Differential Activation of Protein Kinase C Isoforms by Endothelin-1 and Phenylephrine and Subsequent Stimulation of p42 and p44 Mitogen-activated Protein Kinases in Ventricular Myocytes Cultured from Neonatal Rat Hearts*

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The translocation of protein kinase C (PKC) isoforms PKC-α, PKC-δ, PKC-ε, and PKC-ζ from soluble to particulate fractions was studied in ventricular cardiomyocytes cultured from neonatal rats. Endothelin-1 (ET-1) caused a rapid, ET₁ receptor-mediated translocation of PKC-δ and PKC-ε (complete in 0.5–1 min). By 3–5 min, both isoforms were returning to the soluble fraction, but a greater proportion of PKC-ε remained associated with the particulate fraction. The EC₅₀ of translocation for PKC-δ was 11–15 nM ET-1 whereas that for PKC-ε was 1.4–1.7 μM. Phenylephrine caused a rapid translocation of PKC-ε (EC₅₀ = 0.9 μM), but the proportion lost from the soluble fraction was less than with ET-1. Translocation of PKC-δ was barely detectable with phenylephrine. Neither agonist caused any consistent translocation of PKC-α or PKC-ζ. Activation of p42 and p44 mitogen-activated protein kinase (MAPK) by ET-1 or phenylephrine followed more slowly (complete in 3–5 min). Phosphorylation of p42-MAPK occurred simultaneously with its activation. The proportion of the total p42-MAPK pool phosphorylated in response to ET-1 (50%) was greater than with phenylephrine (20%). In addition to activation of MAPK, an unidentified p85 protein kinase was activated by ET-1 in the soluble fraction whereas an unidentified p58 protein kinase was activated in the particulate fraction.

A number of studies have implicated protein kinase C (PKC) in the regulation of ion channels, intracellular ion concentrations, contractility, gene expression, and hypertrophic growth in the cardiac myocyte (reviewed in Ref. 1). The PKC family is a group of protein Ser/Thr kinases which can be divided into three subfamilies that eventually phosphorylate a number of proteins in different respects (2, 3). The subfamilies are the classical PKCs (PKC-α, PKC-β₁, PKC-β₂, and PKC-γ), the novel PKCs (PKC-δ, PKC-ε, and PKC-ζ or L), and the recently identified PKC-θ isoform (4–6), and the atypical PKCs (PKC-ζ, and the recently-identified PKC-ζ₁ (7) and PKC-λ (8) isoforms). The category (novel or atypical) into which the newly identified PKC-λ isoform (9) falls is not entirely clear. This list may not be complete. In myocytes cultured from neonatal rat heart ventricles, examples of isoforms from all three subfamilies (PKC-α, PKC-δ, PKC-ε, and PKC-ζ₁) are known to be expressed as protein (10–12). PKC-β and PKC-γ are not readily detected (11, 12), and the presence of the other isoforms has not been thoroughly investigated.

The consequences of activation of classical and novel PKC isoforms can be studied by treating cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) or its homologs. TPA acts as a long-lived analog of the physiological activator of these PKCs, namely diacylglycerol. In the neonatal ventricular myocyte, exposure to TPA results in translocation/activation of PKC-α, PKC-δ, and PKC-ε (11); however, the physiological substrates of the PKCs are poorly characterized. One consequence of the exposure of myocytes to TPA is the activation of the mitogen-activated protein kinase (MAPK) cascade, i.e., MAPK kinase and MAPK (10, 13). MAPK is a protein Ser/Thr kinase that directly phosphorylates (or mediates activation of protein kinases that eventually phosphorylate) a number of proteins which are important in signal transduction and transcriptional regulation (reviewed in Refs. 14–18).

Our interests lie in the regulation of hypertrophy in cardiac myocytes. The mammalian ventricular myocyte is a terminally differentiated cell that withdraws from cell division at around birth. Thus, although the mass of the adult heart may be increased by suitable stimuli (linked mainly in vivo to increased contractile work), the myocytic contribution to this increase involves predominantly an increase in the size and protein content of pre-existing cells (19). In addition, acquisition of the hypertrophic phenotype is characterized by a number of transcriptional modifications that distinguish hypertrophy from normal maturational growth (19). Ventricular myocytes cultured from neonatal rat hearts represent a useful model system for the study of the myocytic hypertrophic response (19). Agonists which induce a hypertrophic phenotype in cultured cells include tumor-promoting phorbol esters (20–22), endothelin-1 (ET-1) (23–25), and α₁-adrenergic agonists such as phenylephrine (PE) and norepinephrine (26–33). However, the details of the signal transduction pathways through which these agonists induce hypertrophy have not been fully elucidated. A current hypothesis is that ET-1 and the α₁-adrenergic agonists interact with their cell surface receptors to cause a Go₂₃-coupled activation (34) of phospholipase Cβ. The subsequent stimulation of the hydrolysis of membrane phosphatidylinositol bisphosphate (PI), such as phosphatidylinositol-4,5-bisphosphate, raises diacylglycerol concentrations thereby activating PKC. Activation of PKC by
endogenous diaciglycerol (or by exogenous TPA) in some way leads to the hypertrophic response. However, the activation of PKC isoforms expressed in the ventricular myocyte by ET-1 and PE is poorly characterized. ET-1 and PE also activate the 42- and 44-kDa isoforms of MAPK in this cell (13), and we have suggested that this may also be important in the hypertrophic response. Here, we compare the effects of two "physiological" agonists, ET-1 and PE, on the activation of individual PKC isoforms and examine the effects of these agonists on the phosphorylation and activation of MAPK.

**Experimental Procedures**

**Materials**—Sprague-Dawley rats were bred within the National Heart and Lung Institute. Radiochemicals, prestained molecular mass standard proteins, horseradish peroxidase-linked donkey anti-rabbit immunoglobulin, the enhanced chemiluminescence (ECL) Western blotting detection reagents, autoradiography film (Hybrifilm MP), and intensifying screens were from Amersham International. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and reagents for the assay of protein by the Bradford method (35) were from Bio-Rad. Nicktorellase (0.45 μg) was from Schleich & Schuell. ET-1-PE medium 199, Dulbecco's modified Eagle's medium, protease inhibitors, and other biochemicals were purchased from Sigma. Other tissue culture products were from Life Technologies, Ltd. The peptide inhibitor of cAMP-dependent protein kinase was from Bachem. Other laboratory chemicals were from Merck. PE was dissolved in 100 μM β-mercaptoethanol and used fresh daily. Stock solutions of ET-1 and PE were diluted in serum-free medium.

With the exception of anti-αPKC-α, anti-αPKC antiserum was against oligopeptide sequences corresponding to sequences in the C-terminal Vᵢ regions of rat PKC isoforms (36). Antiserum against the PKC-α sequence VNPRYQFPLE and the PKC-β sequence KGSPSYEDLMP and against a Vᵢ peptide sequence in bovine brain PKC-α (AGNKVISPSEDWRQ) were from Life Technologies, Ltd. An antisemur against the bovine brain PKC-β Vᵢ sequence has been shown to cross-react with rat PKC-α (37). Antiserum against the PKC-γ sequence INPLLLE Vᵢ was a gift from Dr. P. J. Parker, Imperial Cancer Research Fund Laboratories, London, United Kingdom. Antiserum 124 raised against the C-terminal peptide sequence KEKRLIFEEETAR from mouse p42 MAPK was a gift from Professor C. J. Marshall, Chester Beatty Laboratories, Institute of Cancer Research, London, UK.

**Ventricular Myocyte Cultures and Preparation of Subcellular Fractions**—Myocytes were isolated and cultured by a method based on that of Iwaki et al. (30). Myocytes were dissociated from the ventricles of 1-2-day-old rat hearts using 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in 116 mM NaCl, 20 mM HEPES, 0.5 mM Na₂HPO₄, 5.6 mM glucose, 0.8 mM MgSO₄, (pH 7.35). The cells were resuspended in Dulbecco's modified Eagle's medium/medium 199 (4:1 v/v) supplemented with 10% horse serum, 5% fetal calf serum, and 100 units/ml of both penicillin and streptomycin. Cells were plated for 30 min on uncoated 60-mm culture dishes (Primaria, Falcon) to deplete fibroblasts, then the myocytes plated at a final density of 1.4 × 10⁶ cells/mm² on 60-mm gelatin precoated dishes (for all experiments except those in which PI hydrolysis was measured) or 10⁷ cells/mm² on 35-mm gelatin precoated dishes (for PI hydrolysis experiments). After 18 h, myocytes were confluent and beating spontaneously. Serum was then withdrawn for 24 h before cells were subsequently exposed to ET-1 or PE in serum-free medium. Cells were washed in ice-cold Dulbecco's Ca²⁺/Mg²⁺-free PBS (3 x 1 ml) and lysed by extraction in 150 μl of Buffer A (12.5 mM Tris-HCl, 2.5 mM EDTA, 1 mM EDTA, 100 mM NaCl, 5 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluoride, 120 μM pepstatin A, 200 μM leupeptin, 10 μM trans-epoxy-succinyl-γ-L-leucyl-aminoo4-quinidine-ketone, pH 7.4) containing 0.5% (w/v) digitonin (36, 39). The extracts were incubated for 5 min at 4°C. For the determination of subcellular distribution, extracts were centrifuged (Eppendorf 5414 m, 15,000 xg, 15 min, 4°C). The soluble fraction was obtained. The particulate fraction was washed in 150 μl of Buffer A, centrifuged, and finally resuspended in the same volume of Buffer A containing 1% (w/v) Triton X-100. Protein concentration in fractions was determined by the Bradford method (35).

**Immunoblotting**—Soluble fractions were heated at 100°C with 0.33 volume of SDS sample buffer (10% (w/v) SDS, 13% (v/v) glycerol, 300 mg Tris-HCl, 130 mM dithiothreitol, 0.2% (w/v) bromophenol blue, pH 6.8) whereas particulate fractions were solubilized by heating in a volume of SDS sample buffer equal to the volume of the original extract. Proteins (10–40 μg) were separated by SDS-PAGE using a 10% (w/v) acrylamide, 0.266% (w/v) bisacrylamide resolving gel with a 6% (w/v) acrylamide, 0.16% (w/v) bisacrylamide stacking gel, and were transferred electrophoretically to nitrocellulose. Non-specific binding sites were blocked with 5% non-fat milk powder in PBST buffer (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl. 0.05% (v/v) Tween 20, pH 7.5) for 30 min at room temperature. Primary antibodies were diluted in blocking solution (1:250 for anti-αPKC-α, 1:500 for anti-αPKC-β, 1:2000 for anti-PKC-ε and anti-PKC-δ, anti-PKC-γ, and anti-PKC-ζ). Nitrocellulose was incubated with primary antibodies overnight at 4°C. Preliminary experiments established the specificity of immunoreactivity by the use of the appropriate competing peptide (2 μg/ml). After washing in PBST (3 x 5 min each), nitrocellulose was incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibody (1:5000 in 1% (w/v) non-fat milk powder in PBST). After repeating the washing procedure described above, bound antibody was detected by the ECL method with exposure to Hyperfilm for 1–15 min (depending on signal strength). Immunoblots were quantitated by scanning laser densitometry.

**Detection of MAPK Activity by the "In Gel" Phosphorylation of Myelin Basic Protein (MBP)**—Denatured soluble or particulate fractions in SDS sample buffer were prepared as described above. Polyacrylamide gels (10% w/v) were formed in the presence of 0.5 mg/ml MBP, thereby cross-linking MBP into the gel matrix. Phosphorylation of MBP was studied by a previously described adaptation (10, 13) of the method of Kametsita and Fujisawa (40). Laser scanning densitometry was used to quantitate the autoradiographs.

**Preliminary and Agonist-Activated Hydrolysis of PI**—Myocytes were incubated with serum-free medium (2 ml) containing 5 μCi of [³²P]inositol for 24 h to prelabel PI pools. Myocytes were exposed to 100 nM ET-1 and BQ123 for 10 min in serum-free medium containing 10 mM LiCl. The incubation was terminated by the removal of medium and addition of 0.8 ml HCO₃⁻. Each plate was scraped and then washed with a further 0.5 ml of 0.8% HCO₃⁻. Precipitated protein was removed by centrifugation, the supernatant fractions were neutralized with KOH-Tris and total [³²P]inositol phosphates determined by a modification (41) of the method of Berridge et al. (42).

**Statistical Methods and Curve Fitting**—Results are presented as mean ± S.E. Statistical significance was tested by a two-tailed Student's t test and was taken as being established at p < 0.05. Fitting of log concentration dependence data to sigmoid curves used the GraphPad Inplot 4 program (GraphPad Software Inc., San Diego, CA).

The equation used was:

\[ y = A + \frac{B - A}{1 + (10^{(x-C)})^D} \]  

where y is the PKC immunoreactivity present in the soluble or particulate fractions (% maximum), a is the log agonist concentration, A and B are the derived minimum and maximum values of y, respectively, C is log EC₅₀, and D is the Hill slope.

**Results**

Increased Association of PKC Isoforms with the Particulate Fraction on Exposure of Myocytes to ET-1 or PE—PKC-α (apparent molecular mass, 85 kDa), PKC-δ (74 kDa), PKC-ε (93 kDa), and PKC-ζ (78 kDa) have been detected in ventricular myocytes cultured from neonatal rat hearts (10–12). For the classical and novel PKC isoforms, increased association with the particulate fraction and concomitant loss from the soluble fraction ("translocation") is equated with activation (43, 44). No translocation of PKC-γ or PKC-δ was detected by immunoblotting of the soluble and particulate fractions of myocytes exposed to high (100 nM) concentrations of ET-1 (Fig. 1A). In contrast, PKC-δ and PKC-ε were lost from the soluble fraction within 15–30 s and appeared in the particulate fraction (Fig. 1A). Over a more prolonged time period (up to 21 min), the subcellular distributions of PKC-δ and PKC-ε returned to those in unstimulated cells (Fig. 1B).

Translocation of PKC-ε from the soluble to the particulate fraction was also detected in myocytes exposed to maximally effective (50 μM) concentrations of PE (Fig. 1C). In contrast to ET-1, there was apparently no loss of PKC-δ from the soluble fraction with PE, although there was an increase in PKC-δ detected to the particulate fraction (Fig. 1C). The failure to
Activation of PKC and MAP Kinase by Endothelin and Phenylephrine

Detect loss of PKC-δ from the soluble fraction (Fig. 1C) was not caused by overexposure of the film during ECL detection. PKC-δ bands were less intense in Fig. 1C than in Fig. 1A when loss of PKC-δ was easily detectable. Puca et al. (12) observed appearance of PKC-ε in the particulate fraction in the absence of its loss from the soluble fraction in neonatal ventricular myocytes exposed to physiological agonists, but the underlying cause was not identified. Changes in immunoreactivity because of phosphorylation were not responsible (12). We conclude that although PE activates PKC-ε and may activate PKC-δ, the responses are not as great as with ET-1. No consistent translocation of PKC-α or PKC-ζ was detected following exposure of myocytes to PE (Fig. 1C).

One difficulty that we initially encountered was related to the distribution of PKC isoforms between soluble and particulate fractions in the absence of agonists. We have found that relatively large proportions of PKC-δ, PKC-ε, and PKC-ζ are associated with particulate fraction in neonatal myocytes...
Fig. 2. Quantification of immunoblots of the time course for the loss of PKC-a, PKC-δ, PKC-ε, and PKC-ζ from the soluble fraction following exposure of myocytes to ET-1 or PE. Immunoblots of denatured soluble fractions (Fig. 1, A and C) were scanned by laser densitometry. Immunoreactive PKC-a (A and C, solid line), PKC-δ (B and D, solid line), PKC-ε (B and D, dashed line), or PKC-ζ (A and C, dashed line) present in the soluble fraction of myocytes exposed to ET-1 (A and B) or PE (C and D) for the times shown is expressed (mean ± S.E., n = three separate preparations of myocytes) as a percentage of the zero time control.

under these basal conditions. For quantitative immunoblotting of the translocation time courses by laser scanning densitometry, we therefore relied primarily on the loss of PKC from the soluble fraction. Quantitation of a series of immunoblots allowed identification of more consistent patterns of behavior (Fig. 2) which largely supported the conclusions from inspection of the representative immunoblots shown in Fig. 1. Although there may have been some loss of PKC-a immunoreactivity from the soluble fractions of cells exposed to 100 nM ET-1, this was never more than 50% of the control (Fig. 2A). Loss of PKC-ζ was likewise difficult to detect (Fig. 2A). In contrast, approximately 90% of PKC-δ and 75% of PKC-ε were lost from the soluble fraction within 30 s (Fig. 2B). The amount of PKC-ε immunoreactivity in the soluble fraction remained depressed (at 3 min, 41 ± 4% of control, mean ± S.E., n = three separate observations) whereas PKC-a immunoreactivity began to reappear (Fig. 2A). PKC-ζ translocation was maximal (Fig. 2A).

Effects of Restimulation of Myocytes with ET-1 on the Translocation of PKC-δ and PKC-ε—Translocation of PKC-δ from the soluble to the particulate fraction was reversed more rapidly than translocation of PKC-ε (Figs. 1B, 2B, and Table I). However, the concentration of ET-1 (100 nM) used in these experiments was about 6-9-fold greater than the EC_{50} for PKC-δ translocation whereas it was 60-70-fold greater than the EC_{50} for PKC-ε translocation (Table II). We were concerned that loss of ET-1 by, for example, peptidase activity could give rise to the more rapid reversal of PKC-δ translocation. Myocytes were therefore re-exposed to a second addition of ET-1 (final concentration 100 nM) at 5 min after the initial exposure to 100 nM ET-1 (Fig. 5). PKC-δ translocated from the soluble to the particulate fraction in 30 s (Fig. 5). By 6 min, this translocation was being reversed. However, re-exposure of myocytes to ET-1 at 5 min did not reverse the reappearance of PKC-δ in the soluble fraction at 6 min. This suggests that loss or destruction

<table>
<thead>
<tr>
<th>PKC isoform</th>
<th>Immunoreactivity in the soluble fraction</th>
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<tr>
<td>PKC-δ</td>
<td>76 ± 3</td>
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<tr>
<td>PKC-ε</td>
<td>34 ± 8</td>
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*p < 0.001 versus PKC-δ at 5 min.

TABLE I

Longer term time course for the loss of PKC-δ and PKC-ε from the soluble fractions of myocytes exposed to ET-1

The concentration dependence of the loss of PKC-δ and PKC-ε from the soluble fraction was increased by improving protein loading to 40 μg/lane for SDS-PAGE. PKC-δ (Fig. 3A) and PKC-ε (Fig. 3B) both translocated in an ET-1 concentration-dependent manner. The calculated EC_{50} values for the loss of PKC-δ and PKC-ε from the soluble fraction were 11 and 1.4 μM, respectively (Table II). These agree well with the corresponding values for the appearance of PKC-δ and PKC-ε in the particulate fraction (15 and 1.7 μM, respectively, Table II). The Hill coefficients for the translocation of PKC-δ by ET-1 (Fig. 3A) were −1.3 (soluble) and 1.1 (particulate). The Hill coefficients for the translocation of PKC-ε by ET-1 (Fig. 3B) were −0.8 (soluble) and 1.1 (particulate).

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FIG. 3. Dependence of the translocation of PKC-6 or PKC-ε on ET-1 or PE concentration. Myocytes were exposed to agonists for 30 s, and denatured soluble and particulate fractions were prepared as described under “Experimental Procedures.” For SDS-PAGE, protein loading was 20 μg/lane for the soluble fraction and 40 μg/lane for the particulate fraction. Following SDS-PAGE and transfer of protein to nitrocellulose, immunoblotting was carried out as described under “Experimental Procedures” with ECL detection. The upper panels of A (effects of ET-1 concentration on PKC-6 translocation), B (effects of ET-1 concentration on PKC-ε translocation), and C (effects of PE on PKC-ε translocation) show representative immunoblots of soluble and particulate fractions (the experiments were carried out on three or four separate occasions). The lower panels of A–C show the quantitation by laser scanning densitometry of the concentration dependence of the translocation for the soluble (●, solid lines) and particulate (■, dashed lines) fractions. Fitting of data to sigmoid curves used the GraphPad Inplot 4 program. For each experiment, the amounts of immunoreactive PKC (expressed as arbitrary absorbance units obtained by laser scanning densitometry) in the soluble or particulate fraction were plotted against log agonist concentration and the constants for the sigmoid line equation obtained. Data were then normalized by dividing by the extrapolated maximal immunoreactivity value, and data for the three or four separate experiments were combined and expressed as means ± S.E. EC50 values (for values, see Table II) were calculated by the Inplot 4 program.

of ET-1 is not responsible for the differences in the time course of translocation between PKC-6 and PKC-ε. To confirm that PKC-δ was still able to translocate, we showed that addition of 1 μM TPA at 5 min after the initial exposure of myocytes to ET-1 resulted in the complete translocation of PKC-δ to the particulate fraction (Fig. 5).

Activation and Phosphorylation of MAPK in Response to ET-1 and PE—In order to study whether the translocation of PKC
was consistent with downstream activation of MAPK, we studied the time courses of activation (by “in gel” MBP phosphorylation) and phosphorylation of MAPK (by its reduced mobility on SDS-PAGE) in soluble fractions. ET-1 (100 nM) detectably activated p42-MAPK and p44-MAPK by 1 min and maximally activated by 2–5 min (Fig. 6A). An unidentified MBP kinase of 85 kDa was also detectably activated by 2 min (Fig. 6A). Other MBP kinases of 115 and 58 kDa were present, but their activity was not detectably stimulated by ET-1. Activation of p115-MBP kinase and p88-MBP kinase could not be detected even when the times for which gels were exposed to film were reduced to avoid saturation (results not shown). Furthermore, the maximum intensity of the p115-MBP kinase signal was less than that for p42-MAPK and p44-MAPK and activation of MAPKs was readily detectable (Fig. 6A).

On phosphorylation, the mobilities of p42-MAPK and p44-MAPK on SDS-PAGE are slightly reduced, and this can be detected by immunoblotting. Using anti-mouse p42-MAPK antiserum 124, reduced mobility of p42-MAPK was detectable by 1 min and maximally by 2–5 min (Fig. 6B). The maximal extent of phosphorylation of total p42-MAPK pool was about 50%. Although antisera 124 detected unphosphorylated (inactive) p44-MAPK, no “reduced mobility” band of p44-MAPK could be detected on exposure of myocytes to ET-1 (Fig. 6B) even though in gel MAPK assays showed activation of p44-MAPK (Fig. 6A). This batch of anti-p42-MAPK antisera may not cross-react well with phosphorylated p44-MAPK.

PE (50 μM) also activated p42-MAPK and p44-MAPK (Fig. 6C), activation being maximal by 3–5 min (cf. ET-1, Fig. 6A). The p85-MBP kinase activated by ET-1 (Fig. 6A) was also activated by PE (Fig. 6C). The degree of activation of MAPK cannot be compared directly with that for ET-1 (Fig. 6A) because of differences in exposure time of the autoradiographs. However, SDS-PAGE immunoblotting (Fig. 6D) showed that although phosphorylation of p42-MAPK was maximal in 2–5 min (cf. ET-1, Fig. 6B), only about 20% of the total p42-MAPK was phosphorylated at these time points (Fig. 6D), i.e. rather less than in ET-1-treated cells (Fig. 6B).

The activity of MAPK in the washed particulate fraction was studied following exposure of myocytes to 100 nM ET-1 (Fig. 7). p42-MAPK and p44-MAPK activities increased with time, but the strongest signal was from the p88-MBP kinase. The events leading to activation of p42-MAPK, p44-MAPK, and p58-MBP kinase in this fraction are not clear.

**DISCUSSION**

*General Points*—A variety of studies have implicated involvement of PKC in pathways that induce the hypertrophic phenotype in the ventricular myocyte (reviewed in Ref. 19). There are two main lines of evidence. First, many known hypertrophic agonists (e.g. ET-1 or α1-adrenergic agonists) stimulate PI hydrolysis (23, 42, 45–50), raise diacylglycerol concentrations (23, 51), and activate PKC (36, 52, 53) in this cell type. Second, direct activation of PKC either with TPA (20–22) or by transfection with plasmids encoding constitutively active PKC constructs (53, 54) leads to development of the hypertrophic phenotype. We have suggested that a consequence of the activation of PKC is the stimulation of the MAPK cascade, and this may also be relevant to the hypertrophic response (10, 13). There have been few detailed quantitative studies on the time courses and concentration dependences of the membrane association of individual translocatable PKC isoforms in response to physiological agonists. The aims of the present study were to investigate whether exposure of myocytes to ET-1 or PE resulted in any differential stimulation of PKC isoforms expressed in ventricular myocytes (PKC-α, PKC-δ, PKC-ε, and PKC-ζ (10–12)) and whether activation of MAPK could be temporally separated from activation of PKC.
Effects of ET-1 on the Partition of PKC-δ and PKC-ε between the Soluble and Particulate Fractions—Experiments with BQ123 indicated that the stimulation of translocation of PKC-δ and PKC-ε (Fig. 4B) by ET-1 is mediated through the ET₄ receptor, as is the stimulation of PI hydrolysis (Fig. 4A). PI hydrolysis is probably at least partly responsible for increasing diacylglycerol concentrations. Maximally effective concentrations of ET-1 caused a rapid translocation of PKC-δ and PKC-ε from the soluble to the particulate fraction (Figs. 1A and 2B). However, PKC-δ returned to the soluble fraction significantly more rapidly than PKC-ε (Figs. 1B, 2B, and Table I). The EC₅₀ value for ET-1 of PKC-ε translocation (calculated from data for either the soluble or particulate fraction) was 1.4–1.7 nM (Fig. 3B and Table II). This EC₅₀ is similar to the Kₐ₅₀ for the binding of ET-1 to the ET₄ receptor in cardiac myocytes (55, 56) and to EC₅₀ values for a variety of ET-1-stimulated phenomena (including PI hydrolysis (23, 42) and MAPK activation (13)) in ventricular myocytes (23–25, 42, 57, 58) reviewed in Ref. 59). Although it is of interest that the EC₅₀ values for the translocation of PKC-ε by ET-1 are similar to values for stimulation of MAPK (13) and the hypertrophic response (23, 25), this finding does not necessarily indicate that PKC-ε is the isoform that regulates these processes.

The EC₅₀ value for ET-1 of PKC-δ translocation (11–15 nM, Fig. 3A and Table II) was about one order of magnitude greater than for PKC-ε. This applied to EC₅₀ values calculated from data for either the soluble or the particulate fraction. Because we were concerned that destruction of ET-1 could give rise to the observed differences in the time courses of translocation for PKC-δ and PKC-ε given the higher affinity of PKC-ε translocation for ET-1, we re-exposed myocytes to ET-1 at 5 min (i.e., after the association of PKC-δ with the particulate fraction was decreasing). No effect of this restimulation could be discerned although PKC-δ is still able to translocate to the particulate fraction when cells are exposed to TPA (Fig. 5). Homologous desensitization of the ET₄ receptor may be occurring (60).

The reasons underlying differences in the time course and concentration dependence of PKC-δ and PKC-ε in myocytes treated with ET-1, and the physiological significance of these differences are difficult to assess. The observations are consistent with PKC-ε having a greater affinity for its physiological activator (presumably diacylglycerol) than PKC-δ. However, the affinities of PKC-δ and PKC-ε for 1-oleyl-2-acetylglycerol are similar, as are those for phorbol-12,13-dibutyrate (61). Alternatively, different diacylglycerol analogs may be responsible for the activation of the two isoforms. Olivier and Parker (62) have suggested that membrane association of PKC-δ and PKC-ε may be dependent on their phosphorylation states and hence the time course or agonist concentration dependence of phosphorylation/dephosphorylation of the two isoforms may differ. Phosphorylation of PKC-ε can be readily detected by its reduced mobility on SDS-PAGE in 7.5% acrylamide, 0.075% bisacrylamide (62). The mobility of PKC-δ is also probably reduced on phosphorylation (62). Our SDS-PAGE method would not have detected phosphorylation of PKC-δ and PKC-ε, and this merits further investigation.

Effects of Agonists on Partition of PKC-δ and PKC-ε between the Soluble and Particulate Fractions—Although TPA promotes a rapid (within 1 min) translocation of PKC-α in neonatal and adult (12) ventricular myocytes, no consistent translocation could be detected in immunoblots of neonatal myocytes exposed to 100 nM ET-1 (Fig. 2A) or 50 μM PE (Fig. 2C). Failure to detect translocation of PKC-α in response to physiological agonists which do cause translocation of PKC-δ/PKC-ε has been reported for neonatal and adult ventricular myocytes (12) and for Swiss 3T3 cells (62). In the latter, no reduction in the mobility of PKC-α could be detected on SDS-PAGE (62), suggesting an unchanged phosphorylation state (66). It is not clear why translocation of PKC-α should not be easily detectable in response to either PE or ET-1, given that it is activated, as is PKC-ε by diacylglycerols (2, 67). Again, as we proposed for PKC-δ, PKC-ε may have a greater affinity for diacylglycerol than PKC-α (but see Ref. 61) or different species of diacylglycerol may be responsible for the activation of the two isoforms. Differences in the regulation of the phosphorylation of PKC-α and PKC-ε may also be responsible.

PKC-ζ is not activated by TPA or diacylglycerol (68–71). Its physiological activator(s) has not unequivocally been identified although a role for phosphatidylinositol 3,4,5-trisphosphate has been proposed (72, 73). Although there has been a report to the contrary (74), neither we nor others (11, 12) have been able to detect translocation of PKC-ζ in cultured neonatal ventricular myocytes in response to TPA, agreeing with results using non-myocytic cells (69, 75, 76). Apparent translocation of PKC-ζ (74, 77, 78) may have resulted from cross-reaction between PKC-α and anti-PKC-ζ antiserum (75). Furthermore, translocation of PKC-ζ has not been detected in response to physiological agonists in neonatal ventricular myocytes (12, PE as agonist) or in fibroblast lines (62, 75). Given these findings, it is not surprising that we were unable to detect translocation of PKC-ζ (Figs. 1, A and C, and 2, A and C). The regulation of

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**Fig. 5.** Effects of restimulation of myocytes with ET-1 on the translocation of PKC-δ. Myocytes were exposed to 100 nM ET-1 for the times indicated. After 5 min, a further addition of 100 nM ET-1 was made to one plate of cells (marked + on 1 min ET-1 line), and an addition of 1 μM TPA was applied to a second plate (marked + on 1 min TPA line). At either 1 min after these further additions (i.e., 6 min of total incubation time) or at 30 s or 6 min after a single exposure to ET-1, denatured soluble and particulate fractions were prepared as described under “Experimental Procedures.” For SDS-PAGE, protein loading was 20 μg/lane for the soluble fraction and 40 μg/lane for the particulate fraction. Following SDS-PAGE and transfer of protein to nitrocellulose, immunoblotting was carried out as described under “Experimental Procedures” with ECL detection. The experiment was repeated three times.
PKC-ζ remains poorly understood. In the time courses of translocation of classical and novel PKCs in fibroblasts have been detected (3, 62, 75). With reference specifically to ventricular myocytes, Pucéat et al. (12) found
that PKC-6 translocated in response to PE or ATP in either neonatal or adult cells and that association of PKC-δ with the particulate fraction in response to agonists was more sustained than PKC-ε. These results differ somewhat from our own (Figs. 1, A and B, and 2, B and D). Translocation studies do not provide any detailed information about the subcellular localization of the PKC isoforms translocated. Using immunocytochemical or subcellular fractionation approaches in non-myocytes, complex patterns of PKC migration have been detected (79–87). With specific reference to neonatal ventricular myocytes, Disatnik et al. (88) extended an earlier immunocytochemical study (89) to show that PKC-α migrated from the cytosol to the perinuclear region, whereas PKC-δ and PKC-ε migrated from an intranuclear location to the perinuclear region (PKC-δ) or to the myofibrillar cross-striations (PKC-ε) in response to norepinephrine or TPA. PKC-ζ translocation was reportedly identical to PKC-α translocation. Immunocytochemical and translocation studies cannot be easily compared because the apparent distribution of PKC may alter on cell lysis (e.g. soluble PKC may represent a pool that is only weakly associated with the particulate fraction in the basal state).

**Activation and Phosphorylation of MAPK—** Treatment of cultured neonatal ventricular myocytes with TPA stimulates MAPK and MAPK kinase, as does treatment with ET-1 or PE (10, 13). Furthermore, down-regulation of PKC-α, PKC-δ, and PKC-ε by a 24-h pre-exposure of cells to TPA partly inhibits the activation of MAPK by ET-1 or α1-adrenergic agonists (10). These results imply that activation of PKC and activation of the MAPK cascade are connected. Translocation of PKC-δ and PKC-ε (maximal in 15–30 s, Fig. 2, B and D) precedes activation (maximal in 3–5 min, Fig. 6, A and C) of p42 MAPK and phosphorylation of p42 MAPK (maximal in 2–5 min, Fig. 6, B and D) when either ET-1 (Figs. 2B and 6, A and B) or PE (Figs. 2D and 6, C and D) is agonist. The maximum proportion of the total p42 MAPK pool that is phosphorylated in cells exposed to high concentrations of ET-1 (50%, Fig. 6B) is greater than in cells exposed to PE (20%, Fig. 6D). We have observed previously that ET-1 is more effective in PE in activating MAPK in crude extracts of myocytes (13). Equally, ET-1 is more effective than PE in bringing about the translocation of PKC-δ and PKC-ε from the soluble to the particulate fraction (Figs. 1, A and C, and 2, B and D). These results are consistent with a scheme whereby activation of PKC leads to activation of the MAPK cascade. It is tempting to speculate that PKC-ε is primarily responsible, the strongest evidence being that only PKC-ε is translocated by PE (Figs. 1C and 2, C and D). However, a role for PKC-δ is not excluded. The intracellular signaling pathway that connects activation of PKC to activation of MAPK is not clear. Distally, MAPK kinase is activated (10). Proximally, the MAPK kinase kinase, c-Raf-1, is reportedly phosphorylated and activated by PKC (90, 91). Whether PKC directly activates a MAPK kinase or whether the situation is more complex has not yet been fully resolved.

We detected activation of p42-MAPK and p44-MAPK in the particulate fraction following exposure of cells to ET-1 (Fig. 7). We do not know whether this results from activation of MAPK pre-existing in the particulate fraction or whether activated MAPK is translocated from the soluble to the particulate fraction. The trivial explanation is that the particulate fraction is contaminated with the soluble fraction. This can probably be excluded because the soluble p85-MAP kinase, which has an activity comparable to the MAPKs (Fig. 6B), is absent from the particulate fraction (Fig. 7). The findings that activated MAPK may be able to enter the nucleus (92–94) or may translocate to other “particulate” locations (95) in other cell types may be relevant.

**Activation of Other MAP Kinases by ET-1 and PE—** ET-1 activated a p85-MAP kinase in the soluble fraction but did not activate the p58- and p115-MAP kinases (Fig. 6B). A similar pattern was observed with PE (Fig. 6D). In contrast, a p58-MAP kinase was the major kinase activated by ET-1 in the particulate fraction. As argued above, it is unlikely that components of the particulate fraction by the soluble fraction explains this result. We do not know whether this enzyme is identical to the soluble p58-MAP kinase. We have observed MAPK kinases of similar molecular masses previously (10). No convincing activation by ET-1 was detected partly because we studied only a limited time course (10). The ability of the in gel assay to reveal these MAPK kinases not detected in crude extracts (removal of inhibitors, unmasking of covert phosphorylation sites in MBP, unmasking of covert MBP binding sites within the protein kinases) has been discussed elsewhere (10). In addition to these, it should be mentioned that the MBP used (Sigma) is only about 50% pure so that covert phosphorylation sites could be unmasked in contaminated proteins.

Although many protein kinases (including PKC (61)) phosphorylate MBP, the in gel assay is carried out in the presence of EGTA (to inhibit Ca++-dependent protein kinases) and the peptide inhibitor of cAMP-dependent protein kinase, the active catalytic subunit of which runs at 40 kDa on SDS-PAGE (96). Activation of MBP kinases presumably requires a covalent modification because non-covalently bound ligands will be removed by the denaturing steps in the in gel protocol. It remains possible that a non-covalent activator could induce protein kinase autophosphorylation and activation in the cell, and autophosphorylation is then sufficient to maintain activation. It is unlikely that PKC-δ or PKC-ε is responsible on the basis of their apparent molecular masses on SDS-PAGE (74 and 93 kDa, respectively). PKC-ζ (78 kDa) can similarly be excluded. The identities of the ET-1-activated p85- and p58-MAP kinases await further clarification.

**Conclusions—** The overall conclusions are that PKC isoforms are differentially activated by physiological agonists, translocation of PKC precedes activation and phosphorylation of MAPK, and ET-1 is more effective in activating both PKC and MAPK than PE. These findings are consistent with activation of PKC initiating events that lead to activation of MAPK, although the intervening steps remain controversial.

**REFERENCES**
