Cardiomyocytes include the conventional PKC-α, -β, and -δ, and nuclear isoforms. The main PKC isoenzymes found in mammalian ventricular cardiomyocytes during physiological and pathological hypertrophy are PKC-α, -β, and -δ. Using replication-defective adenoviruses (AdVs) that express wild-type (WT) and dominant-negative (DN) PKC-α together with phorbol myristate acetate (PMA), which is a hypertrophic agonist and activator of all three PKC isoforms, we studied the role of PKC-α in signaling-specific aspects of the hypertrophic phenotype. PMA induced nuclear translocation of endogenous and AdV-WT PKC-α in neonatal rat ventricular myocytes (NRVMs). WT PKC-α overexpression increased protein synthesis and the protein-to-DNA (P/D) ratio but did not affect cell surface area (CSA) or cell shape compared with uninfected or control AdV β-galactosidase (AdV βgal)-infected cells. PMA-treated uninfected cells displayed increased protein synthesis, P/D ratio, and CSA and elongated morphology. PMA did not further enhance protein synthesis or P/D ratio in AdV-WT PKC-α-infected cells. To assess the requirement of PKC-α for these PMA-induced changes, AdV-DN PKC-α or AdV βgal-infected NRVMs were stimulated with PMA. Without PMA, AdV-DN PKC-α had no effects on protein synthesis, P/D ratio, CSA, or shape vs. AdV βgal-infected NRVMs. PMA increased protein synthesis, P/D ratio, and CSA in AdV βgal-infected cells, but these parameters were significantly reduced in PMA-stimulated AdV-DN PKC-α-infected NRVMs. Overexpression of DN PKC-α enhanced PMA-induced cell elongation. Neither WT PKC-α nor DN PKC-α affected atrial natriuretic factor gene expression. Insulin-like growth factor-I also induced nuclear translocation of endogenous PKC-α. PMA but not WT PKC-α overexpression induced ERK1/2 activation. However, AdV-DN PKC-α partially blocked PMA-induced ERK activation. Thus PKC-α is necessary for certain aspects of PMA-induced NRVM hypertrophy.

signal transduction; heart; adenovirus; translocation

Hypertrophy is an adaptive response of cardiomyocytes to increased hemodynamic load. Hemodynamic overload induces myocardial stretch, which in turn activates intracellular signaling pathways that induce protein synthesis and altered myocardial gene expression (19, 29). The serine-threonine family of protein kinase C (PKC) isoforms has been strongly implicated in the induction of protein synthesis and changes in cardiac gene expression during cardiomyocyte hypertrophy (3, 17). Upon activation, PKC isoforms translocate from the cytosol to various cellular regions including the cell membrane and nucleus. The main PKC isoforms found in mammalian cardiomyocytes include the conventional PKC-α and -β isoforms as well as the novel PKC-δ and -ε isoforms. We recently showed that PKC-δ and -ε each play unique roles in the mediation of certain aspects of cardiomyocyte hypertrophy (15, 31). Both of these PKC isoforms are activated in response to Ca2+ influx via voltage-gated l-type Ca2+ channels (32) and by Gαq subunits of G protein-coupled receptors (8, 9, 27, 28). Signaling via G protein-coupled receptors has been strongly implicated in hypertrophy that results in functional impairment of the myocardium (1, 10). Abnormalities in cardiomyocyte function are thought to be at least partially due to changes in expression levels of myofilament proteins such as myosin heavy chain isoforms and calcium regulatory proteins such as sarcoplasmic reticulum Ca2+-ATPase (SERCA2) and the Na+/Ca2+ exchanger (10, 13, 16, 20). We have previously shown that atrial natriuretic factor (ANF) and SERCA2 gene expression are altered in response to PKC-ε and -δ overexpression in neonatal rat ventricular cardiomyocytes (NRVMs). These alterations indicate the activation of hypertrophic pathways (25, 31). At present, there is little evidence to indicate PKC-α activation in response to G protein-coupled receptor agonists, and so its possible involvement in myocardial hypertrophic pathways remains unclear.

The well-characterized hypertrophic agonist phorbol myristate acetate (PMA) acts downstream of membrane receptors to activate both conventional and novel PKC isoenzymes. We explored the possible role of PKC-α in NRVM hypertrophy using PMA in conjunction with replication-defective adenoviruses (AdVs) that express wild-type (WT) or a kinase-inactive dominant-negative (DN) mutant of PKC-α. We show that PMA induces nuclear translocation of PKC-α, and that PKC-α is both necessary and/or sufficient to induce certain features of cardiomyocyte hypertrophy including increases in protein synthesis, the protein-to-DNA (P/D) ratio, and cell surface area (CSA). We also show that PKC-α does not affect cell shape or ANF gene expression. Studies on other cell types report translocation of PKC-α after stimulation by the physiological growth factor receptor ligand insulin-like growth factor-I (IGF-1), which has been implicated in the induction of physiological hypertrophy (21, 22, 24, 33). Here, we demonstrate nuclear translocation of endogenous PKC-α after IGF-I stimulation in NRVMs. Previously we reported that overexpression of PKC-ε and -δ induced downstream activation of the mitogen-activated protein kinase (MAPK) cascades in NRVMs (15). Here we demonstrate that although comparable overexpression of WT PKC-α fails to activate the MAPK cascade, inhibition of endogenous PKC-α by an adenovirally expressed DN PKC-α mutant partially blocked PMA-induced increases in MAPK.
activation. We conclude that PKC-α produces hypertrophic changes that are distinct from those induced by G protein-coupled receptor-mediated activation of PKC-ε and -δ in NRVMs.

METHODS

Animals were handled in accordance with the “Guiding Principles in the Care and Use of Laboratory Animals” approved by the National Institutes of Health. The methods of care and euthanasia conform to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Cell culture. NRVMs were isolated from cardiac ventricles of 2-day-old Sprague-Dawley rats via collagenase digestion (30). Cells were plated onto collagen-coated plastic dishes and Nunc chamber slides in PC-1 medium. Myocytes attached and spread overnight and were then maintained in a 1:4 solution of serum-free DMEM and medium 199 for up to 72 h. Cells were plated at low, medium, or high density (~100, 600, or 1,600 cells/mm², respectively) depending on experimental requirements.

Subcellular fractionation and Western blotting. Culture medium was removed and homogenization buffer (that contained 2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μM sodium orthovanadate, 1 mM Pefabloc, and 200 mM Tris·HCl, pH 7.5) was added. Cells were frozen in a dry-ice methanol bath, thawed on ice, and scraped into 1.5-ml centrifuge tubes. A nuclear extraction kit (Pierce Endogen) was used to isolate cytosolic, membrane, and nuclear fractions. Briefly, scraped samples were centrifuged (4,000 g for 2–3 min). The supernatant was collected as the cytosolic fraction. The pellet was resuspended in ice-cold cytosol extraction reagent (CER) I buffer with freshly added benzamidine (0.5 mg/ml), Pefabloc (0.75 mM), aprotinin (2 μg/ml), and leupeptin (2 μg/ml). After 10 min of incubation on ice, ice-cold CER II buffer was added to the samples. Samples were centrifuged (14,000 g for 10 min), and the supernatant was collected as the membrane fraction. The pellet was resuspended in nuclear extraction reagent (NER) I buffer with freshly added protease inhibitors. Samples were maintained on ice for 40 min with occasional vortexing. The samples were then centrifuged (14,000 g for 10 min), and the supernatant was collected as the nuclear fraction. Equal volumes of 8% SDS sample buffer were added to the fractions before electrophoresis was performed on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and stained with primary and horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used consisted of anti-PKC-α (BD Biosciences; San Diego, CA) anti-pERK1/2 (Promega; Madison, WI), and anti-ERK1 and anti-ERK2 (Santa Cruz Biotechnology; Santa Cruz, CA). The secondary horseradish peroxidase-conjugated antibodies used consisted of goat anti-mouse and anti-rabbit antibodies (Amersham; Arlington Heights, IL). Protein bands were detected by enhanced chemiluminescence and were captured on X-ray film. Band intensity was quantified by video densitometry.

Adenoviral constructs. AdVs that encode PKC-α, specifically AdV-WT PKC-α, AdV-DN PKC-α, and AdV-WT PKC-α-green fluorescent protein (AdV-WT PKC-α-GFP), were used. AdV PKC-α was constructed as previously described (25). AdV-DN PKC-α (7) was a generous gift of Dr. Trevor Biden (Garvan Institute of Medical Research, St. Vincent’s Hospital, Sydney Australia). AdV-WT PKC-α-GFP was constructed by subcloning the coding sequence for PKC-α-enhanced GFP fusion protein (pPKC-α-enhanced GFP; BD Biosciences) into pACCMVmPlpASR (an adenoviral shuttle plasmid constructed by Dr. R. Gerard). AdV-WT PKC-α-GFP was then produced by homologous recombination of pJM17 and PKC-α-GFP in human embryonic kidney (HEK)-293 cells. Control AdVs that expressed nuclear-encoded (NE) or cytoplasmic (CY) β-galactosidase (AdV-NE βgal and AdV-CY βgal, respectively) and GFP (AdV GFP) were used to control for nonspecific effects of viral infection (14, 15). Each AdV was plaque-purified and amplified in HEK-293 cells. Viruses were purified from cell extracts via double CsCl-gradient centrifugation. The multiplicity of infection (moi) was determined by a viral dilution assay in HEK-293 cells grown in 96-well clusters.

Cell fixation, immunocytochemistry, and confocal microscopy. Uninfected and AdV-WT PKC-α-infected cells in chamber slides were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and blocked in phosphate-buffered saline (PBS) that contained 10% normal goat serum (NGS) for 1 h at room temperature. Cells were then treated with a primary anti-PKC-α antibody (BD Biosciences) in blocking solution (1:30 dilution at 4°C, overnight). Cells were then washed in PBS and treated with a FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes; Eugene, OR) in blocking buffer (1:200 dilution at room temperature for 1 h). Cells were washed in PBS and stained with propidium iodide, mounted in antifade medium (Molecular Probes), and viewed with a Zeiss 410 or 510 laser scanning confocal microscope. NRVMs infected with AdV-WT PKC-α-GFP were fixed, permeabilized, and blocked in PBS with 10% NGS as described. Cells were then treated with anti-MF-20 sarcomeric myosin antibody (heavy-chain, monoclonal antibody (Developmental Studies Hybridoma Bank; Department of Biological Sciences, University of Iowa). Cells were incubated with MF-20 in PBS with 0.1% (wt/vol) bovine serum albumin (a 1:3 dilution at 4°C, overnight). Cells were washed in PBS and treated with rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes) in blocking buffer (1:30 dilution for 1 h). After cells were washed in PBS, they were mounted and viewed by confocal microscopy as described.

CSA and shape measurements. Cardiomyocytes were loaded with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-acetoxymethyl ester [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 0.2% Pluronic F-127 detergent for 1 h and were incubated for 1 h in BCECF-free Krebs buffer. Cells were viewed with a Zeiss LSM 510 laser scanning confocal microscope. Optical sections through the base of the cells (~20 cell field) were stored as digital images and analyzed using Image-1 software (Universal Imaging; West Chester, PA). A binary mask was created by setting the threshold brightness that distinguished the fluorescent cells from the black background. CSA was determined as an exact count of the number of pixels that made up the object’s binary mask multiplied by the area of a unit pixel (31). The shape factor was used to classify objects by degree of roundness, which was derived from the perimeter and area values of the object’s binary mask. The shape factor was calculated by the formula (4πa²/p²), where p is the perimeter (in μm) and a is the CSA (in μm²).

mRNA analysis and real-time RT-PCR. Total cell RNA was isolated using the RNeasy mini kit (Qiagen; Valencia, CA). ANF and GAPDH mRNAs were analyzed by real-time RT-PCR (25). Briefly, cDNA was reverse transcribed from extracted RNA as previously described (25). Real-time RT-PCR was performed using a Bio-Rad iCycler iQ multicolor real-time PCR detection system. For the rat ANF cDNA, the following primers were used: 5′-CTT GCG GTG TGT CAC ACA GC-3′ and 5′-GGG AGA GGT AAC GCC TCA CT-3′. The rat ANF cDNA probe (Integrated DNA Technologies) sequence was 5′-TGG CCA CTC ATG ACA ACC CG-3′. 5′ and 3′ ends of probes were labeled with 6-carboxyfluorescein and di-(tetra-butyl)-1,4-hydroquinone-1, respectively. PCR amplification was performed as previously described (25) using rat ANF primer and probe concentrations of 10 and 1 μM, respectively, and GAPDH as a control. All samples were performed in triplicate and averaged. The mRNA levels, expressed in threshold cycles (Ct), were averaged and converted to an input amount that was standardized to GAPDH and then normalized to the control virus (AdV βgal) samples.

Protein synthesis measurements. NRVMs were labeled with 2 μCi of [3H]leucine for 6 h. Culture medium was then removed, and cells were washed with 1 ml of sterile PBS (pH 7.4). Cold 10% trichloroacetic acid (TCA; 300 μl) was added, and cells were incubated on ice
for 30 min. Cells were scraped into Eppendorf tubes and centrifuged (14,000 g for 5 min). Supernatants were discarded, and pellets were resuspended in cold 10% TCA (200 μL) and centrifuged (14,000 g for 5 min). The supernatants were discarded, and pellets were resuspended in 0.2 N NaOH (200 μL) and incubated at 60°C for 25 min to dissolve pellets. Protein concentration was analyzed in aliquots by bicinchoninic assay, and radioactivity was measured by liquid scintillation spectroscopy.

Protein and DNA measurements. NRVMs were quantitatively scraped from dishes in 1 ml of 0.2 N perchloric acid and collected by centrifugation (10,000 g for 10 min). The precipitate was redissolved by incubation (at 60°C for 20 min) in 250 μL of 0.3 N KOH. Aliquots were used for analysis of total protein by the Lowry method with crystalline human serum albumin as a standard and for DNA using 33258 Hoechst dye and salmon sperm DNA a standard (31).

Fig. 1. Translocation of endogenous protein kinase C (PKC-α) in response to phorbol myristate acetate (PMA). High-density neonatal rat ventricular myocytes (NRVMs; ~1,600 cells/mm²) were maintained in serum-free medium for 72 h and then stimulated with PMA (200 nM) or endothelin-1 (ET-1; 100 nM) for 10 or 30 min (A). Cells were harvested and subjected to differential centrifugation. Resulting subcellular fractions were analyzed for PKC-α distribution by SDS-PAGE and Western blotting. Bar graphs represent data from four separate experiments. Medium-density NRVMs (~600 cells/mm²) were either untreated (B) or stimulated with PMA (200 nM for 10 min; C). Cells were fixed, permeabilized, and immunostained for PKC-α in preparation for mounting and confocal microscopy.
Data analysis. Results are expressed as means ± SE. Normality was assessed using the Kolmogorov-Smirnov test, and homogeneity of variance was assessed using Levene’s test. Data from multiple groups were compared by one-way blocked ANOVA or one-way blocked ANOVA on ranks with subsequent Student-Newman-Keuls test or Dunn’s test where appropriate. Differences among means were considered significant at $P < 0.05$. Data were analyzed using SigmaStat 1.0 (Jandel Scientific; San Rafael, CA).
RESULTS

PMA stimulates nuclear translocation of endogenous and adenovirally overexpressed PKC-α. In initial experiments, we examined the effect of PMA on endogenous PKC-α translocation by subcellular fractionation and Western blotting. As seen in Fig. 1A, PKC-α was distributed in cytosolic and membrane fractions but was absent from the nuclear fraction under basal conditions of high-density, serum-free culture. PMA stimulation caused the translocation of PKC-α from the

Fig. 2. Translocation of adenovirally expressed PKC-α. Medium-density NRVMs (~600 cells/mm²) were maintained in serum-free media and infected with adenovirus (AdV) wild-type (WT) PKC-α [25 multiplicities of infection (moi) for 48 h; A]. Cells were then stimulated with PMA (200 nM for 30 min). After fixation and permeabilization, cells were immunostained for PKC-α. Nuclei were stained with propidium iodide in preparation for mounting and confocal microscopy. Confocal images show the translocation of WT PKC-α (green) in the immunostained cells. Colocalization of PKC-α with the nucleus (yellow) observed at 30 min of PMA treatment is shown. Medium-density NRVMs were maintained in serum-free media and infected with AdV-WT PKC-α-green fluorescent protein (GFP; 50 moi for 48 h; B). Cells were then stimulated with PMA (200 nM for 0–30 min). After fixation and permeabilization, cells were immunostained with MF-20 antibody against sarcomeric myosin heavy chain protein. Confocal images show myosin heavy chain filament staining (red) and PKC-α-GFP localization (green). Without PMA (0 min), PKC-α-GFP was primarily localized in the cytosol. Strong nuclear localization of PKC-α-GFP occurred by 15 min of PMA treatment. By 30 min, PKC-α-GFP was reduced in the cytosol and concentrated in the nucleus and along the plasma membrane.
cytosolic to the nuclear and membrane fractions within 10 min of exposure to the drug. In contrast, the Gαq-coupled receptor agonist endothelin-1 (ET-1) induced only minimal membrane translocation and no nuclear translocation over the same time period. Similar results were obtained with the Gαq-coupled receptor agonist phenylephrine (50 μM for 0–30 min; data not shown). The effect of PMA on PKC-α translocation was confirmed by immunocytochemistry and confocal microscopy (Fig. 1, B and C). As seen in Fig. 1B, endogenous PKC-α was present diffusely throughout the cytoplasm and excluded from the nucleus of unstimulated NRVMs. PKC-α appeared to redistribute from the cytoplasm to the nucleus in response to 10 min of PMA exposure (Fig. 1C).

To verify that adenovirally expressed PKC-α behaved in a similar manner as the endogenous enzyme, we infected NRVMs with AdV-WT PKC-α (25 moi for 48 h) and then stimulated the cells with PMA (200 nM for 30 min). After cell fixation, NRVMs were immunostained first with an anti-PKC-α primary antibody and then with a FITC-conjugated secondary antibody. Nuclei were detected by propidium iodide staining, and colocalization of PKC-α was analyzed by confocal microscopy. In the absence of PMA, WT PKC-α was largely excluded from the nucleus and was localized in the cytoplasm (Fig. 2A). However, some nuclei demonstrated a small degree of colocalization even under basal conditions. After 30 min of PMA treatment, there was a marked increase in the degree of colocalization of WT PKC-α within the nucleus.

As an additional check on the nuclear translocation of adenovirally expressed PKC-α, NRVMs were infected with AdV-WT PKC-α-GFP (50 moi for 48 h; Fig. 2B). In the absence of PMA (0 min), WT PKC-α-GFP was also primarily localized in the cytosol along the myofilaments and in the perinuclear region with only a small amount within the nucleus. At 15 min of PMA treatment, the distribution of WT PKC-α-GFP was altered, which demonstrated strong nuclear localization. By 30 min of PMA treatment, WT PKC-α-GFP was strongly localized within the nucleus as well as along the plasma membrane. This was accompanied by a markedly diminished degree of localization of WT PKC-α-GFP within the cytoplasm. In NRVMs infected with the AdV GFP vector, there was no colocalization of GFP within the nucleus, and GFP distribution was unaltered by PMA treatment (data not shown).

**AdV-WT and AdV-DN PKC-α increase immunoreactive PKC-α levels in NRVMs.** Before examination of the effects of adenoviral overexpression of WT and DN PKC-α on CSA, shape, and protein synthesis in NRVMs, it was necessary to determine whether infection of NRVMs with AdV-WT or AdV-DN PKC-α increased the immunoreactive PKC-α levels. We infected NRVMs with increasing amounts (10–100 moi for 48 h) of AdV-WT PKC-α (Fig. 3). AdV-WT PKC-α increased the amount of immunoreactive PKC-α in a dose-dependent fashion. Likewise, increasing the multiplicity-of-infection level (10–100 moi for 48 h) of AdV-DN PKC-α (Fig. 3B) caused a dose-dependent increase in immunoreactive PKC-α. However, expression levels of endogenous PKC-δ and -ε appeared to be unaffected in NRVMs infected with either AdV-WT or AdV-DN PKC-α.

**PKC-α is necessary but not sufficient to increase CSA.** The contribution of PKC-α to PMA-induced increases in CSA is illustrated in Fig. 4. As seen in Fig. 4A, uninfected cells treated with 200 nM PMA for 48 h demonstrated significantly higher CSA values compared with uninfected cells that did not receive PMA. NRVMs infected with either AdV-WT PKC-α (25 moi for 48 h) or AdV-NE βgal (25 moi for 48 h) also demonstrated CSA values that were significantly higher than uninfected cells. However, there was no significant difference in CSA between NRVMs infected with AdV-WT PKC-α vs. those infected with AdV-NE βgal. Furthermore, PMA increased CSA to a similar extent in both AdV-NE βgal- and AdV-PKC-α-infected NRVMs.

AdV-mediated overexpression of a kinase-inactive mutant of PKC-α (AdV-DN PKC-α) was then used to determine whether inhibition of PKC-α could block the effects of PMA-induced increases in CSA. NRVMs were infected with AdV-CY βgal or AdV-DN PKC-α (100 moi for 72 h) in the presence or absence of PMA (200 nM for 48 h; Fig. 4B).

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**Fig. 4.** Effects of WT and DN PKC-α on cell surface area (CSA). Low-density NRVMs (∼100 cells/mm²) were maintained in control medium [uninfected (UI)] or were infected (25 moi) with AdV-nuclear encoded (NE) βgal or AdV-WT PKC-α (4). Paired cultures were then either maintained in control medium (−PMA) or stimulated with PMA (+PMA; 200 nM for 48 h). CSA was analyzed by 2’7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) dye loading, confocal microscopy, and image analysis. Data are means ± SE of 1,276–1,663 cells for 909–3,934 cells in each group from six different cell isolations. *P < 0.05 vs. non-PMA-treated UI cells; †P < 0.05 vs. UI + PMA. NRVMs were either infected with AdV-cytoplasmic (CY) βgal or AdV-DN PKC-α (100 moi for 72 h) in the presence or absence of PMA (200 nM for 48 h; B). CSA was measured as described (see text). Data are means ± SE of 1,276–1,663 cells from three different cell isolations. *P < 0.05 vs. CY βgal; †P < 0.05 vs. CY βgal + PMA.
AdV-CY βgal-infected cells treated with PMA had significantly higher CSA values compared with their non-PMA-treated counterparts. Overexpression of DN PKC-α alone had no effect on CSA compared with AdV-CY βgal. DN PKC-α overexpression inhibited the PMA-induced increase in CSA, as PKC-α is involved in PMA-induced cell spreading, but after controlling for the nonspecific effects of adenoviral infection, PKC-α overexpression alone is not sufficient to increase CSA in NRVMs.

PKC-α is neither necessary nor sufficient to alter NRVM cell shape. The effects of PMA and adenoviral infection on cell shape are illustrated in Fig. 5. Shape factor values were first compared between uninfected cells, cells infected with AdV-NE βgal, and cells infected with AdV-WT PKC-α (Fig. 5A). Mean shape factor was slightly lower in WT PKC-α-overexpressing cells compared with uninfected myocytes but was not statistically different from cells that expressed NE βgal. Incubation with PMA (200 nM for 48 h) induced a significant alteration in cell shape in all three groups, from a slightly rounded to an elongated morphology. Although mean shape factor values for PMA-treated, NE βgal-infected cells were slightly greater than for PMA-treated, uninfected cells, there was no significant difference in cell shape between PMA-treated, AdV-NE βgal- and PMA-treated, AdV-WT PKC-α-infected NRVMs.

To determine whether inhibition of PKC-α blocked PMA-induced shape changes, cells were infected with either AdV-CY βgal (100 moi for 72 h) or AdV-DN PKC-α (100 moi for 72 h) in the presence or absence of PMA (200 nM for 48 h; Fig. 5, B and C). In the presence of PMA, both AdV-CY βgal- and AdV-DN PKC-α-infected cells showed significantly lower shape factor values (Fig. 5B), which indicates a more-elongated morphology compared with the non-PMA-treated cells (Fig. 5C). In fact, in the presence of DN PKC-α, PMA-induced cellular elongation was enhanced. Together the findings indicate that after the nonspecific effects of adenoviral infection are controlled for, PKC-α does not appear to be involved in PMA-induced cell shape changes in NRVMs.

PKC-α does not increase ANF mRNA levels in NRVM. Similarly, we found that WT PKC-α overexpression did not significantly increase ANF gene expression in NRVMs. Real-
time RT-PCR analysis showed no change in ANF mRNA levels in cells infected with AdV-WT PKCα (25 moi for 48 h) compared with uninfected or AdV-NE βgal-infected NRVMs (data not shown). Furthermore, there was no consistent change in ANF mRNA levels in cells infected with AdV-DN PKCα (100 moi for 48 h) compared with uninfected or AdV-CY βgal-infected cells (data not shown).

PKCα is both necessary and sufficient to increase protein synthesis in NRVMs. We next evaluated whether PKCα overexpression affected the rate of protein synthesis. Protein synthesis was estimated by measuring the rate of [3H]leucine incorporation into TCA-precipitable total protein. A). Data are means ± SE for six or seven different cell isolations. *P < 0.05 vs. non-PMA-treated UI cells; †P < 0.05 vs. NE βgal. NRVMs were infected with AdV-CY βgal or AdV-DN PKCα (100 moi for 72 h) in the presence (+) or absence (−) of PMA (200 nM for 6 h). Protein synthesis was measured as described (B). Data are means ± SE from six or seven different cell isolations. *P < 0.05 vs. CY βgal; †P < 0.05 vs. CY βgal + PMA.

To determine whether inhibition of PKCα could block PMA-induced increases in protein synthesis, NRVMs were infected with AdV-CY βgal or AdV-DN PKCα (100 moi for 72 h) in the presence or absence of PMA (200 nM for 6 h; Fig. 6B). Values were normalized to those for non-PMA-treated AdV-CY βgal-infected cells. Overexpression of DN PKCα had no effect on the basal rate of protein synthesis. PMA increased the rate of protein synthesis by ~20% in AdV-CY βgal-infected cells. However, DN PKCα overexpression blocked the effect of PMA on [3H]leucine incorporation. Thus these results show that PKCα is both necessary and sufficient to regulate protein synthesis in NRVMs.

The effects of PKCα on protein synthesis were verified by analyzing the P/D ratios in similarly treated cultures. As seen in Fig. 7A, both PMA and WT PKCα increased the P/D ratio. PMA increased the P/D ratio in uninfected and NE βgal-infected cells but had little effect in cells infected with AdV-WT PKCα.
AdV-WT PKC-α. Although DN PKC-α overexpression had no significant effect on basal P/D ratio, DN PKC-α partially blocked the increase in protein accumulation in response to PMA (Fig. 7B).

**IGF-I activates PKC-α in NRVMs.** As discussed (see PMA stimulates nuclear translocation of endogenous and adenovirally overexpressed PKC-α), PKC-α was not substantially activated by the hypertrophic agonists (e.g., ET-1 and phenylephrine) that activate Goq-coupled receptors. However, PKC-α does undergo membrane and nuclear translocation in response to PMA as well as to physiological agonists that activate receptor tyrosine kinases in cardiomyocytes and nonmuscle cells (21, 22, 24). Therefore, we examined whether PKC-α undergoes membrane and nuclear translocation in response to IGF-I, which is a physiological ligand and known hypertrophic agonist in NRVMs. As seen in Fig. 8, PKC-α levels decreased in the cytosolic fraction and increased in the membrane and nuclear fractions of NRVMs treated with IGF-I (50 ng/ml for 10–30 min).

**PKC-α is necessary but not sufficient to induce ERK1/2 activation.** To determine whether the PKC-α-mediated effects were accompanied by activation of the ERK cascade, NRVMs were infected with AdV-WT PKC-α (25 moi for 4–48 h), and ERK1/2 phosphorylation was assessed by Western blotting. As seen in Fig. 9, acute stimulation of NRVMs with PMA and ET-1 significantly increased the level of ERK1/2 phosphorylation, whereas IGF-I only modestly increased ERK1/2 phosphorylation (Fig. 9, A and B). Infection of NRVMs with AdV-WT PKC-α (25 moi for 4–48 h) did not increase ERK1/2 phosphorylation compared with the control, agonist stimulation, or infection with AdV-NE βgal (25 moi for 4–48 h; Fig. 9, A and C). Additionally, AdV-WT PKC-α did not increase c-Jun NH2-terminal kinase (JNK)1/2 and p38 phosphorylation in NRVMs (data not shown). To determine whether the inhibition of PKC-α blocked PMA-induced ERK1/2 activation, NRVMs were infected with AdV-DN PKC-α (100 moi for 48 h) and then acutely stimulated with PMA (200 nM for 10 min). As seen in Fig. 10, A and B, PMA-induced ERK1/2 phosphorylation was partially inhibited by DN PKC-α compared with PMA-treated uninfected cells or AdV-CY βgal-infected NRVMs.

**DISCUSSION**

In this report, we have elucidated the contribution of PKC-α to certain aspects of PMA-induced NRVM hypertrophy. We demonstrated nuclear translocation of both endogenous PKC-α and adenovirally overexpressed WT PKC-α in response to PMA stimulation. By adenoviral overexpression of WT and DN mutants of PKC-α, we showed that PKC-α is involved in PMA-induced increases in CSA, protein synthesis, and P/D ratio. We demonstrated the specificity of these hypertrophic changes to PKC-α by showing that overexpression of WT or DN PKC-α did not alter endogenous expression levels of PKC-ε and -δ. We showed that the physiological ligand IGF-I induced membrane and nuclear translocation of endogenous PKC-α in a manner similar to PMA. Finally, we examined the effects of PKC-α on downstream activation of the MAPK cascades. We found that although overexpression of WT PKC-α did not significantly activate ERK1/2, inhibition of PKC-α by AdV-DN PKC-α was able to partially block PMA-induced ERK1/2 phosphorylation.

We observed nuclear translocation of both endogenous PKC-α and adenovirally expressed WT PKC-α in response to acute PMA stimulation. Our findings differ from those of Braz et al. (4), who reported PMA-induced translocation of AdV-WT PKC-α to the perinuclear region in confocal images. Braz et al. (4) also reported that PMA induced the translocation of endogenous PKC-α to the membrane fraction. However, PKC-α content in the nuclear fraction was not examined. The differential findings may be due to differences in cell culture methods that affect the basal state of growth regulation at the

![Fig. 8. Insulin-like growth factor-1 (IGF-I) induces nuclear translocation of endogenous PKC-α. High-density NRVMs (~1,600 cells/mm²) were maintained in serum-free medium for 72 h and then stimulated with IGF-I (50 ng/ml) for 10 or 30 min. Cells were harvested and subjected to differential centrifugation. Resulting subcellular fractions were analyzed for PKC-α distribution by SDS-PAGE and Western blotting. Bar graph represents data from four separate experiments.](http://ajpheart.physiology.org/)

**Fig. 8.** Insulin-like growth factor-1 (IGF-I) induces nuclear translocation of endogenous PKC-α. High-density NRVMs (~1,600 cells/mm²) were maintained in serum-free medium for 72 h and then stimulated with IGF-I (50 ng/ml) for 10 or 30 min. Cells were harvested and subjected to differential centrifugation. Resulting subcellular fractions were analyzed for PKC-α distribution by SDS-PAGE and Western blotting. Bar graph represents data from four separate experiments.
time of agonist stimulation. In their study, NRVMs were stimulated for 24 h in serum-containing medium to induce hypertrophy before agonist treatment (4). Of note, Pass et al. (23) have shown that induction of cardiac hypertrophy by PKC-δ/H9280 overexpression affects the expression of receptors for activated C kinase (RACK)-1 and increases the membrane translocation of PKC-ε/H9252 in transgenic mice. Thus it is conceivable that induction of a hypertrophic phenotype in NRVMs before agonist stimulation alters the intracellular environment that may have affected the pattern of PKC isoenzyme translocation. Our NRVM cultures were maintained in serum-free conditions, which minimized the likelihood of a preexisting hypertrophic phenotype at the time of PMA treatment.

We compared the effects of PMA on translocation of endogenous PKC-α to those of the Gqα-coupled receptor agonist ET-1. PMA induced membrane and nuclear translocation of PKC-α, whereas ET-1 did not. This concurs with findings in other studies that show the failure of Gqα-coupled receptor stimulation to activate PKC-α in cardiomyocytes (2, 8, 9, 26–28). Our observations, however, disagree with findings of Braz et al. (4) and Kerkela et al. (18), who showed translocation of PKC-α in response to the α1-adrenergic, Gqα-coupled receptor agonist phenylephrine. These differential findings may also be due to the preexistence of hypertrophy, owing to the pretreatment of cells with serum before phenylephrine stimulation. Kerkela et al. (18) have shown that antisense downregulation of PKC-α blocked phenylephrine-induced increases in total PKC activity in NRVM, which implies that PKC-α is activated by phenylephrine. Furthermore, they assessed PKC activity by an enzymatic radioassay after stimulation of NRVMs with a 100-μM concentration of phenylephrine (18). In contrast, Puceat et al. (26) showed that stimulation of NRVMs with a 100-μM concentration of phenylephrine in the presence of propranolol to block β-adrenergic receptors failed to induce translocation of PKC-α. Furthermore, Clerk et al. (8) demonstrated that neither phenylephrine nor ET induced PKC-α translocation in NRVMs, which concurs with our findings (see Fig. 1; data not shown).

The present investigation is the first to directly demonstrate nuclear translocation of a PKC isoenzyme in NRVMs. The implications of nuclear translocation of PKC isoenzymes in cardiomyocytes are of obvious interest. There is evidence to

**Fig. 9.** ERK activation in response to agonist stimulation vs. AdV-WT PKC-α overexpression. High-density NRVMs (~1,600 cells/mm²) were acutely stimulated for 10 min with PMA (200 nM), ET-1 (100 nM), or IGF-1 (50 ng/ml) or were infected with either AdV-WT PKC-α or AdV-NE βgal (25 moi for 4–48 h). Cells were harvested, and cell lysates were analyzed for ERK1/2 phosphorylation by SDS-PAGE and Western blotting. Western blots (A) probed for pERK1/2 (top) and total ERK1/2 (bottom). Bar graph (B) shows the effects of agonist stimulation on pERK2-to-total ERK2 ratio, where data are representative of four separate experiments. *P < 0.05 vs. control. Bar graph (C) compares pERK2-to-total ERK2 ratio in AdV-WT PKC-α- and AdV-NE βgal-infected cells, where data are representative of three separate experiments.
indicate that several nuclear targets of PKC isoenzyme phosphorylation exist. These include transcription factors, topoisomerasers, and nuclear lamins (6). Recently Xiao et al. (35) showed that upstream stimulatory factor (USF)-1, a transcription factor that upregulates α-myosin heavy chain gene expression, has PKC-specific-phosphorylation sites. Their study suggests that USF-1 may be phosphorylated by a calcium-sensitive PKC isoenzyme (35). Thus PKC-α is a likely candidate for USF-1 phosphorylation in cardiomyocytes.

We demonstrated the effects of AdV-WT PKC-α and AdV-DN PKC-α overexpression on CSA and cell shape. We showed that overexpression of control AdV-NE βgal increased CSA to values similar to those in cells that overexpress AdV-WT PKC-α. This is consistent with previous findings from our laboratory, where we have observed that overexpression of a control AdV alone can induce hypertrophic changes in NRVMs (12). However, the findings of Braz et al. (4) suggest that AdV βgal overexpression does not increase CSA in NRVMs. Although the reason for the differential findings are unclear, our method of analyzing CSA was not biased by observer selection and used data acquired from large numbers of cells within each treatment group. Such large sample sizes are necessary for rigorous statistical analyses to account for the large variations in CSA within NRVM cultures and to obtain a realistic representation of changes within individual treatment groups. To determine whether PKC-α was necessary for PMA-induced increases in CSA, we analyzed the effect of DN PKC-α overexpression in PMA-treated NRVMs. By showing that DN PKC-α had an inhibitory effect on PMA-induced increases in CSA, we demonstrated that PKC-α was necessary but not sufficient to increase CSA. This is a new and important aspect of PKC-α function that is unique to our study. The present findings also indicate that PKC-α is not involved in PMA-induced changes in cell shape. PMA treatment caused an alteration in cell shape in NRVMs that was not observed in response to WT PKC-α overexpression and was not inhibited by DN PKC-α overexpression. The elongated morphology observed in cardiomyocytes in response to PMA treatment was also observed in NRVMs that overexpressed a constitutively active mutant of PKC-ε (31). Because PMA activates both conventional and novel PKC isoenzymes, the altered morphology of NRVMs after PMA stimulation is thus most likely due to PKC-ε activation.

We found ANF mRNA levels to be unchanged by WT PKC-α or DN PKC-α overexpression in NRVMs. Our findings coincide with those of Kerkela et al. (18), who reported that downregulation of PKC-α with antisense oligonucleotides did not block agonist-induced upregulation of ANF mRNA in NRVMs. Studies by Decock et al. (11) using transient transfection methods to overexpress PKC isoenzymes in NRVMs showed that upregulation of ANF expression was greater in cardiomyocytes that express PKC-ε than PKC-α. Similarly, Strait et al. (31) revealed an upregulation of ANF expression in NRVMs that overexpress a constitutively active mutant of PKC-ε. The upregulation of ANF expression is a common feature of pathological hypertrophy. Another feature of pathological hypertrophy is the downregulation of SERCA2 expression. Recently we showed that overexpression of either AdV-WT PKC-ε or AdV-WT PKC-δ downregulated SERCA2 mRNA levels, whereas AdV-WT PKC-α had no effect (25). In a recent study, however, Braz et al. (5) demonstrated that PKC-α may have a regulatory role with respect to SERCA2 activity whereby PKC-α affects dephosphorylation of phospholamban, the SERCA2 pump inhibitory protein. The hearts of transgenic PKC-α-knockout mice were hypercontractile and showed increased phospholamban phosphorylation compared with nontransgenic hearts. In contrast, hearts from transgenic mice that overexpressed PKC-α were hypotcontractile, which demonstrates decreased phospholamban phosphorylation. The study showed that PKC-α increased the activity of protein phosphatase-1, which dephosphorylates phospholamban and in turn enhances its interaction with SERCA2 and prevents SERCA2 activity, thereby resulting in a hypercontractile phenotype. Hypocontractile and hypercontractile phenotypes were also reported by Braz et al. (5) in adult rat cardiomyocytes that had been infected with AdV-WT PKC-α (100 moi for 2 h) and AdV-DN PKC-α (100 moi for 2 h), respectively. Although it is possible that this may also be true in NRVMs, it is difficult to measure contractile parameters in individual neonatal rat cardiomyocytes, because the cells must be plated at a low density that in itself would result in diminished contractility.
ally, the sarcoplasmic reticulum in neonatal cardiomyocytes is poorly developed, and the levels of SERCA2 mRNA and protein expression are also reduced compared with adult cardiomyocytes (34).

In the absence of PMA, overexpression of WT PKC-ε increased both protein synthesis and the P/D ratio in NRVMs. These results are consistent with observations by Braz et al. (4) and Kerkela et al. (18). In the presence of PMA, however, overexpression of WT PKC-ε blunted PMA-induced enhancement of protein synthesis as well as P/D ratio. The reason for this is presently unclear and requires additional investigation.

With respect to the other two major PKC isoenzymes present in NRVMs (PKC-ζ and -δ), the ability to increase protein synthesis appears to be a role unique to PKC-ε. We have previously shown that overexpression of a constitutively active mutant of PKC-ζ actually decreased the P/D ratio in NRVMs (31). To date, there have been no published studies to indicate the involvement of PKC-δ in regulating protein synthesis. Heidkamp et al. (15) have shown that overexpression of WT PKC-δ in NRVMs resulted in apoptosis, which is a scenario that would argue against a protein synthetic role for PKC-δ.

Although PKC-ε is a nonphysiological activator of PKC-α, other studies report that ligand stimulation of tyrosine kinase growth-factor receptors can activate PKC-α in several cell types. Pecherskaya and Solem (24) demonstrated that the growth factor IGF-I activated PKC-α in adult rat cardiomyocytes, and Neri et al. (21, 22) showed that PKC-α translocates to the nucleus in fibroblasts stimulated with IGF-I, EGF, and PDGF. As seen in Figs. 1 and 8, we demonstrate that in addition to PMA, the physiological ligand IGF-I also induces nuclear translocation of PKC-α in contrast with the effects of ET-1.

Downstream activation of the MAPK cascades has been previously observed in our laboratory after PKC-ε and -δ overexpression in NRVMs (15). Heidkamp et al. (15) demonstrated an increase in ERK1/2 activation in NRVMs after 8 h of PKC-ε (25 moi) overexpression. In the present study, although we observed strong ERK1/2 activation after PMA and ET-1 stimulation and modest activation after IGF-1 stimulation, marked ERK1/2 activation was not observed after WT PKC-α overexpression in NRVMs. In the present study, we examined ERK1/2 activation at 4, 8, 24, and 48 h after AdV-WT PKC-α infection and observed no marked increase in ERK1/2 activation compared with uninfected control or βgal-infected NRVMs. The increase in ERK1/2 activation in response to PKC-α overexpression reported by Braz et al. (4) reflects only a modest increase in ERK1/2 phosphorylation after a 24-h infection of NRVMs with a fourfold higher dose (100 moi) of AdV PKC-α. However, in concurrence with the observations of Braz et al. (4), we report that inhibition of endogenous PKC-α activity by AdV-DN PKC-α partially inhibited PMA-induced increases in ERK1/2 phosphorylation. Our results suggest that although PKC-α alone was not sufficient to enhance ERK1/2 activation, it appears to be necessary in PMA-induced increases in ERK activation. Although the specific mechanism by which DN PKC-α inhibits PMA-induced increases in ERK activation is unclear, it is possible that this may occur by DN PKC-α inhibiting PKC-ε activity (15). Although we did not see a change in total endogenous levels of PKC-ε in NRVMs overexpressing DN PKC-α, it is possible that DN PKC-α inhibits PKC-ε activity by hindering PKC-ε translocation and binding to its specific RACK protein (23). We did not observe any increase in JNK1/2 or p38MAPK phosphorylation in NRVMs infected with AdV-WT PKC-α. In contrast, Heidkamp et al. (15) demonstrated a marked increase in JNK1/2 and p38 phosphorylation after 24 h of PKC-δ overexpression.

The present investigation of the role of PKC-α in hypertrophy indicates that PKC-α is both necessary and sufficient for certain aspects of PMA-induced cardiomyocyte hypertrophy but not others. The specific contributions of PKC-α to cardiomyocyte hypertrophy are distinct from those of PKC-ζ and -δ (15, 31). The activation of PKC-α by the physiological ligand IGF-I is of much interest, whereby IGF-I has been demonstrated to be beneficial to cardiomyocytes (33). Thus it is important to further evaluate the possible role of PKC-α in various cardioprotective aspects of IGF-I-mediated hypertrophy.

REFERENCES


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