Role of protein kinase C-ε in hypertrophy of cultured neonatal rat ventricular myocytes

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Role of protein kinase C-ε in hypertrophy of cultured neonatal rat ventricular myocytes. Am J Physiol Heart Circ Physiol 280: H756–H766, 2001.—Using adenovirus (Adv)-mediated overexpression of constitutively active (ca) and dominant-negative (dn) mutants, we examined whether protein kinase C (PKC)-ε, the major novel PKC isoenzyme expressed in the adult heart, was necessary and/or sufficient to induce specific aspects of the hypertrophic phenotype in low-density, neonatal rat ventricular myocytes (NRVM) in serum-free culture. Adv-caPKC-ε did not increase cell surface area or the total protein-to-DNA ratio. However, cell shape was markedly affected, as evidenced by a 67% increase in the cell length-to-width ratio and a 17% increase in the perimeter-to-area ratio. Adv-caPKC-ε also increased atrial natriuretic factor (ANF) and β-myosin heavy chain (MHC) mRNA levels 2.5 ± 0.3- and 2.1 ± 0.2-fold, respectively, compared with NRVM infected with an empty, parent vector (P < 0.05 for both). Conversely, Adv-dnPKC-ε did not block endothelin-induced increases in cell surface area, the total protein-to-DNA ratio, or upregulation of β-MHC and ANF gene expression. However, the dominant-negative inhibitor markedly suppressed endothelin-induced extracellular signal-regulated kinase (ERK) 1/2 activation. Taken together, these results indicate that caPKC-ε overexpression alters cell geometry, producing cellular elongation and remodeling without a significant, overall increase in cell surface area or total protein accumulation. Furthermore, PKC-ε activation and downstream signaling via the ERK cascade may not be necessary for cell growth, protein accumulation, and gene expression changes induced by endothelin.

effectors and inhibitors of cell signaling; heart development; heart; myocardial hypertrophy; signaling; signal transduction; adenovirus

HYPERTROPHIC GROWTH is a common response of the myocardium to ischemic injury, hypertension, or valvular and congenital heart disease. Although the chronically increased wall stress that accompanies these disorders results in an initially beneficial adaptation to maintain normal cardiac function, the development of left ventricular hypertrophy (LVH) poses an independent risk factor for subsequent cardiovascular morbidity and mortality (3). Hypertrophic cardiac growth is characterized by the activation of various cell signaling kinases, the induction of immediate early gene expression, the reexpression of a fetal gene program of secondary response genes, and the ultimate development of increased cardiomyocyte cell volume and protein accumulation in the absence of cell division. Despite a wealth of experimental and clinical data regarding the development and progression of cardiac hypertrophy, the intracellular mechanisms whereby cardiomyocytes sense the increased hemodynamic load and convert mechanical stimuli into growth-promoting biochemical processes are only now being elucidated.

Investigators interested in the signal transduction cascades responsible for the induction of cardiomyocyte hypertrophy have made extensive use of cultured neonatal rat ventricular myocytes (NRVM). These cells display many characteristics of the hypertrophic phenotype when subjected to either neurohormonal or mechanical stimuli that induce cardiomyocyte hypertrophy in vivo (42). A common feature of many of these hypertrophic stimuli is the activation of one or more of the isoenzymes of protein kinase C (PKC). PKCs are a family of phosholipid-dependent, serine-threonine kinases that are divided into three subfamilies (conventional, novel, and atypical) based on their activation requirements for Ca2+ and diacylglycerol and their sensitivity to phorbol esters. The PKC family includes at least twelve members. NRVM express only PKC-α, PKC-δ, PKC-ζ, and PKC-ɛ (35), which may be differentially regulated and have specific functions in the cardiomyocyte (31). This specificity is likely due to their differential activation by hypertrophic stimuli (5, 13, 31) and their differential localization within the cell (16). Nevertheless, the role of each isoenzyme in the induction of specific aspects of the hypertrophic phenotype remains unknown.

PKC-ɛ is one of the three phorbol-ester-sensitive PKC isoenzymes found in NRVM and is the most abundant novel (i.e., Ca2+-insensitive) PKC isoenzyme found in adult rat cardiac myocytes (5). Previous studies investigating the importance of PKC-ɛ in cardiomyo-
oocyte hypertrophy have demonstrated that, of the ma-
minor PKC isoenzymes expressed in rat cardiomyocytes,
only PKC-ε translocates in response to acute pressure
overload (27). Additionally, Clerk et al. (13) and Jiang
et al. (24) have suggested that PKC-ε is an upstream
regulator of the Ras-Raf-mitogen/extracellular signal-
regulated kinase (MEK)-extracellular signal-regulated
kinase (ERK) signaling cascade. This signal transduc-
tion pathway has been implicated in mediating both
cardiomyocyte gene expression changes and cytoskel-
etal alterations in response to hypertrophic agonists (1,
10, 20, 21, 29, 44, 45). On the basis of these previous
reports, the objective of the present study was to utilize
adenoviral vectors expressing constitutively active and
dominant-negative mutants of PKC-ε to critically ana-
yze whether this signaling kinase is necessary and/or
sufficient for the induction of specific aspects of the
cardiomyocyte hypertrophic phenotype.

METHODS

Reagents. PC-1 tissue culture medium was obtained from
BioWhittaker (Walkersville, MD). DMEM was obtained from
GIBCO-BRL (Grand Island, NY). Medium 199, Ca2+-free and
Mg2+-free Hanks’ balanced salts (HBSS; modified), acid-
soluble calf skin collagen, and antibacterial/antimycotic solution
were obtained from Sigma Chemical (St. Louis, MO). Per-
manox chamber slides were obtained from Nunc (Naperville,
IL). [32P]ATP and [32P]dCTP were purchased from Amer-
sham (Arlington Heights, IL). Monoclonal antibodies to
PKC-ε and paxillin were obtained from Signal Transduction
Laboratories (Lexington, KY). Rabbit polyclonal antibodies to
ERK1 and ERK2 were obtained from Santa Cruz Biotechnol-
gy (Santa Cruz, CA). Rabbit polyclonal antibodies to the
phosphorylated forms of ERKs were obtained from Promega
(Madison, WI). Goat anti-mouse rhodamine-conjugated IgG
and FITC-phalloidin were obtained from Molecular Probes
(Eugene, OR). Horseradish peroxidase-conjugated goat anti-
rabbit and goat anti-mouse IgGs were from Bio-Rad (Her-
cules, CA). PKC-ε specific substrate peptide (Gl-His-Arg-Met-
Arg-Pro-Arg-Lys-Gly-Ser-Val-Arg-Arg-Arg-Val) was
obtained from Biomed (Plymouth Meeting, MA). All other
reagents were of the highest grade commercially available
and were obtained from Sigma and Baxter (McGaw Park, IL).

Adenoviral constructs. Constitutively active (ca) PKC-ε
adenoavirus (Adv) was constructed by first subcloning
capPKC-ε cDNA (kindly provided by Drs. Peter Parker and
Peter Sugden, Imperial College of Science Technology and
Medicine, Cambridge, UK) into a pAC-CMV-pLpA-SR (SR)
plasmid. The enzyme was made constitutively active by de-
letion of residues 154–163 of its inhibitory pseudosubstrate
domain (47). The subcloned construct was then cotransfected
into HEK-293 cells. After homologous recombination, the
 deletion of residues 154–163 of its inhibitory pseudosubstrate
domain [amino acid 159 (A to E)], thereby destroying the construct’s kinase activity but main-
taining the enzyme in an active conformation. The double-
mutant PKC-ε was then used in the generation of a replica-
tion-defective Adv as described above. Adv were amplified
and purified using HEK-293 cells, as previously described (18). Viral titers were estimated by absorbance at 260 nm

Ventricular dissociation and cardiac myocyte isolation. An-
imals used in these experiments were handled in accordance
with the Guiding Principles in the Care and Use of Animals,
approved by the Council of the American Physiological Soci-
ety. Ventricular myocytes were isolated from the hearts of
2-day-old Sprague-Dawley rats by collagenase digestion, as
previously described (36). Released cells were collected by
centrifugation, resuspended in PC-1 medium, plated at a
density of 400 cells/mm² on collagen-coated tissue culture
dishes or chamber slides, and left undisturbed in a 5% CO₂
incubator for 14–18 h. Unattached cells were removed by
aspiration and washed two times in HBSS, and the attached
cells were maintained in a solution of DMEM medium 199
(4:1) containing antibiotic/antimycotic solution. Thereafter,
media were changed daily. Cardiomyocytes were infected (60
min, 25°C with gentle agitation) with various concentrations
of replication-defective Adv diluted in DMEM medium 199.
Medium was then replaced with virus-free DMEM medium
199, and the cells were cultured for an additional 48–72 h.
Under these cell culture conditions, cardiomyocytes display
little or no spontaneous intracellular Ca²⁺ concentration
[(Ca²⁺)₅] transients or beating activity (18, 19).

Surface area and cell shape analysis. Cardiomyocytes were
loaded with 2',7'-bis(2-carboxethyl)-5(6)-carboxyfluorescein
(BCECF)-AM (2 µM in a modified Krebs medium in (mM): 135
NaCl, 5.9 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 11.5 glucose, and 11.6
HEPES, pH 7.3) supplemented with 0.1% BSA and 0.02%
Pluronic F-127 detergent) for 1 h followed by incubation in
BCECF-free Krebs buffer for 1 h. Cells were then viewed
using a Zeiss model LSM 410 laser scanning confocal micro-
scope. Optical sections through the bottom of the cells (~10
cells/field) were then stored, and the digital images were
subjected to image analysis using the Image-1 Software
Package (Universal Imaging, West Chester, PA). A binary
mask was created by setting a threshold brightness that
distinguished the fluorescent cells from the black back-
ground. The area of each cell was then determined as an
exact count of the number of pixels that make up the object’s
binary mask multiplied by the area of a unit pixel. Cell
perimeter was measured as the length of the outline of the
object’s binary mask. The shape factor classified objects
based on the extent of their roundness, which was derived
from the measured perimeter and area of the object’s binary
mask, and was calculated according to the following formula

\[ \text{shape factor} = \left( \frac{4\pi a}{p^2} \right) \]

where \( p \) is the perimeter in \( \mu m \), and \( a \) is the area in \( \mu m^2 \).

Immunofluorescence. Cells grown on Permanox chamber
slides were fixed (10 min, room temperature) with 2% (wt/vol)
paraformaldehyde in sodium PBS, washed (15 min) in 1%
(wt/vol) glycine in PBS, and permeabilized (15 min) with
0.5% (vol/vol) Triton X-100 in PBS. Samples were first incu-
ated in blocking solution (PBS + 0.1% Triton X-100 + 1%
goat serum) for 1 h. Myocytes were then stained with anti-
paxillin monoclonal antibody (1:1,000 in blocking solution)
for 1 h and then were stained with a rhodamine-conjugated,
donkey anti-rabbit secondary antibody (1:30 in blocking
solution) for 1 h. Cells were also stained with FITC-conjugated
phalloidin (1:40 in PBS) for 1 h to visualize F-actin filaments
and myofibrillar structure. The stained cells were viewed

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using a Zeiss model 510 laser scanning confocal microscope. Multiple optical sections (~1 μM thick were taken of each sample to eliminate out-of-focus fluorescence of the intensely stained myocytes.

**Cellular composition.** For the quantitative analysis of total cellular protein and DNA content, 0.2 N perchloric acid (1 ml) was added, and the cells were then scraped from the dishes and collected by centrifugation (10,000 g, 10 min). The precipitate was redissolved by incubation (60°C, 20 min) in 250 μl of 0.3 N KOH. Aliquots were then used for analysis of total protein by the Lowry method using crystalline human serum albumin as standard and for DNA using 33258 Hoechst dye and salmon sperm DNA as standard, as previously described (36). Data were the means of duplicate wells for each cell isolation and were expressed as microgram total protein per microgram DNA.

**mRNA analysis.** Total cellular RNA was isolated by the method of Chomczynski and Sacchi (11). RNA was quantified by absorbance at 260 nm, and its integrity was determined by examining the 28S and 18S rRNA bands in ethidium bromide-stained agarose gels. Rat a-myosin heavy chain (MHC), β-MHC, atrial natriuretic factor (ANF), and sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 2 mRNAs and rat 18S rRNA were then quantitatively analyzed by Northern blotting and scintillation spectroscopy (Instant Ímager, Hewlett-Packard), as previously described (8).

**Subcellular fractionation.** Subcellular fractionation was performed as previously described (41) with minor modifications. Briefly, NRVM grown on 35-mm dishes were washed one time with PBS, and 200 μl of homogenization buffer (2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 500 μM sodium orthovandate, 1 mM Pefabloc, and 20 mM Tris-HCl, pH 7.5) were added. Cells were then frozen in a dry ice-methanol bath, thawed on ice, and scraped in a plastic tube, and sonicated. The cell homogenate was then centrifuged (100,000 g, 60 min, 4°C), and the supernatant fraction (representing the cytosolic fraction) was stored at -80°C. The pellet was resuspended by sonication in 200 μl of extraction buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 5 mM β-mercaptoethanol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefabloc, and 0.5% Triton X-100). After centrifugation, the supernate [representing the membrane-bound (P₁) fraction] was stored at -80°C.

**PKC activity assay.** PKC-ε enzyme activity was measured in total cell extracts and subcellular fractions as follows. For total cell extracts, NRVM were scraped in 200 μl of extraction buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 5 mM β-mercaptoethanol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Pefabloc, and 0.5% Triton X-100), lysed via sonication, and centrifuged (16,000 g, 30 min). Subcellular fractions were prepared as described above. The reaction mixture consisted of 25 μg lysate protein in 20 μl of PKC assay buffer [50 mM Tris-HCl, pH 7.4, 250 μg/ml BSA, 1 mM EGTA, 80 μg/ml phosphatidyethanolamine, 20 μg/ml phosphatidyleserine, 7.5 mM magnesium acetate, 200 mM phosphol 12-myristate 13-acetate (PMA), and 10 μM PKC-ε specific substrate peptide]. The reaction was begun by the addition of 25 μM ATP (containing 0.5 μCi/assay \(^{32}P\)/ATP) and incubation at 30°C for 10 min. Next, 25 μl of the reaction mixture were spotted onto a P81 phosphocellulose filter and air-dried. Filters were washed three times for 10 min each with 0.5% phosphoric acid and then one time with acetone. \(^{32}P\) radioactivity was measured using a scintillation counter.

**Mitogen-activated protein kinase and PKC Western blotting.** NRVM were washed one time with PBS, and 300 μl of lysis buffer (50 mM sodium pyrophosphate, 50 μM NaF, 50 μM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM sodium orthovandate, 10 μg leupeptin/ml, 10 μg aprotinin/ml, 1 mM Pefabloc, 0.01% Triton X-100, and 10 mM HEPES, pH 7.4) were added. Cells were then frozen on a dry ice-methanol bath, thawed on ice, and scraped in a plastic tube. Samples were sonicated one time and centrifuged (14,000 g, 30 min), and the supernatant fractions were stored at -80°C. ERK1/2 activation was assessed by separating equal amounts of cellular protein (20–50 μg, as determined by the bicinchoninic acid-Bradford method) by SDS-PAGE and Western blotting with polyclonal antibodies specific for the phosphorylated forms of ERK1/2. Primary antibody binding was visualized using enhanced chemiluminescence and was quantified by laser densitometry. In some experiments, ERK1/2 activation and abundance were assessed by gel-shift analysis, as previously described (18, 41). PKC abundance was assessed using similar SDS-PAGE and Western blotting techniques, except that cells were scraped in a modified lysis buffer (150 mM NaCl, 10% glycerol, 1.5 mM MgCl\(_2\), 1 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 500 μM sodium orthovandate, and 1 mM Pefabloc, pH 7.5), and nitrocellulose membranes were probed with PKC-ε-specific monoclonal antibodies.**

**Data analysis.** Results were expressed as means ± SE. Normality was assessed using the Kolmogorov-Smirnov test, and homogeneity of variance was assessed using Levene’s test. Data were compared by one-way blocked ANOVA on Ranks followed by the Student-Newman-Keuls test, paired or unpaired t-tests, or Mann-Whitney Rank Sum or Wilcoxon Signed Rank tests, where appropriate. Data were analyzed using the SigmaStat Statistical Software Package (Jandel Scientific, San Rafael, CA).

**RESULTS**

Overexpression of caPKC-ε alters NRVM morphology but does not induce NRVM hypertrophy. To determine whether PKC-ε overexpression was sufficient to induce hypertrophic growth, we overexpressed PKC-ε, which was made constitutively active by deletion of its pseudosubstrate domain (47). Overexpression was achieved by use of an adenoviral vector (Adv-caPKC-ε) that was generated in our laboratories. Infection of NRVM with this Adv resulted in a large, dose-dependent increase in PKC-ε immunoreactivity, as shown in the representative immunoblot in Fig. 1A. Subcellular fractionation of Adv-caPKC-ε-infected cells indicated that the majority of the constitutively active enzyme was in a Triton X-100 soluble membrane fraction under basal conditions (data not shown). PKC enzyme activity was measured in total cell extracts using a PKC-ε-specific substrate peptide (Fig. 1B). Based on the near-maximal level of PKC-ε activity in cells infected with 100 viral particles/cell, this amount of Adv-caPKC-ε was used in all subsequent experiments.

We have previously shown that agonists that activate PKC isoenzymes, such as phenylephrine (PE), endothelin-1 (ET), and PMA, increase NRVM cell surface area and total protein content (18, 32, 36, 37, 41). As shown in Table 1, however, overexpression of caPKC-ε alone did not increase overall cell surface area, nor did it increase total protein content or the total protein-to-DNA ratio. In fact, total protein/DNA
Western blotting with a monoclonal antibody specific for PKC-ε.

By 32P incorporation using a PKC-ε-specific peptide substrate. cpm, Counts/min.

Counts/min. PKC-ε enzyme activity in whole cell extracts was analyzed using a PKC-ε-specific peptide substrate.

Overexpression of constitutively active (ca) protein kinase C (PKC-ε) in neonatal rat ventricular myocytes (NRVM). A: NRVM were maintained under control conditions (Uninfected) or were infected with 10–1,000 viral particles/cell (ppc) of replication-defective adenovirus (Adv) encoding caPKC-ε (Adv-caPKC-ε) or a control adenovirus containing no insert (Adv-SR). After 48 h, cells were harvested for analysis of immunoreactive PKC-ε by SDS-PAGE and Western blotting with a monoclonal antibody specific for PKC-ε. B: NRVM were infected with 10–1,000 ppc of replication-defective adenovirus encoding bacterial β-galactosidase (Adv-βgal) or Adv-caPKC-ε. PKC-ε enzyme activity in whole cell extracts was analyzed by 32P incorporation using a PKC-ε-specific peptide substrate. cpm, Counts/min.

Fig. 1. Overexpression of constitutively active (ca) protein kinase C (PKC-ε) in neonatal rat ventricular myocytes (NRVM). A: NRVM were maintained under control conditions (Uninfected) or were infected with 10–1,000 viral particles/cell (ppc) of replication-defective adenovirus (Adv) encoding caPKC-ε (Adv-caPKC-ε) or a control adenovirus containing no insert (Adv-SR). After 48 h, cells were harvested for analysis of immunoreactive PKC-ε by SDS-PAGE and Western blotting with a monoclonal antibody specific for PKC-ε. B: NRVM were infected with 10–1,000 ppc of replication-defective adenovirus encoding bacterial β-galactosidase (Adv-βgal) or Adv-caPKC-ε. PKC-ε enzyme activity in whole cell extracts was analyzed by 32P incorporation using a PKC-ε-specific peptide substrate. cpm, Counts/min.

Table 1. NRVM infected with Adv-caPKC-ε showed a 67% increase in the cell length-to-width ratio and a 17% increase in the perimeter-to-area ratio. Quantification of the shape factor, an index of cell roundness, similarly revealed that cells overexpressing caPKC-ε were significantly less round compared with NRVM infected with Adv-SR.

Overexpression of caPKC-ε alters ANF and MHC mRNA levels. Previous studies have shown that transient transfection of constitutively active mutants of PKC-α, PKC-β, PKC-ε, and PKC-ζ all transactivated ANF promoter activity in NRVM (15, 39), whereas constitutively active mutants of PKC-α and PKC-β transactivated β-MHC promoter activity, albeit to different extents (25). All of these studies employed promoter-reporter gene constructs in low-efficiency, transient transfection assays to analyze transcriptional induction of these marker genes. Therefore, as a further check on the effects of caPKC-ε overexpression on the induction of the hypertrophic phenotype, we examined steady-state mRNA levels of α-MHC, β-MHC, ANF, and SERCA2 48 h after viral infection. As shown in Fig. 3, Adv-caPKC-ε increased ANF and β-MHC mRNA levels 2.5 ± 0.3- and 2.1 ± 0.2-fold, respectively, compared with Adv-SR-infected NRVM (P < 0.05 for both). In contrast, α-MHC mRNA levels were significantly reduced by 43 ± 8% compared with Adv-SR-infected cells (P < 0.05), but SERCA2 mRNA levels were unaffected (1.2 ± 0.1-fold change; P > 0.5).

Table 1. Overexpression of caPKC-ε causes alterations in cell shape

<table>
<thead>
<tr>
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<th>Adv-SR</th>
<th>Adv-caPKC-ε</th>
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<tbody>
<tr>
<td>Total protein, µg/dish</td>
<td>24 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>DNA, µg/dish</td>
<td>0.58 ± 0.07</td>
<td>0.67 ± 0.09*</td>
</tr>
<tr>
<td>Total protein-to-DNA ratio, µg/µg</td>
<td>45 ± 5</td>
<td>40 ± 5*</td>
</tr>
<tr>
<td>Surface area, µm²</td>
<td>819 ± 48</td>
<td>754 ± 38</td>
</tr>
<tr>
<td>Length-to-width ratio, µm/µm</td>
<td>2.97 ± 0.27</td>
<td>4.95 ± 0.48*</td>
</tr>
<tr>
<td>Perimeter-to-area ratio, µm⁻¹</td>
<td>0.21 ± 0.01</td>
<td>0.25 ± 0.01*</td>
</tr>
<tr>
<td>Shape factor</td>
<td>0.47 ± 0.02</td>
<td>0.38 ± 0.02*</td>
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Data are means ± SE for 200–250 cells in each group, obtained from 3 different cell experiments. Low-density neonatal rat ventricular myocytes (NRVM) were cultured in serum-free medium and infected (1 h) with 100 viral particles/cell (ppc) of replication-defective adenovirus (Adv) encoding constitutively active (ca) protein kinase C (PKC-ε) or a control adenovirus containing no insert (Adv-SR). Virus-containing medium was replaced, and cells were maintained in serum-free medium for an additional 48 h. Total protein, DNA content, and total protein-to-DNA ratio was measured in cell extracts obtained from 6 different cell isolations. Similarly infected cells were also loaded with 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein and visualized by laser scanning confocal microscopy. Digital fluorescent images were subjected to image analysis. Changes in cell shape were quantified as changes in length-to-width ratio, perimeter-to-area ratio, and shape factor. *P < 0.05 by paired t-test, or Wilcoxon signed rank test.
Characterization of Adv-dnPKC-ε. Next, we made use of an adenoviral vector encoding a dominant-negative mutant of PKC-ε (Adv-dnPKC-ε; see Ref. 30). This mutant form of PKC-ε was generated by creating point mutations in both the ATP-binding site and the pseudosubstrate domain. The mutations rendered the enzyme catalytically inactive while maintaining it in an active conformation, thereby enabling the mutated enzyme to localize to appropriate intracellular sites.

As seen in Fig. 4A, infection of NRVM with Adv-dnPKC-ε resulted in a dose-dependent increase in PKC-ε expression, as measured by Western blotting of extractable total cell protein with monoclonal antibodies that recognized both endogenous and adenovirally expressed dnPKC-ε. Viral concentrations in the range of 250–750 particles/cell produced high levels of transgene expression. Subcellular fractionation revealed that the majority of the immunoreactive PKC-ε was found in a Triton X-100 soluble membrane fraction of Adv-dnPKC-ε overexpressing cells under basal conditions (Fig. 4B). In contrast, cells infected with Adv-SR showed that the majority of the endogenous enzyme was present in the cytosolic fraction. Stimulation of Adv-SR-infected cells revealed the typical translocation of endogenous PKC-ε from the cytosolic to the membrane (P1) fraction within 10 min of ET treatment. In contrast, there was no detectable increase in membrane-bound, immunoreactive PKC-ε in ET-treated, Adv-dnPKC-ε overexpressing cells. These results are consistent with the notion that overexpression of dnPKC-ε blocked translocation of the wild-type, endogenous PKC-ε. However, translocation of the endogenous, wild-type PKC-ε may have been masked by the abundant overexpression of “exogenous” dnPKC-ε transgene. Therefore, endogenous PKC-ε activity was measured in the P1 fraction of Adv-SR- and Adv-dnPKC-ε-infected cells (Fig. 4C). Membrane extracts from cells infected with Adv-SR showed a statistically significant 3.5 ± 0.9-fold increase in PKC-ε activity in response to ET treatment for 10 min. Adv-dnPKC-ε had no significant effect on basal PKC-ε activity compared with Adv-SR-infected cells. However, there was no ET-induced increase in PKC-ε activity in the membrane fraction of cells infected with Adv-dnPKC-ε. There was also no significant difference in protein content of the membrane fractions of the two groups of cells (200 ± 14 vs. 223 ± 25 μg protein in the P1 fraction of Adv-SR- and Adv-dnPKC-ε-infected cells, respectively; P = 0.45). Thus overexpression of dnPKC-ε did not falsely “dilute” the active enzyme in the activity assay. Taken together, these data indicate that overexpression of high levels of dnPKC-ε indeed interfered with the translocation and activation of endogenous, wild-type PKC-ε.

Activation of PKC-ε is not necessary for ET-induced NRVM hypertrophy. We next examined whether PKC-ε activation was necessary for the induction of cellular hypertrophy in response to ET. In these experiments, we compared the effects of ET treatment (100 nM, 48 h) on cell surface area and total protein/DNA ratio in uninfected cells, cells infected with the control Adv-SR, and NRVM infected with Adv-dnPKC-ε. In the case of
In the case of the Adv-infected cells, ET treatment was begun 24 h after adenoviral infection and continued for an additional 48 h. As seen in Fig. 5, ET resulted in a significant increase in cell surface area (Fig. 5A) and total protein/DNA ratio (Fig. 5B) in uninfected NRVM, which is consistent with the ability of this peptide growth factor to induce cellular hypertrophy in low-density NRVM (5, 19, 23, 40). Infection with either Adv-SR or Adv-dnPKC-ε had no significant effect on cell surface area or total protein/DNA ratio in unstimulated NRVM. As expected, ET significantly increased both indexes of NRVM hypertrophy in cells infected with Adv-SR, although the response was somewhat reduced compared with uninfected cells. Surprisingly, ET also stimulated cell spreading and total protein accumulation in NRVM infected with Adv-dnPKC-ε. The response was virtually identical to that observed in Adv-SR-infected myocytes.

We then examined whether PKC-ε-dependent signaling was critical for the gene expression changes observed in ET-treated NRVM. Here too we compared the effects of ET on MHC, ANF, and SERCA2 mRNA levels in uninfected cells, cells infected with the control Adv-SR, and NRVM infected with Adv-dnPKC-ε. As shown in Fig. 5C, adenoviral infection with either SR or Adv-dnPKC-ε substantially reduced basal mRNA levels encoding α-MHC and SERCA2 compared with uninfected NRVM. Other transcripts, such as β-MHC and ANF mRNA and 18S rRNA were less affected. ET treatment increased β-MHC and ANF mRNA levels in Adv-SR-infected cells, although the response was somewhat reduced compared with uninfected cells. Surprisingly, ET also stimulated cell spreading and total protein accumulation in NRVM infected with Adv-dnPKC-ε. The response was virtually identical to that observed in Adv-SR-infected myocytes.
three- to fivefold in uninfected NRVM while substantially reducing α-MHC and SERCA2 mRNA levels. ET treatment reproduced the same “fetal” pattern of gene expression in Adv-SR-infected cells, although the responses were somewhat blunted. Surprisingly, ET also stimulated this fetal gene program in NRVM infected with Adv-dnPKC-ε. Again, the response was virtually identical to that observed in Adv-SR-infected myocytes.

**Overexpression of dnPKC-ε blocks ERK activation.** In light of these somewhat unexpected findings, we examined whether dnPKC-ε had any demonstrable effects on acute signaling events in NRVM. Ping et al. (30) previously demonstrated that infection of cultured adult rabbit cardiomyocytes with Adv-dnPKC-ε blocked ERK activation in response to simulated ischemic preconditioning, providing further evidence implicating PKC-ε as an upstream regulator of the ERK signaling cascade in cardiomyocytes. Because ET is a potent activator of ERK1/2 in NRVM (13) and AT-1 cardiac myocytes (24), we examined whether overexpression of dnPKC-ε blocked ERK1/2 activation in re-

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**Fig. 5.** Overexpression of dnPKC-ε does not prevent ET-induced NRVM hypertrophy. A: NRVM were maintained under control conditions (uninfected) or infected with Adv-SR or Adv-dnPKC-ε (750 ppc each). After 24 h, cells were then chronically stimulated with ET (100 nM, +ET) or diluent (-ET) for an additional 48 h. Cell surface area was then assessed by 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) dye loading and image analysis. Data are means ± SE of 90–250 cells from each treatment group. *P < 0.05 vs. unstimulated cells in each group by Mann-Whitney Rank Sum test. B: total protein/DNA was assessed in similarly treated cultures. Data are means ± SE for 4–5 different cell isolations. **P < 0.05 vs. unstimulated cells in each group by paired t-test. C: total RNA was isolated from similarly treated NRVM. The resulting Northern blot was sequentially probed with 32P-labeled oligodeoxynucleotide and cDNA probes specific for α-MHC, β-MHC, ANF, and SERCA2 mRNAs and 18S rRNA.
response to this hypertrophic agonist. Of note, we compared the effects of acute exposure to ET (100 nM, 10 min) on ERK1/2 phosphorylation in NRVM infected with Adv-dnPKC-e vs. cells infected with the control Adv-SR. In both cases, acute ET stimulation was performed 24–48 h after adenoviral infection to ensure high levels of dnPKC-e expression at the time of ET stimulation. As seen in Fig. 6A, ET increased ERK1/2 phosphorylation in Adv-SR-infected NRVM, as identified by Western blotting with an antibody that recognized only the phosphorylated forms of ERK1/2. In contrast, increasing concentrations of Adv-dnPKC-e markedly reduced ET-induced ERK1/2 activation. These results were confirmed by gel-shift analysis, as depicted in Fig. 6B. As is evident from the Fig. 6B, ET induced an upward shift in the apparent molecular weight of both ERK1 and ERK2 in Adv-SR-infected NRVM. However, overexpression of dnPKC-e markedly suppressed this gel shift but did not affect the total amount of ERK1 or ERK2 in the cells. Quantitative analysis of six separate phospho-ERK Western blotting experiments is depicted in Fig. 6C. As is evident from Fig. 6C, basal levels of ERK2 phosphorylation were similar in Adv-SR- and Adv-dnPKC-e-infected NRVM. As expected, ET significantly increased ERK2 phosphorylation in Adv-SR-infected NRVM. However, ERK2 phosphorylation in ET-stimulated, Adv-dnPKC-e-infected NRVM was considerably reduced compared with ET-stimulated, Adv-SR-infected cells (2.9 ± 0.6–vs. 1.6 ± 0.04-fold; P < 0.05). Nevertheless, dnPKC-e overexpression did not completely abrogate ERK2 phosphorylation. Furthermore, doubling the Adv-dnPKC-e concentration did not eliminate this residual ERK2 phosphorylation (see Fig. 6A), suggesting that PKC-e-independent pathways may play a role in regulating ERK2 phosphorylation in NRVM. Taken together, these data indicate that PKC-e is indeed upstream of the ERK cascade in cardiomyocytes but that acute activation of neither PKC-e nor ERK1/2 may be necessary for ET-induced protein accumulation, gene expression changes, and cellular growth.

**DISCUSSION**

Our results indicate that caPKC-e overexpression in low-density NRVM was not sufficient to increase cell surface area or total protein/DNA ratio, although cell shape was altered markedly. Conversely, overexpression of dnPKC-e did not prevent NRVM hypertrophy induced by the hypertrophic agonist ET but markedly suppressed ERK activation. These somewhat unexpected results suggest that PKC-e and downstream signaling via the ERK cascade are not necessary for the induction of cardiomyocyte hypertrophy. It should be pointed out, however, that overexpression of mutant forms of PKC-e in NRVM maintained in low-density culture may not be analogous to the activation of endogenous PKC-e in response to neurohormonal and mechanical stimuli in the adult heart in vivo. First, PKC-e overexpression was accomplished under culture conditions in which NRVM display no or only infrequent [Ca$^{2+}$]$_i$ transients and mechanical activity (18). These conditions are quite distinct from the situation encountered in vivo, wherein cardiomyocytes encounter both intrinsic and extrinsic mechanical loading, display numerous cell-cell contacts, and continuously cycle [Ca$^{2+}$]. Second, the dnPKC-e studies used a mutant form of the rabbit enzyme to inhibit downstream signaling of the endogenous rat enzyme. Although dnPKC-e overexpression of the rabbit enzyme prevented ET-induced increases in PKC-e activity in NRVM membranes, it is conceivable that, due to species differences, more subtle actions of the endogenous enzyme were not prevented. Third, overexpression of caPKC-e and dnPKC-e was accomplished by adenoviral infection. However, infection with high titers of Adv, even in the absence of an inserted transgene, produced significant effects on cardiomyocyte gene expression and partially suppressed ET-induced cell growth. Thus...
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caution should be exercised in relating these studies to cardiomyocytes undergoing hypertrophy in vivo.

Nevertheless, these cell culture results are interesting in light of recent studies that specifically examined the role of PKC-\(\varepsilon\) in cardiomyocyte growth and function in vivo. Takeishi et al. (43) and Mochly-Rosen et al. (28) recently described two different transgenic mouse lines resulting in enhanced PKC-\(\varepsilon\)-dependent signaling. Both groups of animals demonstrated only mild concentric LVH with normal left ventricle function. \(\beta\)-MHC mRNA levels were increased in both studies, but other marker genes were less affected. In one of the lines, single cell capacitance, an index of cell volume, was actually decreased, suggesting that the observed increase in left ventricle mass was actually the result of cellular hyperplasia, rather than an overall increase in the size of individual muscle cells (28). Conversely, Khasar et al. (26) have described a line of mutant mice lacking PKC-\(\varepsilon\). These animals have abnormal nociceptor regulation, increased sensitivity to acute behavioral effects of ethanol (22), but normal cardiac development, and as adults they display no obvious cardiac phenotype. Taken together, these results and our own suggest that PKC-\(\varepsilon\) may have a complex role in overall growth regulation during cardiomyocyte hypertrophy and remodeling.

Despite its lack of effect on overall cell surface area, the shape of NRVM expressing caPKC-\(\varepsilon\) was markedly affected. There is very little known about how cardiomyocytes convert mechanical and neurohormonal signals into biochemical responses that induce alterations in cardiomyocyte shape. We were particularly interested in whether the long, cell processes induced by caPKC-\(\varepsilon\) overexpression contained actin filaments and whether these projections also contained focal adhesions and/or costameres. Elegant studies by Dabiri and coworkers (14) have indicated that myofibrillogenesis begins at the cell surface by the organization of focal adhesion proteins, actin filaments, and nonsarcomeric myosin. As shown in Fig. 2, the elongated cell processes observed in Adv-caPKC-\(\varepsilon\)-infected NRVM indeed contained at least one focal adhesion component (paxillin), which was arranged in a striated pattern along F-actin filaments, consistent with the structural organization of focal adhesions and costameres (19, 38). One can speculate that PKC-\(\varepsilon\) may in some way be involved in the regulation of sarcomeric assembly, especially at the ends of the growing myofibril. PKC-\(\varepsilon\) has in fact been localized by immunocytochemical techniques to regions of the cell adjacent to or within costameres (7) and within intercalated discs (17). Nevertheless, additional studies will be required to further investigate the functional role of PKC-\(\varepsilon\) in the assembly and maintenance of these structures.

In agreement with the transgenic overexpression studies described above, we found that adenovirally mediated caPKC-\(\varepsilon\) overexpression produced selective alterations in NRVM gene expression. Our results confirm previous findings of Decock et al. (15) who demonstrated that overexpression of a constitutively active mutant of PKC-\(\varepsilon\) increased ANF promoter activity. Of note, these investigators used transient, cotransfection of an ANF promoter-reporter gene construct along with the identical expression cassette used to construct the Adv-caPKC-\(\varepsilon\) for the present report. Our results extend these findings by using an Adv to induce overexpression, rather than to rely on the relatively low transfection efficiency provided by the calcium phosphate precipitation method. Both cell culture studies indicate that caPKC-\(\varepsilon\) overexpression was sufficient to increase ANF expression two- to threefold over control levels, which is contrary to that observed in the two transgenic lines discussed above (28, 43). In addition, we showed that mRNA levels encoding \(\alpha\)- and \(\beta\)-MHC were markedly affected. caPKC-\(\varepsilon\) overexpression resulted in the typical MHC isoenzyme “switch” from \(\alpha\)- to \(\beta\)-MHC predominance associated with contraction-induced (33, 34, 36) and agonist-induced (23, 33, 46) NRVM hypertrophy in vitro and with pressure overload-induced cardiomyocyte hypertrophy in vivo (9). Interestingly, SERCA2 mRNA levels were unaffected, which is in agreement with both of the previously mentioned transgenic animal experiments. It should be pointed out, however, that ANF and \(\beta\)-MHC mRNAs are readily detected in NRVM, even under basal conditions, in which cardiomyocytes display little or no spontaneous [\(\mathrm{Ca}^{2+}\)]\(_{i}\) transients and mechanical activity and have <5% of their total PKC-\(\varepsilon\) in the \(\mathrm{P}_{1}\) membrane fraction (41). Stimulation of [\(\mathrm{Ca}^{2+}\)]\(_{i}\) transients and contractile activity by membrane depolarization in the absence of other factors was sufficient to translocate PKC-\(\delta\), and to a much lesser extent PKC-\(\varepsilon\), and also to increase ANF and \(\beta\)-MHC promoter activities (41). Conversely, overexpression of dnPKC-\(\varepsilon\) (as described in the present report) markedly suppressed the translocation of endogenous PKC-\(\varepsilon\) into the membrane fraction but failed to block the ET-induced alterations in ANF and MHC gene expression. Because ET increases [\(\mathrm{Ca}^{2+}\)]\(_{i}\), transients (19), and activates both PKC-\(\varepsilon\) and PKC-\(\delta\) (13, 31), there are likely to be other PKC-\(\varepsilon\)-independent signaling pathways that regulate ANF and MHC gene expression in NRVM, even under conditions in which basal [\(\mathrm{Ca}^{2+}\)]\(_{i}\), transients and mechanical activity are minimized.

As demonstrated in Fig. 6, overexpression of dnPKC-\(\varepsilon\) markedly suppressed ET-induced ERK activation, thus providing additional, strong evidence that PKC-\(\varepsilon\) is an upstream regulator of the Ras-Raf-MEK-ERK cascade in cardiomyocytes (10, 13, 24, 29, 30). ERKs are acutely activated by a variety of neurohormonal agonists that induce NRVM hypertrophy, although their specific role in the induction of various aspects of the hypertrophic phenotype remains controversial (1). Using adenovirally mediated overexpression of constitutively active and dominant-negative mutants of MEK, Ueyama et al. (45) recently demonstrated that NRVM indeed utilize the ERK cascade to induce hypertrophic responses, including upregulation of ANF gene expression. However, they indicated that interruption of only one pathway may be insufficient for complete inhibition of the hypertrophic responses induced by ET, PE, or mechanical stretch. Similarly,
we recently showed that electrical stimulation of contraction increased ANF and β-MHC promoter activity and induced NRVM hypertrophy, which was associated with the acute activation of Jun NH2-terminal kinase (JNK) 2 and JNK3 but not ERK1 or ERK2 (41). Because ET acutely activates both JNKs (6, 12) and ERKs (4, 13, 24), as well as PKC-ε and PKC-δ (4, 13, 24, 31), it seems reasonable to conclude that there is substantial cross talk between the novel PKCs and their downstream targets during hypertrophic signaling. This conclusion is, of course, highly dependent on the completeness of the block of PKC-ε-dependent signaling by Adv-dnPKC-ε overexpression. Furthermore, it should be pointed out that we were unable to completely abrogate ET-induced ERK activation with the dominant-negative Adv. Thus it is conceivable that the observed, residual level of ET-induced ERK phosphorylation was sufficient to trigger the cell growth and gene expression changes observed in Fig. 5.

In summary, the results described in this report indicate that caPKC-ε overexpression is sufficient to induce selective changes in cardiomyocyte gene expression indicative of the hypertrophic phenotype. Adv-caPKC-ε altered cell geometry, producing cellular elongation and remodeling without a significant, overall increase in cell size or total protein accumulation. Furthermore, PKC-ε activation and downstream signaling via the ERK cascade may not be necessary for cell growth, protein accumulation, and gene expression changes induced by the hypertrophic agonist ET-1. Future studies will be necessary to determine whether the other PKC isoenzymes expressed in cardiomyocytes are necessary and/or sufficient alone or in combination to induce specific aspects of this complex phenotype.

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