Characterization of Protein Kinase C Isotype Expression in Adult Rat Heart

Protein Kinase C-ε Is a Major Isotype Present, and It Is Activated by Phorbol Esters, Epinephrine, and Endothelin

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The pattern of protein kinase C (PKC) isotype expression in whole extracts of dispersed, freshly isolated adult rat ventricular myocytes and adult rat heart ventricle was examined by immunoblot analysis using antisera specific for PKC-α, -β1, -γ, -δ, -ε, -ζ, or -η isotypes. This analysis revealed significant levels of expression of the Ca²⁺-independent isotype PKC-ε, which was detected as a band of 97-kd molecular mass. PKC-ζ was detected principally as a 66-kd band that probably represented a proteolytic product of the holoenzyme. PKC-η was detected only in whole ventricle as a doublet at 75 and 81 kd and was therefore probably present in nonmyocytic cells. PKC-α, -β1, -γ, and -δ could not be detected. Because of our inability to detect PKC-α, -β1, -γ, and -δ in whole extracts, PKC isotypes were partially purified from whole heart by DEAE Sephacel chromatography. PKC-α, -β1, -γ, and -δ could still not be detected in the appropriate fractions. All PKC isotypes were detectable in appropriate positive control extracts (brain or certain cultured cell lines). In unstimulated isolated cardiomyocytes, the majority (80-95%) of the PKC-ε immunoreactivity was present in the soluble fraction of the extract. On exposure of the cardiomyocytes to 1 μM phorbol 12-myristate 13-acetate (PMA), PKC-ε undergoes a rapid (<30 seconds), sustained (at least 60 minutes), and virtually complete association with the Triton X-100-soluble membrane fraction. There was an associated loss of PKC-ε from the soluble fraction. The EC₅₀ for PMA of the translocation event was 15-37 nM. Exposure of cardiomyocytes to 1 μM 4β-phorbol 12,13-didecanoate or 1 μM phorbol 12,13-dibutyrate also resulted in translocation of PKC-ε to the membrane fraction, whereas exposure to 1 μM 4α-phorbol 12,13-didecanoate was without effect. PKC-ε also translocated on exposure of cardiomyocytes to 50 μM epinephrine or 100 nM endothelin-1. However, in both cases, the extent of translocation was significantly less than that after exposure to PMA. We conclude that interventions that lead to hypertrophy of cardiomyocytes (phorbol esters, epinephrine, and endothelin-1) activate PKC-ε. (Circulation Research 1993;72:757-767)

KEY WORDS • protein kinase C • immunoblotting • protein kinase C-ε • phorbol esters • epinephrine • endothelin

The activation of the multifunctional Ser-Thr protein kinase, protein kinase C (PKC), has been implicated in the action of a number of hormones and growth factors in a variety of tissues.¹-⁵ In vivo, the major physiological activator of the enzyme is thought to be diacylglycerol (DG), which is formed after the stimulation of membrane phospholipid hydrolysis by agonists. Molecular cloning and biochemical analysis have revealed that PKC exists as a family of at least eight related enzymes that can be further divided into two subfamilies. The first subfamily comprises the α, β1, β2, and γ isotypes that were originally isolated from the brain.⁶⁻⁷ These isotypes are dependent on the presence of Ca²⁺ for activity and readily phosphorylate model substrates, such as histone III-S. The second group, consisting of the δ, ε, ζ, and η isotypes, differ from the first group in that they lack the Ca²⁺-binding domain present in the first subfamily; hence, their activity is independent of Ca²⁺-¹,³,⁸⁻¹¹ This subfamily only poorly phosphorylates histone III-S. It has been suggested that the second subfamily may be important in tissues such as muscle because it may regulate intracellular events independent of the oscillating Ca²⁺ concentrations associated with contractile activity.¹⁰ However, very little more is known about the specific enzymatic properties (e.g., protein substrate specificity in vivo and differential activation by the various DG species) or tissue expression of many of these isotypes.²

The tumor-promoting phorbol esters, which act as long-lived DG analogues and activate enzymes of the PKC family,¹² have been used extensively to demonstrate involvement of PKC in the regulation of many cellular processes. In the adult heart, these include effects on contractility¹³⁻¹⁵ and the stimulation of protein synthesis.¹⁶ Additionally, in myocytes cultured from neonatal rat hearts, phorbol esters have been shown to...
induce hypertrophy.\textsuperscript{17-19} In the heart, the activation of a number of receptors (including α\textsubscript{1}-adrenergic,\textsuperscript{20-24} muscarinic,\textsuperscript{20,25} endothelin,\textsuperscript{26-28} purinergic,\textsuperscript{25} and angiotensin II\textsuperscript{28-31} receptors) stimulates the hydrolysis of membrane phosphoinositides, resulting in rises in DG concentrations.\textsuperscript{27,32} Although the rise in DG concentration should, in turn, lead to activation of PKC in the heart, this has been demonstrated directly only in a few cases.\textsuperscript{17,24,33,34} One problem has been that previous studies of cardiac PKC activation have relied largely on measurements of PKC activity with histone III-S as a substrate.\textsuperscript{13,14,17,19,24,33} Measurements of this type would reliably detect only PKC-α, -β, and -γ, and there seems to be little agreement concerning which, if any, of these isotypes are present in the heart.\textsuperscript{35-39} The expression of Ca\textsuperscript{2+}-independent isotypes in the heart has not been previously examined. Characterization of PKC isotype profiles in cardiac myocytes is of particular interest to us in view of the putative role of the enzyme in cardiac hypertrophy.\textsuperscript{40} The characterization of PKC expression in the heart is an essential prelude to understanding its importance in this process. Therefore, we have investigated the expression of PKC in adult rat heart tissue preparations using immunoblotting. Furthermore, we have examined the acute regulation of PKC-ε by potentially hypertrophic stimuli: phorbol esters,\textsuperscript{17-19} epinephrine,\textsuperscript{41,42} and endothelin-1 (ET-1).\textsuperscript{27,43}

Materials and Methods

Animals and Materials

Adult male Sprague-Dawley rats were from Harlan-Olac, Bicester, UK. All reagents were obtained from Merck, Dagenham, UK, or Sigma, Poole, UK, unless otherwise stated. Aqueous solutions were prepared using double-distilled deionized water. Phorbol esters were dissolved in dimethyl sulfoxide at a concentration of 1 mM. All were stored at −20°C and were diluted to the appropriate concentration with incubation medium (modified Krebs-Henseleit saline containing 25 mM NaHCO\textsubscript{3}, 4.7 mM KCl, 118.5 mM NaCl, 1.2 mM MgSO\textsubscript{4}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 2% [wt/vol] bovine serum albumin [BSA], 10 mM glucose, and 1 mM added CaCl\textsubscript{2} equilibrated with 95% O\textsubscript{2}-5% CO\textsubscript{2}. Epinephrine was freshly prepared at a concentration of 25 mM in 0.1 mM l-ascorbic acid and subsequently dialyzed in incubation medium. Collagenase ( Worthington type 1) was from Lorne Diagnostics, Twyford, UK. Sodium dodecyl sulfate (SDS) and polyacrylamide gel electrophoresis (PAGE) reagents were from Bio-Rad, Hemel Hempstead, UK. Prestained protein molecular mass standards, horseradish peroxidase–linked donkey anti-rabbit immunoglobulin, horseradish peroxidase–linked sheep anti-mouse immunoglobulin, the PKC-α/β monoclonal antibody (MCS), the enhanced chemiluminescence Western blotting detection system, and autoradiography film (Hyperfilm MP) were from Amersham International, Amersham, UK. Nitrocellulose (0.45 μm BABB, Schleicher & Schuell) was from Anderman & Co., Kingston-Upon-Thames, UK. Peptides corresponding to isotype-specific sequences in the V\textsubscript{c} Terminal regions of PKC species were prepared, and antibodies to these peptides were raised as described previously.\textsuperscript{44} Briefly, the peptide sequences used (with residue numbers in parentheses) were as follows: PKC-α, PQFVHPILQSAV (661-672); PKC-β\textsubscript{1}, SEFLKPEVKS (664-673); PKC-δ, VNPKYEQFLE (664-673); PKC-ε, NQEEFKGFSYF GEDLMP (721-737); PKC-ζ, INPLLSAEESV (581-592); and PKC-η, QDEFRNFYYSVPELQL (668-683). PKC-γ was detected with a mouse monoclonal antibody.\textsuperscript{45}

Preparation of Whole-Cell and Tissue Extracts

Ventricular myocytes were isolated by collagenase digestion of hearts from adult male rats (250–300 g) as described in detail previously\textsuperscript{46} and were immediately used for experiments. Samples of cardiac myocytes were washed in BSA-free incubation medium three times before the cell pellet was resuspended in an equal volume of boiling SDS-PAGE sample buffer (10% [wt/vol] SDS, 13% [vol/vol] glycerol, 300 mM Tris-HCl (pH 6.8), 0.2% [wt/vol] bromophenol blue, and 0.13 M dithiothreitol) and boiled for 5 minutes.

Hearts and brains were rapidly removed from male Sprague-Dawley rats. After a brief retrograde perfusion to remove blood, heart ventricles were "freeze-clamped" using aluminum tongs cooled in liquid N\textsubscript{2}, immediately pulverized under liquid N\textsubscript{2}, resuspended in boiling SDS-PAGE sample buffer, and boiled for 5 minutes. No attempts were made to separate the left and right ventricles. Brains were placed on ice, quickly cut into small pieces, immediately homogenized in boiling SDS-PAGE sample buffer using a hand-held ground-glass homogenizer, and boiled for 5 minutes. The COS-1 cells, which were transiently transfected with PKC-δ DNA to induce overexpression of this PKC isotype, and the HeLa cells, which expressed PKC-ζ, have been described elsewhere.\textsuperscript{47,48} All cells were prepared for SDS-PAGE and immunoblotting as described above. Protein was determined by the method of Bradford\textsuperscript{49} using BSA as a standard.

Partial Purification of PKC Isotypes

From Heart and Brain

Partial purification of PKC was carried out essentially as described by Allen and Katz\textsuperscript{50} and Koide et al.\textsuperscript{50} Hearts and brains were rapidly removed from adult male Sprague-Dawley rats. Hearts were perfused briefly with Krebs-Henseleit saline to remove blood. All procedures were then carried out at 4°C. Each tissue was homogenized already in ground-glass homogenizers in 4 vol of 20 mM Tris-HCl, 2 mM EDTA, and 2 mM EDTA (pH 7.5) containing 20 μM leupeptin, 10 μM trans-epoxysuccinyl-l-leucyl-aminodi(4-guanidino)-butane (E64), 200 μM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol, and 1% [vol/vol] Triton X-100. The homogenates were centrifuged for 10 minutes at 10,000 g and 2 ml supernatant fraction was then applied to a DEAE Sepharose column (3-mL bed volume) equilibrated with 20 mM Tris-HCl, 2 mM EDTA, and 2 mM EDTA (pH 7.5). The column was washed with 3 mL of the column equilibration buffer containing 20 μM leupeptin, 10 μM E64, 200 μM PMSF and 5 mM dithiothreitol and then with 7.5 mL of the same buffer containing 250 mM NaCl (a concentration sufficient to elute all PKC isotypes).\textsuperscript{50} The salt wash was retained and concentrated to 0.5 mL by ultrafiltration (Centricon 10 concentrator, Amicon, Beverly, Mass.). Samples of the total homogenate, Triton X-100 soluble supernatant fraction, and the concentrated PKC-enriched column
fractions were taken for protein determination and processing for immunoblotting as described elsewhere in “Materials and Methods.”

Subcellular Redistribution of PKC-ε in Cardiac Myocytes

Freshly isolated cardiac myocytes (0.7–1 mg myocyte protein per tube, i.e., approximately 140,000–200,000 myocytes per tube) were washed twice and resuspended in incubation medium. After exposure at 37°C to phorbol esters, ET-1, or epinephrine, the myocytes were centrifuged for 3–5 seconds (Eppendorf centrifuge, Merck, Dagenham, UK), and the cell pellet was washed three times in BSA-free incubation medium at room temperature. Two methods were used to disrupt the cells. In the first method (freeze-thaw disruption), the cell pellet was resuspended by vortexing in 0.6 ml ice-cold lysis buffer composed of (mM) Tris-HCl 25, EGTA 5, EDTA 2, and NaF 100 (pH 7.4) containing (μM) leupeptin 20, E64 10, pepstatin A 120, and PMSE 200, along with 5 mM dithiothreitol. Samples were then snap-frozen in liquid N2 and freeze-thawed three times. A sample was taken for estimation of the total cellular PKC-ε immunoreactivity before the remaining sample was centrifuged for 10 minutes at 10,000g and 4°C. The pellet was further extracted in lysis buffer containing 1% (vol/vol) Triton X-100 for 10 minutes at 4°C and was again centrifuged for 10 minutes at 10,000g and 4°C. Boiling SDS-PAGE sample buffer was added to all samples, which were then boiled for a further 5 minutes.

For studies on the effects of epinephrine and ET-1 on the subcellular distribution of PKC-ε, a second method (digitonin disruption51) was used. The advantages of this method of cell disruption have been discussed. The cell pellet was resuspended by gentle inversion in 0.6 ml of 0.05% (wt/vol) digitonin in ice-cold lysis buffer and incubated with mixing by inversion for 4°C for 5 minutes. A sample was taken for estimation of total PKC-ε before the remaining sample was centrifuged for 2 minutes at 10,000g and 20°C. The membrane fraction was extracted with lysis buffer containing Triton X-100 as above. The resulting fractions were prepared for SDS-PAGE as described above.

Immunoblot Analysis of PKC

Protein samples (typically 50–100 μg whole tissue samples or 20–50 μg myocyte subcellular fractions) were separated by SDS-PAGE using a 10% (wt/vol) acrylamide separating gel and 6% (wt/vol) acrylamide stacking gel. Protein was then electrophoretically transferred to nitrocellulose using a Semi-Dry Transfer Cell (Bio-Rad). The transfer buffer used was 25 mM Tris-HCl and 192 mM glycine (pH 8.3) containing 20% (vol/vol) methanol. After blocking nonspecific sites with blocking solution containing 5% (wt/vol) nonfat milk and 0.05% (vol/vol) Tween 20 in phosphate-buffered saline (PBS, consisting of [mM] Na2HPO4 80, NaH2PO4 20, and NaCl 100 [pH 7.5]) for 1 hour at room temperature, the nitrocellulose was incubated with primary antibodies (1/1,000–1,250 dilution in the blocking solution) with or without appropriate competing peptide (2 μg/ml) overnight at 4°C. After washing (5 minutes each) in 0.05% (vol/vol) Tween 20 in PBS, in 0.05% (vol/vol) Tween 20 plus 0.5 M NaCl in PBS, and again in 0.05% (vol/vol) Tween 20 in PBS, filters were incubated for 1 hour at room temperature with the horseradish peroxidase–linked secondary antibody (1/5,000 dilution in 0.05% [vol/vol] Tween 20 and 1% [wt/vol] nonfat milk in PBS). After repeating the washing as described above, the bound antibody was detected by the enhanced chemiluminescence method according to the manufacturer’s (Amersham International) instructions. This method depends on the production of light after the oxidation of luminol by horseradish peroxidase in the presence of H2O2 and is, according to the manufacturer, at least 10 times more sensitive than other detection systems. Exposure times of immunoblots to Hyperfilm were 1–60 minutes, as appropriate (see the following section). Quantitation of PKC-ε translocation was performed by laser-densitometric analysis of suitable autoradiographs.

Data Analysis and Expression of Results

Because of the nature of the enhanced chemiluminescence detection method, detailed quantitation is only valid for any single PKC isotype examined in a single immunoblot (e.g., in the translocation of PKC-ε shown in Figures 4 and 5). Results for experiments such as these are therefore expressed as percentages of suitable control experiments performed and analyzed simultaneously. It is not possible to compare quantitatively different immunoblots of the same PKC isotype. The data are expressed as mean±SEM of at least three independent observations. Two-tailed Student’s t tests for unpaired data were performed, with significance established at p<0.05. Curve fitting to sigmoid curves was carried out using GRAPHPAD software (ISI, San Diego, Calif.). No constants were set in the fitting procedure, and intermediate weighting was used.

Results

Expression of Ca2+-Dependent PKC Isotypes in Whole Tissue Extracts

The heart is a heterogeneous tissue in terms of cell type, with myocytes constituting only approximately 25% of the cell number (although they constitute approximately 75% of cardiac protein). Thus, to obtain some information concerning the expression of PKC in both myocytic and nonmyocytic cells, we have examined freshly isolated cardiac myocytes and whole heart ventricular samples by immunoblotting with PKC isotype–specific antisera. Extracts of rat brain tissue or transformed cell lines known to overexpress defined PKC isotypes were run simultaneously as positive controls. In the first series of experiments, we attempted to detect Ca2+-dependent isotypes of PKC in cardiac myocytes and whole heart ventricles. Typical results of the immunoblot analysis for the Ca2+-dependent PKC isotypes in brain, cardiac myocyte, and heart samples (50 μg total protein in each lane) are shown in Figure 1. Polyclonal antiserum to PKC-α detected a 79-kd protein in brain, which was effectively competed by the PKC-α peptide that was used as the antigen (Figure 1a). The molecular mass of PKC-α from rat brain is 77–80 kD.144 However, anti–PKC-α antiserum failed to react with cardiac myocyte and whole heart ventricular samples (Figure 1a). Overexposure of the immunoblots did not improve detection of any PKC-α bands of 70–80-kd molecular mass. Although anti–PKC-β1 antiserum reacted strongly
and specifically with protein in brain samples of 82-kd molecular mass (corresponding to the reported molecular mass of PKC-β). It reacted only weakly with cardiac myocytes or heart samples (Figure 1b). Long exposure times (up to 60 minutes) were required to detect protein bands in the region of 70–80-kd molecular mass, and these bands were not consistently competed by PKC-β peptide. Some lower molecular mass proteins were also specifically detected in brain samples (molecular mass, 40–50 kD), and these might represent the catalytic domain of the proteolytically cleaved PKC-β. In addition, a commercially available monoclonal antibody to an epitope common to both PKC-α and PKC-β (MCS, Amersham International) failed to react with up to 200-μg samples of heart or cardiac myocyte protein (results not shown). With respect to PKC-γ, our monoclonal antibody reacted with a protein of appropriate molecular mass (80 kD) in brain extracts. No signal was detected in 50 or 100 μg cardiac myocyte or whole heart ventricular protein. These results indicate that PKC-α, -β, and -γ may be expressed at only very low levels in adult rat cardiac myocytes and whole heart ventricular tissue and, as such, confirm the results of a previous immunoblot study of rat heart.35

Expression of Ca**²⁺**-Independent PKC Isotypes in Whole Tissue Extracts

The expression of PKC-δ, -ε, -ζ, and -η in adult rat heart preparations was examined. In agreement with previous studies, PKC-δ was detected as a 74–78-kd protein in brain extracts (100 μg protein loaded) and COS-1 cells overexpressing PKC-δ (Figure 2a). Two protein bands of lower molecular mass (44 and 46 kD), which were competed by the PKC-δ peptide antigen, were also detected in COS-1 cells (Figure 2a). These were presumably catalytic domain fragments of PKC-δ formed during sample processing. No such small molecular mass proteins were present in brain samples. There was no reaction of PKC-δ antisem with whole heart ventricular or cardiac myocyte samples (100 μg protein loaded).

In brain, cardiac myocytes, and whole heart ventricular samples, PKC-ε was detected as a 97-kd protein that was competed by PKC-ε antigen (Figure 2b). The relative abundance of PKC-ε in the heart was very much less than in the brain. Although loading of heart protein for SDS-PAGE was 10-fold greater than brain, the subsequent immunoblot showed a much greater abundance of immunoreactivity in brain (Figure 2b). This result confirms that PKC-ε mRNA detected previously in heart and brain10 is expressed as protein.

PKC-ζ was detected in brain extracts (100 μg protein loaded) and HeLa cells expressing this isotype as a doublet with a prominent band at approximately 75 kD and a fainter band at 79 kD. Both bands were effectively competed by PKC-ζ peptide antigen, although the HeLa cell signal was so great that some immunoreactivity remained. It is possible that this doublet might represent different phosphorylation states of PKC-ζ. In addition, bands of lower molecular mass that were competed by PKC-ζ peptide antigen were detected in brain extracts at 67, 64, 56, and 34 kD and in HeLa cell extracts at 64 and 34 kD. The bands of <75-kD molecular mass presumably represent proteolytically produced fragments. In contrast to brain and HeLa cell extracts, PKC-ζ antisem reacted only weakly with proteins in samples of cardiac myocytes and heart (100 μg protein loaded). Proteins of 81–76-, and 66-kd molecular mass were competed effectively by incubation with PKC-ζ peptide antigen. The most prominent band was detected at 66 kD, which, because of the effectiveness of the competition reaction, is probably a fragment of the PKC-ζ protein. The molecular mass of PKC-ζ was originally reported to be 64 kD. However, more recent studies have provided evidence for an 80-kD PKC-ζ protein.62,63 The consistency with which PKC-ζ fragments with molecular masses of approximately 66 kD are found in the extracts examined here probably reflects the sensitivity of PKC-ζ to proteolytic cleavage.

Finally, expression of PKC-η was examined. In whole heart ventricular extracts, two bands with molecular masses of 81 and 75 kD were detected (50 μg protein loaded), and these were both competed by the PKC-η
antigen peptide (Figure 2d). No bands were detectable in cardiac myocytes or brain extracts (50 μg protein loaded in each case). The molecular mass of PKC-η calculated from the cDNA sequence is 78 kd, but the expressed protein has an apparent molecular mass of 82 kd11 (L. Dekker, P.J. Parker, and P. McIntyre, unpublished observations). The PKC-η doublet observed in whole heart ventricular extracts may represent different phosphorylation states of PKC-η. We conclude that, in whole heart ventricle, PKC-η may be expressed in a nonmyocytic cell type(s) that is absent from brain.

**Immunoblot Analysis of Partially Purified PKC**

To assess whether the inability to detect PKC-α, -β1, -γ, and -δ was related to the use of whole tissue extracts, PKC isotype expression pattern was analyzed in fractions concentrated by ultrafiltration after DEAE Sepharose chromatography of tissue supernatant fractions (Figure 3). Brain was used as a positive control. The specificity of the immunoblotting protocol was established by competition of the antiserum reaction with the appropriate peptide antigen (results not shown for this series of experiments). We were unable to detect any significant levels of expression of PKC-α (Figure 3a) or PKC-β1 (Figure 3b) in spite of being able to establish that this procedure enriched these PKC isotypes in fractions derived from brain. In contrast, PKC-ε was readily detected and enriched in heart and brain fractions (Figure 3c). For PKC-ζ, brain fractions showed a weak signal at 75 kd and a strong signal at 57 kd (Figure 3d). Heart fractions showed a weak signal at 75 kd and a strong signal at 65 kd (Figure 3d). These bands probably correspond to the bands at 75 and 56 kd in whole brain supernatant fractions and 76 and 66 kd in whole heart supernatant fractions (see Figure 2c). For PKC-β1 and PKC-ζ, the differences in appearance between Figures 1, 2, and 3 were related to the time of exposure of the film to the immunoblots, which was much shorter in Figure 3, to demonstrate the effects of partial purification for brain fractions. Longer times of exposure of immunoblots of partially purified PKC did not improve the detectability of PKC-α and PKC-β1 in heart (results not shown). In addition, PKC-γ and PKC-δ could not be detected in the heart fractions, but results are not shown because of difficulties with signal-to-noise ratio in these immunoblots.

In summary, cardiac myocytes express PKC-ε and may express PKC-ζ, although the sensitivity of the latter to proteolysis may present a problem in studies of its regulation in freshly isolated cardiac myocytes or in the heart. Although we could not detect other PKC isotypes using our protocols, we cannot claim to have definitively excluded the possibility that these isotypes are expressed at some level in the adult rat heart.

**Regulation of PKC-ε**

The relatively high level of expression of the holoenzyme form of PKC-ε in adult rat cardiac myocytes allows the regulation of this isotype to be studied specifically in physiologically functional freshly isolated cells. We did not attempt to detect the translocation of other PKC isotypes because of the low levels of detectability in our system. The activation of PKC-ε was studied using a translocation assay followed by immunoblotting. Approximately 5–20% (five observations) of the total

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**Figure 2. Expression of Ca2+-independent protein kinase C (PKC) isotypes in adult rat brain, whole heart ventricle, and cardiac myocytes. Immunoblots show extracts of brain (lane B), cardiac myocytes (lane C), or heart ventricle (lane H) prepared as described in "Materials and Methods," and immunoblot analysis was performed using polyclonal antiserum to PKC-δ at a sample loading of 100 μg protein per lane (panel a), polyclonal antiserum to PKC-ε at a sample loading of 5 μg protein per lane for brain or 50 μg protein per lane for cardiac tissues (panel b), polyclonal antibody to PKC-ζ at a sample loading of 100 μg protein per lane (panel c), or polyclonal antibody to PKC-η at a sample loading of 50 μg protein per lane (panel d). Where lanes are marked with a plus sign, samples of cultured cells overexpressing PKC-δ (COS-1 cells, panel a) or PKC-ζ (HeLa cells, panel c) were included as positive controls. Protein loading was not determined but was that found empirically to be sufficient to give a suitable signal. The left blots show results obtained in the absence of the appropriate competing peptide antigen; the right blots show results in the presence of the antigen (2 μg/ml). Antisera were used at a dilution of 1/1,000. Bound antibody was detected by the enhanced chemiluminescence method (see "Materials and Methods"), and immunoblots were exposed to film for 5 minutes (panels a and b), 1 minute (panel c), or 60 minutes (panel d). The numbers on the right of the figure indicate the positions of the prestained molecular mass standards in kilodaltons.
immunoreactive PKC-\(\epsilon\) is associated with the Triton X-100–soluble cell membrane fraction of freshly isolated cardiac myocytes (results not shown). The remainder is associated with the soluble fraction. Although these findings agree with recent reports that 10–20% of measured PKC activity (histone III-S as substrate) was in the particulate fraction of adult or neonatal cardiac myocytes, it should be noted that the activity of PKC measured in this way may not be attributable to PKC-\(\epsilon\).

The tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), activate PKC and have been used extensively to implicate the involvement of PKC in many cellular functions. On exposure to 1 \(\mu\)M PMA, PKC-\(\epsilon\) in cardiac myocytes underwent a rapid (detectable in <30 seconds) and virtually complete association with the Triton X-100–soluble membrane fraction (Figure 4). Control experiments confirmed that the carryover of the dimethyl sulfoxide solvent (final concentration, 0.1% [vol/vol]) did not affect the subcellular distribution of PKC-\(\epsilon\) (results not shown). The redistribution was sustained during incubation of the cardiac myocytes for 60 minutes in the presence of 1 \(\mu\)M PMA (Figure 4). The sustained redistribution of other PKC isoforms or PKC activity induced by phorbol esters has been previously reported for a number of cell types. However, there was a relative loss in membrane-associated PKC-\(\epsilon\) immunoreactivity after 60 minutes in comparison with the 1- or 5-minute time point. There was no detectable production of proteolytic fragments of PKC-\(\epsilon\) during these incubations. One (but not the only) explanation of these findings is that there may be downregulation of PKC-\(\epsilon\) after exposure of cardiac myocytes to PMA but that any proteolysis leads to the formation of fragments that are not detectable by the protocol used.

The translational of PKC-\(\epsilon\) was dependent on the concentration of PMA. The results of one such experiment in which cardiac myocytes were exposed to PMA for 5 minutes are shown in Figure 5. The combined results of densitometric analyses of three such experiments are shown in Figure 6. The data in Figure 6 were fitted to sigmoid curves. For the composite curve describing the association of PKC-\(\epsilon\) with the membrane fraction, maximal association was 325% of the control value, and the \(EC_{50}\) value was 37 nM (2 degrees of freedom, \(r^2=0.989\)). For the loss of PKC-\(\epsilon\) from the soluble fraction, the minimum amount remaining was 28%, and the \(EC_{50}\) value was 15 nM (2 degrees of

**Figure 3.** Immunoblot analysis of partially purified protein kinase C (PKC) isotypes from rat brain and heart. Brain and heart PKC isotypes were partially purified by DEAE Sepharose chromatography and immunoblotted with specific antisera to PKC-\(\alpha\) (panel a), PKC-\(\beta_1\) (panel b), PKC-\(\epsilon\) (panel c), and PKC-\(\xi\) (panel d) as described in "Materials and Methods." Sample protein loading for the total homogenates (lanes B1 and H1), Triton X-100–soluble supernatant fraction (lanes B2 and H2), and the concentrated DEAE Sepharose column fractions (lanes B3 and H3) was 100 \(\mu\)g except for brain samples in panel c, for which only 5 \(\mu\)g of the brain (lane B1), brain supernatant fraction (lane B2), and brain column fractions (lane B3) was loaded. All antisera were used at a dilution of 1/500 to increase the sensitivity of the detection system. Bound antibody was detected by the enhanced chemiluminescence method, and in all cases the immunoblots were exposed to Hyperfilm for 2 minutes. This exposure time was chosen specifically to demonstrate the enrichment of PKC isoforms in brain and heart samples. Longer exposure times did not improve the detection of PKC-\(\alpha\) and PKC-\(\beta_1\) in the heart samples. PKC-\(\gamma\) and PKC-\(\delta\) remained undetectable in the heart samples (results not shown). The numbers on the right of the figure indicate the positions of the prestained molecular mass standards in kilodaltons. The partial purification protocol was repeated with similar results.

**Figure 4.** Immunoblot analysis of the time course of translocation of protein kinase C-\(\epsilon\) (PKC-\(\epsilon\)) induced by phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes. Freshly isolated adult rat cardiac myocytes were incubated with 1 \(\mu\)M PMA for the times indicated. Cytosol and Triton X-100–soluble membrane fractions were prepared after disruption of the cells by freeze-thawing as described in "Materials and Methods." Cytosolic or solubilized membrane protein (20–30 or 15–25 \(\mu\)g, respectively) derived from an equal number of cells was used in each case, and these loadings were sufficient to obtain autoradiographs suitable for scanning after enhanced chemiluminescence detection (see "Materials and Methods") for 1–5 minutes. Immunoblot analysis was performed using anti–PKC-\(\epsilon\) antiserum at a dilution of 1/1,000. Results are representative of data from three separate experiments.
freedom, \( r^2 = 0.959 \). In view of the problems of quantitation and data analysis of logarithmic plots, the two EC_{50} values agree well.

Translocation of PKC-e in response to high (1 \( \mu \)M) concentrations of other phorbol esters was examined (Table 1). 4\beta-Phorbol 12,13-didecanoate, which activates PKC\(^{12}\) induced changes in the subcellular distribution of PKC-e that were indicative of activation, whereas the isomeric 4\alpha phorbol 12,13-didecanoate, which does not activate PKC\(^{12}\) was without effect. Phorbol 12,13-dibutyrate also caused translocation of PKC-e consistent with its ability to activate the enzyme.\(^{12}\)

Epinephrine stimulates phosphoinositide hydrolysis in adult rat cardiac myocytes through the \( \alpha_1 \)-adrenoceptor.\(^{23}\) Endothelin also stimulates phosphoinositide turnover in these cells.\(^{66}\) Both agents therefore potentially raise DG concentrations and activate PKC. It is possible that the intracellular signaling pathway thus initiated may be important in the development of cardiac hypertrophy (reviewed in Reference 40). Therefore, the activation of PKC-e by these physiological agonists was next examined by either digitonin disruption or freeze-thawing (Table 2). Although both methods of disruption gave qualitatively similar results, we observed that translocation of PKC-e as assessed after digitonin disruption was proportionally greater as compared with freeze-thawing. Both epinephrine and ET-1 caused translocation of PKC-e to the membrane fraction after exposure of the myocytes to these agents for 1 minute. However, epinephrine or ET-1 was markedly less effective than PMA. In contrast to PMA (Figure 4), the

![Figure 5](http://circres.ahajournals.org/)

**FIGURE 5.** Immunoblot analysis of the dependence of protein kinase C-e (PKC-e) translocation on the concentration of phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes. Freshly isolated adult rat cardiac myocytes were treated for 5 minutes with the concentrations of PMA indicated. After disruption of the cells by freeze-thawing, the cytosol and Triton X-100-soluble membrane fractions were prepared as described in “Materials and Methods.” Whole extracts of brain (lane B, 2 \( \mu \)g protein loaded) were included as positive controls. Loading of cardiac myocyte protein and autoradiography were as described in the legend to Figure 4. Immunoblot analysis of each fraction was performed using anti-PKC-e antiserum at a dilution of 1:1,000. Results are representative of data from three separate experiments.

![Figure 6](http://circres.ahajournals.org/)

**FIGURE 6.** Graph showing dependence of the translocation of protein kinase C-e (PKC-e) immunoreactivity on the concentration of phorbol 12-myristate 13-acetate (PMA) in cardiac myocytes. Composite data for three separate experiments carried out as in Figure 5 are shown. Immunodetection of PKC-e in Triton X-100-soluble membrane fractions (■, solid line) and soluble fractions (▲, broken line) was performed as described in “Materials and Methods” and in the legend to Figure 5. Quantitation was by laser densitometry. Results are expressed as percentages of control values from each scan.

### Table 1. Effects of Phorbol Esters on the Subcellular Distribution of Protein Kinase C-e in Cardiac Myocytes

<table>
<thead>
<tr>
<th>Addition</th>
<th>PKC-e immunoreactivity associated with the membrane fraction (percent of control value)</th>
<th>PKC-e immunoreactivity associated with the cytosol fraction (percent of control value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>315±20% ( ^* )</td>
<td>3±3% ( ^* )</td>
</tr>
<tr>
<td>4\beta-PDD</td>
<td>336±80% ( ^\ddagger )</td>
<td>40±8% ( ^\ddagger )</td>
</tr>
<tr>
<td>4\alpha-PDD</td>
<td>124±34%</td>
<td>103±9%</td>
</tr>
<tr>
<td>PDB</td>
<td>195±36%</td>
<td>39±21% ( ^\ddagger )</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>117±29%</td>
<td>92±26%</td>
</tr>
</tbody>
</table>

PKC-e, protein kinase C-e; PMA, phorbol 12-myristate 13-acetate; PDD, phorbol 12,13-didecanoate; PDB, phorbol 12,13-dibutyrate. Values are mean±SEM.

Cardiac myocytes were exposed to the phorbol esters (each at a concentration of 1 \( \mu \)M) for 5 minutes, as described in “Materials and Methods.” The concentration of dimethyl sulfoxide carried over into the incubations was 0.1% (vol/vol). Myocytes were subsequently disrupted by freeze-thawing, and the membrane fraction was extracted with Triton X-100, as described in “Materials and Methods.” There were three independent observations in each group. Statistical significance of the difference from the control result (taken as 100%) was assessed by an unpaired two-tailed \( t \) test.

\( ^* p<0.001, ^\ddagger p<0.05, \) and \( ^\ddagger p<0.01 \) vs. control value.
TABLE 2. Effects of Phorbol 12-Myristate 13-Acetate, Endothelin-1, or Epinephrine on the Subcellular Distribution of Protein Kinase C-e in Cardiac Myocytes

<table>
<thead>
<tr>
<th>Addition</th>
<th>PKC-e immunoreactivity associated with the membrane fraction (percent of control value)</th>
<th>PKC-e immunoreactivity associated with the cytosol fraction (percent of control value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitonin disruption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA (1 μM)</td>
<td>1,160±121%*</td>
<td>4±24%*</td>
</tr>
<tr>
<td>Epinephrine (50 μM)</td>
<td>260±65%†</td>
<td>40±8%*</td>
</tr>
<tr>
<td>Endothelin-1 (100 nM)</td>
<td>285±71%†</td>
<td>25±3%*</td>
</tr>
<tr>
<td>Freeze-thawing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA (1 μM)</td>
<td>541±187%‡</td>
<td>0±0%*</td>
</tr>
<tr>
<td>Epinephrine (50 μM)</td>
<td>247±107%</td>
<td>53±2%*</td>
</tr>
<tr>
<td>Endothelin-1 (100 nM)</td>
<td>172±60%</td>
<td>62±10%‡</td>
</tr>
</tbody>
</table>

PKC-e, protein kinase C-e; PMA, phorbol 12-myristate 13-acetate. Values are mean±SEM.

Cardiac myocytes were exposed to PMA, endothelin-1, or epinephrine for 1 minute, as described in "Materials and Methods." They were then disrupted by lysis in buffer containing 0.05% (vol/vol) digitonin or by freeze-thawing. The membrane fraction was extracted with Triton X-100, as described in "Materials and Methods." There were four or five independent observations in each group for digitonin disruption. Three of these cell preparations were also analyzed after disruption by freeze-thawing. Statistical significance of the difference from the control result (taken as 100%) was assessed by an unpaired two-tailed t test.

* p<0.001 and †p<0.05 for each separate method of disruption.‡p<0.05 for comparison of the methods of disruption.

translocation of PKC-e in response to epinephrine or ET-1 was not sustained. After 5 minutes, the pattern of PKC-e distribution reverted to that of the control cells (results not shown). These results are consistent with the findings of Henrich and Simpson, who showed that norepinephrine was markedly less effective in comparison with PMA in causing translocation of PKC activity in cultured neonatal rat cardiac myocytes and that translocation in response to norepinephrine was not sustained. These results suggest a potential role for epinephrine and ET-1 in the regulation of PKC-e activity in cardiac myocytes in vivo.

Discussion

The different isotypes constituting the PKC family have been mainly identified by molecular cloning techniques. The availability of CDNA probes has led to the investigation of the tissue distribution of these isotypes by Northern blot analysis, with the apparent assumption that the relative abundance of the mRNA for a particular PKC isotype provides an indication of the relative abundance of the protein expressed in each tissue. In addition, many studies, including the early studies on the expression of the PKC-α, -β, and -γ isotypes, have concentrated on the brain. Mizuno et al. concluded that the PKC-δ mRNA was ubiquitously expressed in all mouse tissues examined but that expression was significantly less in the heart than in many other tissues (brain, skin, lung, kidney, or intestine). Schaap et al. reported that mRNA for PKC-e was expressed in a variety of rat tissues, with relative abundance being highest in the brain and next highest in the lung and heart. The expression of PKC-ζ mRNA was demonstrated in the brain, kidney, and lung but has not been examined in the heart. Most recently, PKC-η mRNA expression has been reported in the lung, skin, and whole heart, and our results (Figure 2d) agree with this finding, even though the isotype is probably expressed in a nonmyocytic cell type. (It should be noted that PKC-η is the rodent homologue of the human PKC-L.) In contrast to other members of the PKC family, PKC-η is only weakly expressed in the brain.

Although we cannot accurately quantitate the relative amounts of the PKC isotypes present in adult rat cardiac myocytes by immunoblotting, we have presented evidence that PKC-e is a major isotype of PKC expressed in these cells (Figure 2b). It is possible that there is also significant expression of PKC-ζ (Figure 2c). The other PKC isotypes may be expressed in the cardiac myocyte but are below the levels of detection using our protocols. In addition, there is no certainty that all members of the PKC family have so far been identified. PKC-e has not been knowingly purified from or studied in the heart. This may, in part, result from the use of activity assays with histone substrate during the PKC purification protocols. The model substrate most frequently used (histone III-S) is poorly phosphorylated by PKC-e, and the presence of the Ca²⁺-independent PKC-e isotype emphasizes the importance of the activation of phospholipid hydrolysis and the production of DG (as opposed to regulation in conjunction with changes in Ca²⁺ concentrations) in the activation of PKC in the heart.

We were unable to detect PKC-α, -β, or -γ in extracts of isolated cardiac myocytes or whole heart ventricle (Figure 1). In contrast, Kosaka et al have reported the presence of a PKC species containing PKC-α isotype sequence in the rat heart. These researchers first separated PKC activities (histone H₁ as substrate) by hydroxyapatite chromatography. Most (approximately 90%) of the heart PKC activity eluted as a peak (with a shoulder) at a potassium phosphate concentration of approximately 0.15 M. This species (peak III in the nomenclature used in Reference 37) was cross-reacted with an antibody that was raised against residues 4–19 in the V₁ region of the PKC-α sequence. A minor peak of activity eluting at 0.09 M potassium phosphate (peak II in the nomenclature used in Reference 37) did not cross-react with antibodies raised against PKC-α, -β, or -γ sequences. The possibility that peak II might contain the Ca²⁺-independent PKC isotypes PKC-δ, -ε, or -ζ (or even the not-yet-identified PKC-η) was raised. The presence of the Ca²⁺-independent isotypes is only clearly demonstrable by an immunological approach or by the use of isotype-specific model substrates (were these available).

In contrast to the results of Kosaka et al, a more recent study reported that three peaks of PKC activity (histone III-S as substrate) could be resolved from the soluble fraction of adult rat heart tissue by hydroxyapatite chromatography. These eluted at approximately 0.06, 0.10, and 0.13 M potassium phosphate. These were considered to correspond to peak I (the minor peak of activity), peak II (the major peak of activity), and peak III (the intermediate peak of activity) in the nomenclature described in Reference 37. On the basis of their elution after hydroxyapatite chromatography and be-
cause peaks II and III cross-reacted with monoclonal antibody MC5 (Amersham International), which recognizes a sequence common to PKC-α and PKC-β, peak I was identified as PKC-γ, peak II as PKC-β, and peak III as PKC-α. Our failure to detect any PKC-α, -β, or -γ immunoreactivity in heart or cardiac myocyte samples with the isotype-specific antisera or with the MC5 antibody could suggest a requirement for partial purification and concentration of PKC from these sources before detection is possible. However, as Figure 3 shows, this is not the case. The relatively low abundance of PKC-α, -β, and -γ isotypes in the heart is also suggested by the finding that the PKC-specific activity in the supernatant fraction of a crude heart homogenate is approximately 5 pmol of phosphate transferred per minute per milligram protein with histone III-S as substrate. In comparison, the specific activity of PKC in supernatant fractions from brain is 14,300 pmol of phosphate transferred per minute per milligram protein.44

In related studies, PKC-α and PKC-β have been detected in partially purified extracts of cultured neonatal rat cardiac myocytes.38 PKC-γ was not detected.38 In addition, a non-PKC-α/non-PKC-β PKC of 70-kd molecular mass was detected38 by a monoclonal antibody (CK1.4). On the basis of their differing molecular masses, the PKC identified by CK1.4 cannot be PKC-ε. The relevance of the situation in cultured neonatal cardiac myocytes to the adult situation is not clear at this stage. It is entirely possible that transitions in PKC isotype expression may occur in the heart during maturation. Indeed, our recent studies (M.A. Bogoyevitch, P.J. Parker, and P.H. Sudgen, unpublished observations) have revealed detectable levels of expression of PKC-α in heart ventricles of 1–3-day-old rats. The levels of expression of PKC-α decline during maturation. In addition, we have detected expression of PKC-α in the atria of adult rat hearts (M.A. Bogoyevitch, P.J. Parker, and P.H. Sudgen, unpublished observations).

In addition to their effects on contractility, tumor-promoting phorbol esters, α1-adrenergic stimulation, and endothelin are hypertrophic in cultured neonatal cardiac myocytes (reviewed recently in Reference 40). α1-Adrenergic stimulation and endothelin also stimulate phosphoinositide hydrolysis33,24,26–28,32 in cardiac myocytes, thereby raising DG concentrations.32,77 Transfection of cultured neonatal rat cardiomyocytes with DNA encoding constitutively active forms of PKC-βII or either PKC-α or PKC-βIII induced changes in gene expression typical of the hypertrophic response. The implication is that PKC may play a role in the regulation of cardiac myocyte growth. Therefore, it was of interest to examine the regulation of cardiac myocyte PKC-ε, i.e., a major PKC isotype present in these cells. The tumor-promoting phorbol esters, for which PKC is the only well-characterized cellular receptor, have been extremely useful in delineating the involvement of PKC in cellular responses. The PKC family of enzymes is either soluble or only loosely associated with intracellular membranes in their inactive state. On activation by DG or suitable phorbol esters, PKC becomes more tightly associated with the membrane fraction.70,71 We have demonstrated that this response is intact for PKC-ε in cardiac myocytes (Figures 4–6, Table 1). However, there have been conflicting reports on the effects of phorbol esters on the activation of PKC-ε in other cells. In Chinese hamster ovary cells overexpressing PKC-ε, the enzyme was mostly particulate, would not translocate on exposure to 0.02 μM PMA, and was resistant to downregulation.61 Furthermore, Heidenreich et al.72 were unable to detect any effects of 0.1 μM PMA on the subcellular distribution or total concentration of PKC-ε in cultured neurons. In contrast, 0.2 μM phorbol 12,13-dibutyrate caused a rapid redistribution of PKC-ε in GH4C1 rat pituitary cells and subsequent downregulation of the enzyme within 1 hour.73 Similar studies indicate that PKC-ε translocates and downregulates in Swiss 3T3 cells in response to tumor-promoting phorbol esters.74 Thus, we do not consider it likely that PKC-ε is abnormally regulated in cardiac myocytes.

To the best of our knowledge, no attempts have been made to detect activation of cardiac myocyte PKC in a translocation assay by an immunoblotting protocol, although Mochly-Rosen et al.38 have investigated intracellular redistribution of PKC isotypes after exposure of cultured neonatal rat cardiac myocytes to PMA or norepinephrine by immunocytochemistry. Other groups13,14,17,19,34 have demonstrated a translocation of total PKC activity to the membrane fraction after exposure of freshly isolated adult cardiac myocytes or cultured neonatal cardiac myocytes to appropriate phorbol esters. The EC50 for this effect was approximately 30 nM, which agrees with the values that we obtained in this study.14 A lesser degree of translocation was also seen on exposure of these cells to high concentrations (10–100 μM) of 1,2-dioctanoyl-sn-glycerol,13 Norepinephrine24 or phenylephrine33,34 cause small increases in the association of total PKC activity with the cardiac membrane fraction. We are not aware of any direct demonstration of PKC activation by ET-1 in cardiac ventricular myocytes, although this has been demonstrated in smooth muscle preparations.64,75 Phorbol esters, α1-adrenergic agonists, and ET-1 induce responses typical of hypertrophy in adult and neonatal cardiac myocytes. Our general conclusion from the translocation studies is that a common mechanism of action exists for these agents through the activation of a major isotype of PKC in the heart, PKC-ε. The finding that epinephrine and ET-1 each induce PKC-ε translocation indicates that this PKC isotype may be involved in the coupling of the actions of these agents to the cellular responses in cardiac myocytes.

References


Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominao M, Kuroda T, Nishizuka Y: Limited proteolysis of pro-
Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin.

M A Bogoyevitch, P J Parker and P H Sugden

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