.0%; mean ± S.D., n = 2), but consistent with the effects of ET-1 (10 min) on the PKCβ activity in total extracts (Fig. 8D), there was an increase in lipid-independent activity in fractions 24–29 (peak IIB of PKCβ immunoreactivity) (Fig. 10, E and F). Most interestingly, the lipid-independent activity appeared to be equally distributed between soluble and particulate fractions. In contrast to PKCβ in total extracts (Fig. 8), we failed to detect any significant PKCβ activity in peak III following separation into soluble and particulate fractions. The reason for this is not clear, but it is possible that this activity was distributed between the soluble and particulate fractions, and the activity in each was below the detectable level for this assay. Alternatively, the slightly different buffer composition may have resulted in inefficient extraction of this subspecies.

**DISCUSSION**

PKCβ was first identified almost 20 years ago (25, 26) and has been implicated in key cellular responses such as the regulation of proliferation and apoptosis (27). In relation to this study, PKCβ is one of the PKCs that has been implicated in cardiac myocyte hypertrophy (28) and ischemic preconditioning cardioprotection (29), although equally its inhibition has been reported to be beneficial in cardiac ischemia (30). However, the regulation of this kinase is still not well understood. Like all c/nPKCs, PKCβ interacts with DAG, which brings the kinase to the plasma membrane, and this plays a part in its activation. More recent studies indicate that other events, including phosphorylation, are associated with activation of PKCβ (8). In addition, PKCβ interacts with other proteins (e.g. receptors for activated C kinase), which are assumed to play a role in subcellular targeting of the enzyme for activation and/or substrate phosphorylation (28). How this relates to DAG production is rarely considered.

The identification of phorbol esters as “DAG mimetics,” and therefore activators of c/nPKCs, resulted in wide usage of these compounds for the investigation of the mode of activation and function of c/nPKCs within cells. However, although PMA (for example) obviously does activate c/nPKCs, activation of PKCβ by PMA does not necessarily resemble the activation by physiological generation of DAG. Specifically, in cardiac myocytes (as in other cells), PMA promotes sustained translocation of PKCβ to the particulate fraction of the cell followed by down-regulation of the protein (14), whereas physiological agonists, including ET-1, induce transient translocation (15), and we have failed to detect any significant subsequent to down-regulation. This transient translocation raises the question of whether PKCβ is active only when tethered by DAG to the membrane. Alternatively, like other protein kinases (e.g. protein kinase B (31) and c-Raf (32)), PKCβ may be recruited to the membrane for activation and then retain its activity (presumably now independent of DAG/ThrSer) as it migrates to other areas of the cell. Here, potentially following interaction with targeting proteins, PKCβ phosphorlylates specific substrates. Our data are strongly supportive of the latter scenario because PKCβ recovered after 10 min of stimulation with ET-1 showed an increase in lipid-independent activity (Fig. 8, D, F, and G), which would be required for the enzyme to remain active in other areas of the cell. We have attempted to study the subcellular localization of PKCβ in cardiac myocytes by immunostaining using antibodies to the total enzyme and the different phosphorylated forms. However, these experiments have proved unsuccessful because (we suspect) the levels of expression of endogenous PKCβ are below the threshold for detection. An increase in lipid-independent activity was also seen in response to PMA (Fig. 8, C, F, and G), but it is possible that the detergent used to prepare whole cell extracts for FPLC resulted in extraction of PKCβ associated with PMA and membrane lipids. A similar argument could apply for ET-1, but for this physiological agonist, there was retrotranslocation of PKCβ back into the soluble fraction of the cell by 10 min (Fig. 1D). This coincided with an increase in lipid-independent activity in the soluble extract (Fig. 10F) in addition to the particulate extract (Fig. 10F). The mechanisms by which PKCβ attains

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4 A. Clerk and P. H. Sugden, unpublished data.
Regulation of PKCδ in Cardiac Myocytes

its lipid independency are not entirely clear but may relate to its phosphorylation status.

By analogy with cPKCs, phosphorylation of Thr-505 (activation loop), Ser-643 (turn motif), and Ser-662 (hydrophobic domain) were predicted to be maturational events required for PKCδ to be primed for activation by its allosteric regulators (7). Although subsequent reports suggested that phosphorylation of Thr-505 is not absolutely essential for PKCδ activity (9), other groups found that, in the absence of Thr-505 phosphorylation, PKCδ activity was substantially reduced (10). To confuse matters further, a study in cardiac myocytes showed that PMA promotes phosphorylation of Thr-505, with an associated increase in lipid-independent activity and possible change in substrate specificity (11). Our studies are consistent with all these observations, but the identification of different subspecies of PKCδ provides further insight.

ET-1, like PMA, promoted an increase in phospho-PKCδ(Thr-505) and the potential autophosphorylation site Ser-643 in cardiac myocytes (Fig. 1). However, following separation of PKCδ subspecies on Mono Q FPLC (Fig. 4), it was clear that these phosphorylations principally affected a fraction (~25%) of PKCδ that eluted at lower NaCl concentrations than the rest (peak I). Because we could not detect significant PKCδ activity in these fractions (Fig. 8A), phosphorylation of PKCδ(Thr-505/Ser-643) would appear to be essentially the maturational events as originally proposed (7). However, in cardiac myocytes, peak I of PKCδ immunoreactivity was present in the soluble fraction (Fig. 9), and this contrasts with studies of maturing PKCs that appear to be expressed in the particulate (0.2% Triton X-100-insoluble) fraction (33, 34). This suggests that the "immature" PKCδ in cardiac myocytes is not simply being processed de novo subsequent to synthesis. Consistent with this concept, PMA or ET-1 rapidly recruited this pool of PKCδ for activation; there was increased phosphorylation of Thr-505 and Ser-643, with an associated increase (~25%) in total recoverable PKCδ activity. The increase in phosphorylation of PKCδ(Thr-505/Ser-643) occurred on or immediately subsequent to translocation of the global pool of PKCδ to the particulate fraction of the cell and was inhibited by the selective PKC inhibitor, GF109203X (Fig. 3). However, it is not clear if the phosphorylations occurred at the membrane or resulted from activated PKCs phosphorylating immature PKCδ in the soluble fraction. Whatever the mechanism, our data are consistent with phosphorylation of PKCδ(Thr-505/Ser-643) being required for significant activity.

Phosphorylation of PKCδ(Thr-505/Ser-643) did not correlate with the increase in lipid-independent activity of the enzyme because PKCδ phosphorylated on these residues was detected in fractions across peak II (21–28) following FPLC separation (Fig. 5, A–F), but lipid-independent activity was detected principally in fractions 24–28 (Fig. 8). However, the increase in phosphorylation of PKCδ on Tyr residues did correlate with the increase in lipid-independent activity (Fig. 5, G–J). Furthermore, Tyr phosphorylation of PKCδ induced by PMA or ET-1 occurred subsequent to its translocation to the particulate fraction (Fig. 2, A and B) and, for ET-1, correlated with its retrotranslocation to the soluble fraction of the cell (Fig. 28). These data suggest that Tyr phosphorylation of PKCδ may not only result in lipid-independent activity of the enzyme but may also lead to its release from the membrane. Although our studies have not directly addressed which of the Tyr residues may be involved in these events, it would appear unlikely that Tyr-311 plays a significant role because phosphorylation of this residue was detected even in peak I of PKCδ immunoreactivity following FPLC separation (Fig. 5, C–F), which had no significant activity (Fig. 8A). Stimulation with PMA or ET-1 over 10 min reduced the immunoreactivity of PKCδ for immunoprecipitation with a monoclonal antibody raised to residues 114–289 (Fig. 7), suggesting that PKCδ may be phosphorylated within this region. Of the two Tyr residues in this region known to be phosphorylated (8), Tyr-155 is located C-terminal to the pseudosubstrate site, and Tyr-187 is within the C1A domain required for DAG binding. It is interesting to speculate that phosphorylation of Tyr-155 may prevent the protein from resuming a "closed" conformation with the pseudosubstrate sequence embedded in the active site, and thus eliminate the requirement for allosteric modulators for activity. It is also interesting to consider whether phosphorylation of Tyr-187 could be incompatible with DAG binding and promote the release of PKCδ from the membrane. Such a scenario could account for the transient translocation of PKCδ to the membrane with physiological agonists as opposed to the sustained translocation induced by PMA, given that (as has been reported (5, 6)) PMA interacts with the C1B domain rather than C1A. Further studies of the specific effects of phosphorylation of different Tyr residues induced by physiological agonists in PKCδ are clearly required.

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Note Added in Proof—While this manuscript was under revision, Rybin and Steinberg (Rybin, V. O., and Steinberg, S. F. (2005) Am. J. Physiol. Cell Physiol. Epub ahead of print, October 19) also noted that phosphorylation of PKCδ may reduce its immunoprecipitation with the BD Bioscience antibody, as we have shown here (Fig. 7). Although we suggested that this may result from Tyr-phosphorylation of PKCδ, Rybin and Steinberg attribute this to an event that can be inhibited by GF109203X (i.e. it may be caused by Ser-/Thr-phosphorylation).

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