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Identification of PKCα Isoform-Specific Effects in Cardiac Myocytes Using Antisense Phosphorothioate Oligonucleotides

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ABSTRACT

Members of the mammalian protein kinase C (PKC) superfamily play key regulatory roles in multiple cellular processes. In the heart, PKC signaling is involved in hypertrophic agonist-induced gene expression and hypertrophic growth. To investigate the specific function of PKC signaling in regulating cardiomyocyte growth, we used antisense oligonucleotides to inhibit PKC α, the major isozyme present in the neonatal heart. Transfection of cultured neonatal cardiomyocytes with antisense PKCα oligonucleotides resulted in a marked reduction in both PKCα mRNA and protein levels. PKCα antisense treatment also reduced phenylephrine (PE)-induced PKC activity and perinuclear translocation of PKCα. Antisense inhibition of PKCα led to reduction of PE-induced increase in skeletal α-actin mRNA levels and atrial natriuretic peptide (ANP) secretion but had no significant effects on PE-induced β-myosin heavy chain, ANP, or B-type natriuretic peptide (BNP) gene expression. On the other hand, antisense PKCα treatment attenuated endothelin-1–induced increase in ANP and BNP peptide secretion, whereas endothelin-1–induced gene expression of ANP and BNP remained unchanged. The hypertrophic agonist-induced growth of cardiomyocytes, characterized by increased [^{3}H]leucine incorporation, was not affected with antisense PKCα treatment. Furthermore, we found that PE-induced increase in extracellular signal-regulated kinase (ERK) activity was partially inhibited by antisense PKCα treatment, implicating ERK as a downstream mediator for PKCα signaling. These results indicate that PKCα isozyme is involved in hypertrophic signaling in cardiomyocytes and provide novel strategies for future studies to identify other cellular targets controlled selectively by PKCα or other PKC isozymes.

Hypertrophic phenotype in neonatal cardiac myocytes is characterized by an increase in cell size and protein synthesis, increased sarcomere organization, and induction of genes, which largely recapitulate the fetal pattern of gene expression; among those are skeletal α-actin (α-*SkA*) and β-myosin heavy chain (β-*MHC*), and the natriuretic peptide genes atrial natriuretic peptide (*ANP*) and B-type natriuretic peptide (*BNP*) (Ruskoaho, 1992; Sugden and Clerk, 1998). Hypertrophic cardiac growth is also characterized by the activation of various cell signaling cascades, including protein kinase C (PKC), mitogen activated protein kinases, and phosphatases, such as calcineurin (Sugden and Clerk, 1998; Molkentin and Dorn, 2001). Activation of PKC and its various isoforms has been shown to exert multiple cardiovascular functions, including regulation of ion channels, intracellular ion concentration, contractility, activity of transcription factors (c-jun and c-fos), gene expression, and hypertrophic cardiomyocyte growth (Shubeita et al., 1992; Clerk et al., 1994; Naruse and King, 2000). In cardiac myocytes, several autocrine/paracrine growth factors and neurotransmitters, such as angiotensin II, endothelin-1 (ET-1), and α_1-adrenergic agonist phenylephrine (PE), can activate PKC.

Cardiac myocytes express members of all three subfamilies of PKC: one classic isoform (PKCα), two novel isoforms (PKCδ and PKCϵ), two atypical isoforms (PKCζ and PKCλ/ι), and possibly PKCβI and PKCβII (Rybin and Steinberg, 1994; Sugden and Clerk, 1998; Mackay and Mochly-Rosen, 2001). Direct activation of classic and novel PKCs by phorbol esters, mimicking the actions of ET-1 and PE, is known to promote cardiomyocyte hypertrophy (Sugden and Clerk, 1998). Moreover, studies with pharmacological inhibitors of PKC have

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ABBREVIATIONS: α-SkA, skeletal α-actin; β-MHC, β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; PKC, protein kinase C; PE, phenylephrine; PMA, phorbol 12-myristate 13-acetate; ET-1, endothelin-1; ODN, oligodeoxynucleotide; DMEM, Dulbecco's modifed Eagle's medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; ERK, extracellular signal-regulated kinase; RT-PCR, reverse-transcriptase polymerase chain reaction; PD98059, 2′-amino-3′-methoxyflavone; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; PAGE, polyacrylamide gel electrophoresis.

revealed a major role for PKC in regulation of hypertrophic response in cardiac myocytes (Uusimaa et al., 1992; Hanford and Glembotski, 1996). Despite studies with transgenic mice and ventricular myocytes overexpressing PKC isoforms (Sugden and Clerk, 1998; Molkentin and Dorn, 2001), the downstream targets of the different isozymes remain largely unknown.

Translocation of PKC isoforms to distinct intracellular sites is recognized as an essential step in activation of different PKC isoforms (Mackay and Mochly-Rosen, 2001). PKCα, the major calcium-dependent PKC isozyme expressed in neonatal cardiac myocytes, is located in the soluble fraction in resting cells, and an increase in calcium concentration selectively translocates PKCα to the particulate fraction (Rybin and Steinberg, 1994). Exposure of cardiac myocytes to phorbol 12-myristate 13-acetate (PMA) or the α-adrenergic receptor agonist norepinephrine has been shown to translocate PKCα to the perinuclear membrane (Disatnik et al., 1994). Hypertrophic agonists PE and ET-1, in turn, have been shown to increase the proportion of membrane-associated PKCε and PKCδ, whereas translocation of PKCα to the cell membrane has not been detected (Clerk et al., 1994; Puceat et al., 1994). In previous studies, overexpression of PKCα has been shown to induce hypertrophic growth in neonatal cardiac myocytes (Shubeita et al., 1992; Braz et al., 2002). Calcium-independent isozyme PKCε has a significant role in regulating hypertrophy in adult rat cardiac myocytes, whereas the role of PKCα in adult cardiomyocytes is less important (Sugden and Clerk, 1998; Mackay and Mochly-Rosen, 2001).

Antisense oligonucleotides (ODNs) can be used to disrupt gene function in a variety of in vitro culture systems and in vivo, and they have proven potential in clinical use [e.g., in treating AIDS and leukemias (Beltinger et al., 1995)]. In the present study, we used antisense approach to inhibit PKCα, the major PKC isozyme present in neonatal heart. To evaluate the significance of PKCα in cardiomyocyte hypertrophy, *α-SkA* and *β-MHC* gene expression, as well as natriuretic peptide secretion and gene expression, was studied. To test the hypothesis of PKCα signaling interconnecting with other signaling pathways, we also measured possible downstream targets of PKCα, such as ERK (extracellular signal-regulated kinase) and calcineurin. Our results indicate that PKCα participates in hypertrophic signaling in cardiomyocytes and that there is a high selectivity in the proximal signaling pathways activated by hypertrophic agonists.

Materials and Methods

Cell Culture, Transfections, and Immunocytochemistry. Ventricular cardiomyocytes were prepared from 2- to 4-day-old Sprague-Dawley rats (Tokola et al., 1994). Cells were plated at a density of $2 \times 10^5/cm^2$ onto Falcon wells from 15 to 35 mm in diameter. After a 16-h incubation, myocytes were subjected to liposome-mediated transfection with FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche), or Tfx-50 (Promega, Madison, WI) for 6 h. Oligonucleotides (20 bases) used were phosphorothioated to increase nuclease resistance and support RNase H cleavage of hybridizing RNA. Sequence for antisense PKCα ODN was 5′-ATTATCTCTGGTGATTTGGA-3′ targeting to 3′ untranslated sequence (As1) on the rat PKCα cDNA, and for scrambled ODN, 5′-GTGATATGTGCAGTTATTTC-3′. Two other antisense PKCα oligonucleotides used were 5′-TAAACGTCAGCCATGGTCCC-3′, targeting to 5′ untranslated sequence (As2), and 5′-TTAGCGATGACCAGCTGATC-3′, targeting to coding sequence (As3) on the rat PKCα cDNA. After transfection, cells were washed twice with DMEM and cultured in complete serum-free medium. When appropriate, 100 μM PE or 100 nM ET-1 (Sigma, St. Louis, MO) were added to culture medium on the third day in culture. The doses of PE (100 μM) and ET-1 (100 nM) (Kerkelä et al., 2002) have previously been shown to result in cardiomyocyte hypertrophy.

For immunocytochemistry, cardiomyocytes were grown on glass coverslips. Cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde containing 0.2% Triton X-100, and then washed three times in PBS and once with pure ethanol at −20°C. Cells were next placed in ice bath, washed three times with cold PBS, and blocked in PBS containing 10% fetal bovine serum and 0.02 M glycine. Sarcomeric organization was visualized by labeling α-actin filaments with Alexa Fluor 568 Phalloidin (Molecular Probes, Eugene, OR). PKCα was assessed using antibody against PKCα (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:400 in blocking solution. Subsequently, cells were washed twice with cold PBS-glycine, and anti-mouse 3′-fluorescein isothiocyanate (FITC)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at a dilution of 1:200 in blocking solution. Finally, cells were washed twice with PBS, once with H_2O, and subjected to fluorescence microscopy.

Confocal Microscopy. Cardiac myocytes were cultured on glass-bottomed wells and transfected with complexes of FITC-labeled ODNs and FuGENE 6 as described above. The localization of 3′ FITC-labeled ODNs in spontaneously beating ventricular myocytes was examined using laser scanning confocal microscopy (LSM 510; Zeiss, Thornwood, NY) equipped with argon laser and attached to a Zeiss Axiovert 100 TV microscope. Differential interference contrast and green fluorescence channels were used.

Immunoblot Analysis. Cells were washed twice with ice-cold PBS and collected by scraping into 500 μl of lysis buffer, which consisted of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 50 mM NaF. Extracts were further lysed with sonication and supernatant was collected after centrifugation. For western blot analysis, cell lysates were matched for protein concentration (10–20 μg), loaded on SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5% nonfat milk and then incubated with PKCα (Upstate Biotechnology), PKCδ, PKCε, PKCζ or PKCβII (Sigma) antibodies overnight at 4°C. Amount of protein was detected by enhanced chemiluminescence using hyperfilm *MP* from Amersham International (Amersham, Bucks, UK).

Kinase Assays. After treatment with appropriate agonists, myocytes (approximately 1×10^6) were washed with ice-cold PBS at room temperature. Samples for PKC activity and ERK activity assays were collected by scraping into 100 μl of lysis buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin, and 5 mM benzamidine. The extracts were sonicated and supernatant was collected after centrifugation. Protein extract (15 μl) was incubated at 30°C for 15 min with 10 μl of substrate buffer containing specific substrate peptide in the presence of 1 μCi of [γ-^{32}P]ATP. Each reaction was terminated and blotted on separate peptide-binding paper discs, which were repeatedly washed with 75 mM orthophosphoric acid. The incorporated radioactivity was measured with a scintillation counter (Rackbeta II; PerkineElmer Wallac, Turku, Finland). The Biotrak assays for PKC and ERK activity were provided by Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). The PKC assay lysis buffer contains EGTA, but calcium is added back to the assay buffer, which also includes lipid

cofactors. The Biotrak assays contain the substrates, which are synthetic peptides specific for PKC and ERK, respectively.

Radioimmunoassay. Assay of immunoreactive ANP and BNP was performed as described previously (Pikkarainen et al., 2002). The sensitivity of both assays was 1 fmol/tube. The intra- and inter-assay variations were <10 and <15%, respectively. Serial dilutions of the tissue extracts showed parallelism with the standards. The ANP antiserum fully cross-reacts with pro-ANP, but there is no cross-reaction with BNP or C-type natriuretic peptide (< 0.1%). The BNP antiserum does not cross-react with ANP or C-type natriuretic peptide (< 0.1%).

RT-PCR Analysis. PKCα mRNA levels for rat samples were measured by quantitative reverse transcription-PCR analysis using TaqMan chemistry. Forward and reverse primers for PKCα mRNA detection were AGACCACAAATTCATCGCCC and CAAACCCCCA-GATGAAGTCG, respectively. PKCα amplicon was detected using fluorogenic probe 5′–5-carboxyfluorescein–CCCACCTTCTGCAGC-CACTGCA–5-carboxytetramethylrhodamine–3′. The results were normalized to 18S RNA quantified from the same samples using the forward and reverse primers TGGTTGCAAAGCTGAAACTTAAAG and AGTCAAATTAAGCCGCAGGC, respectively. The probe for the 18S amplicon was 5′–VIC–CCTGGTGGTGCCCTTCCGTCA–5-carboxytetramethylrhodamine–3′.

RNA Extraction and Northern Blot Analysis. RNA was isolated from ventricular myocytes by the guanidine thiocyanate-CsCl method. For Northern blot analysis, 6 μg of RNA sample was transferred to nylon membrane. Full-length ANP and BNP probes were labeled with [^{32}P]dCTP using T^7 Quick Prime Kit (Amersham Biosciences). The ANP Probe was a kind gift from Dr. Peter L. Davies (Queen's University, Kingston, ON, Canada). cDNA probes for rat α-SkA and rat β-MHC were made by RT-PCR technique. Sequencing showed that the probes correspond to bases 2950 to 3184 (GenBank/EMBL accession number v01218) and 5794 to 5923 (GenBank/EMBL accession number x15939), respectively. The membranes were hybridized overnight at 42°C in 5× saline sodium citrate, 0.5% SDS, 5× Denhardt's solution, 50% formamide, and 0.1 mg/ml sheared salmon sperm DNA. After hybridizations, membranes were washed in 0.1× saline sodium citrate and 0.1% SDS three times at 65°C and subjected to analysis with the Molecular Imager FX (Bio-Rad, Hercules, CA).

Protein Synthesis. [^{3}H] Leucine incorporation was measured as described previously (Kerkelä et al., 2002). When appropriate, PE (100 μM) and ET-1 (100 nM) were added, and after 24 h, cells were lysed and processed for measurement of incorporated [^{3}H]leucine (Amersham Biosciences) by liquid scintillation counting.

Statistics. Differences between data groups were evaluated for significance using a Student's t test of unpaired data or one-way analysis of variance and Bonferroni's post-test. Results are expressed as mean ± S.E.M.

Results

Antisense Delivery. Transfecting cardiac myocytes has been found difficult compared with many other cell types. Different transfection methods, such as calcium phosphate precipitation and electroporation, have been applied to transfect DNA into the cells. Several different cationic liposome carriers have also been designed to increase cellular uptake of ODNs, and they have been demonstrated to increase the transfection efficiency up to 1000-fold (Bennett et al., 1992). In the current study, we used three different cationic lipids to optimize the transfection method and to ensure that there was no interference of the lipid treatment with the results. In multiple experiments, highest transfection efficiency was achieved with FuGENE 6, which also showed least toxicity analyzed with microscope imaging of the cells. To examine the transfection efficiency of the antisense PKCα ODNs using the cationic liposome delivery system, fluorescent-tagged ODNs (500 nM) corresponding to PKCα were added with FuGENE 6, and the cells were subjected to confocal microscopy. The data in Fig. 1 demonstrate that in the presence of FuGENE 6, both antisense and scrambled ODNs were effectively delivered into the cells. Proportion of fluorescent-tagged ODN transfected myocytes was ~60 to 80%. The strongest fluorescence was seen in the areas around the nucleus. On the second day after the transfection, fluorescence staining was decreased but still substantial. In the absence of cationic liposome carrier, only minimal transfection efficiency was observed (data not shown).

Effects of Antisense PKCα ODN Treatment on PKC. Several oligonucleotides, each 20 bases in length, were designed to hybridize to different regions of rat PKCα mRNA. Myocytes were transfected by using FuGENE 6 as cationic lipid. To verify that the results were not influenced by the lipid treatment, the experiments were re-

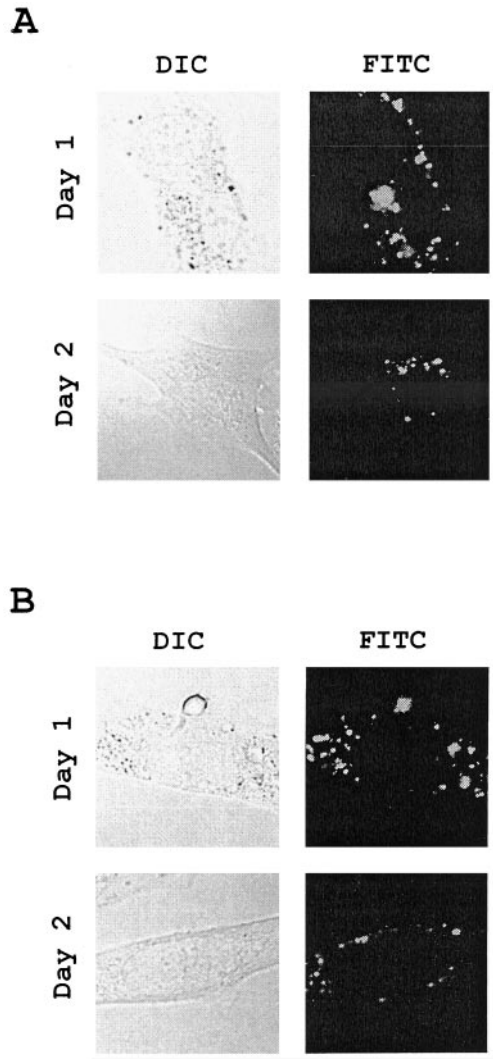

Fig. 1. Cellular uptake of 3′ FITC-labeled oligonucleotides. Neonatal rat ventricular myocytes were transfected with antisense (A) and scrambled (B) ODNs using FuGENE 6 as liposome carrier. After a 6-h transfection period, cellular internalization of FITC-labeled oligonucleotides was examined using an LSM 510 laser scanning confocal microscopy (day 1). After a 24-h incubation period, confocal microscope imaging of the cells was repeated (day 2). DIC, differential interference contrast image; FITC, laser confocal fluorescence image.

peated using also *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate and Tfx-50 as lipids. Treatment of myocytes with the PKCα antisense ODN (0.5 μM) targeted to 3′ untranslated sequence (As1) on rat PKCα cDNA reduced PKCα mRNA levels by 50% compared with scrambled ODN (Fig. 2A).

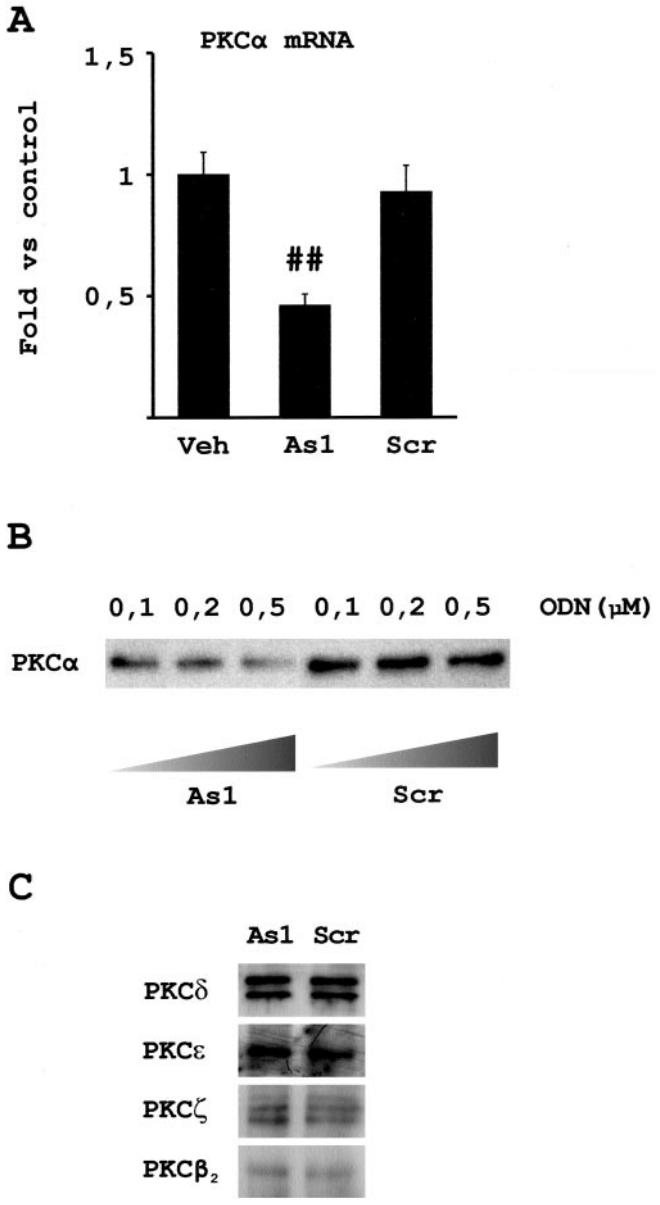

Fig. 2. PKCα synthesis. A, RT-PCR analysis showing the effect of antisense PKCα (As1) and scrambled (Scr) ODN treatments on PKCα mRNA levels. mRNA results are expressed as ratio to 18S, as determined by RT-PCR analysis. Each bar represents results of four to six separate experiments. Similar results were obtained in three independent cell cultures. ##, $p < 0.01$ compared with scrambled ODN-treated cells. B, representative immunoblots of neonatal rat cardiomyocyte lysates resolved by SDS-PAGE and probed with PKCα-specific antibody. Detection was with horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence. Neonatal ventricular myocytes were transfected with three different concentrations of antisense PKCα or scrambled ODNs (0.5, 0.2, and 0.1 μM). FuGENE 6 was used as a cationic lipid in the ratio of 1:2 (ODN/FuGENE 6). C, representative immunoblots of neonatal rat cardiomyocyte lysates resolved by SDS-PAGE and probed with PKCδ-, PKCε-, PKCζ-, and PKCβII-specific antibodies. Neonatal ventricular myocytes were transfected with antisense PKCα or scrambled ODNs (0.5 μM).

The levels of 18S RNA were unaffected by the PKCα antisense ODN treatment.

In agreement with changes in PKCα mRNA levels, treatment with the antisense PKCα ODN reduced PKCα protein levels by more than 60%. Dose-response studies in multiple experiments revealed that maximal reduction in PKCα protein levels was achieved at 0.5 μM, whereas control ODN with the same base composition as the antisense ODN, but with a scrambled sequence, had no effect (Fig. 2B). On the other hand, no difference was found between samples from antisense PKCα and scrambled ODN-treated myocytes when subjected to Western blot analysis using antibodies against PKCδ, PKCε, PKCζ, or PKCβII (Fig. 2C). In untreated control cells, PKCα staining was diffuse around the cell, and treatment with PE (100 μM) for 5 min translocated PKCα to the perinuclear region (Fig. 3) as observed previously (Disatnik et al., 1994; Braz et al., 2002). In antisense PKCα ODN-treated cells, fluorescence staining for PKCα was dramatically decreased, whereas in control ODN-treated cells significant amount of PKCα was seen around the nucleus (Fig. 3).

Effect of PKCα antisense treatment on PE-induced PKC activity was studied by measuring the transfer of a phosphate group to a peptide substrate highly selective for protein kinase C. As reported previously (Clerk et al., 1994), PE (100 μM) was found to be a strong activator of PKC. PE

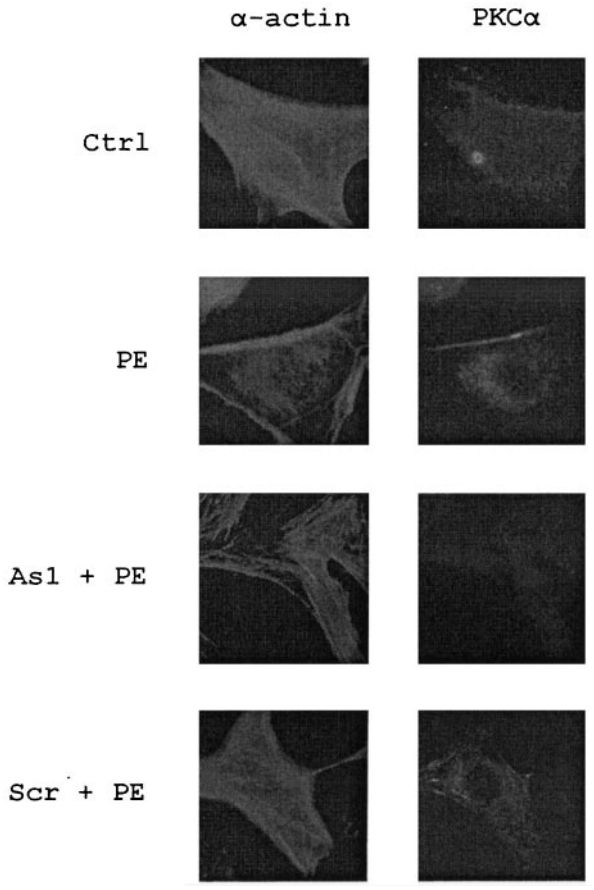

Fig. 3. PKCα expression. Myocytes were plated onto glass coverslips and transfected with PKCα antisense ODNs (AS) or corresponding scrambled ODNs (Scr). After liposomal transfection, cells were washed, incubated in serum-free medium for 48 h, and, when indicated, exposed to PE (100 μM) for 5 min. Cells were then fixed and stained for α-actin with Alexa Fluor 568 Phalloidin and for PKCα with anti-PKCα antibody.

treatment evoked a 2.8-fold increase in PKC activity at 4 min, which was significantly inhibited with pretreatment of cells with PKCα antisense ODNs (Fig. 4A). A similar result was also seen at 30 min, although the response of PKC to PE had already markedly decreased (Fig. 4B). The two other antisense PKCα sequences targeting to 5′ (As2) and coding (As3) sequence of the rat PKCα cDNA had no effect on the PE-induced PKC activity (Fig. 4B).

Effect of Antisense ODN Inhibition of PKCα on Hypertrophic Phenotype. To assess whether treatment of myocytes with PKCα antisense oligonucleotide was sufficient to influence cardiomyocyte hypertrophy, we measured expression of two hypertrophic genes, *β-MHC* and *α-SkA*. Treatment with PE for 48 h induced a 1.6-fold increase in α-SkA and a 2.1-fold increase in β-MHC mRNA levels (Fig. 5, A and B). Inhibition of PKCα was sufficient to inhibit *α-SkA*

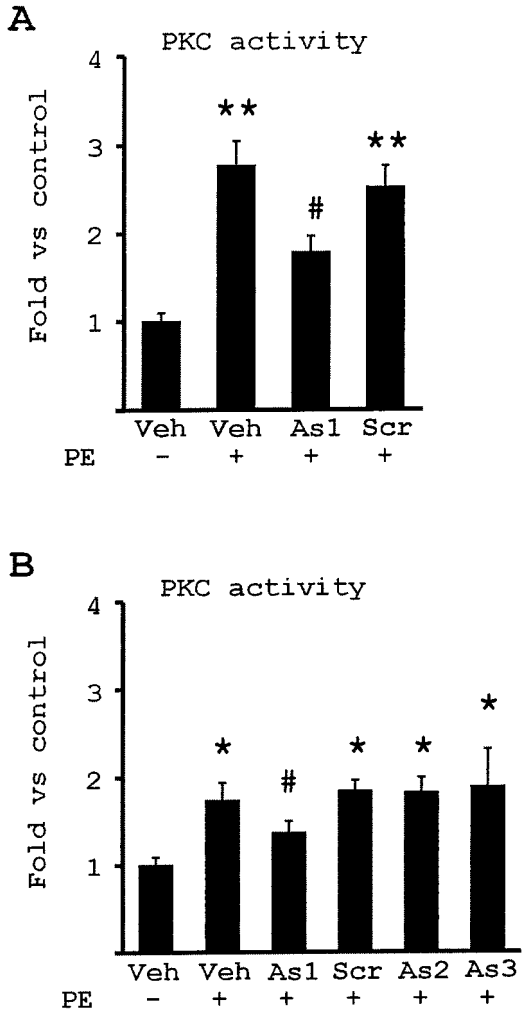

Fig. 4. PKCα activity. Myocytes were transfected with PKCα antisense ODNs (As1), corresponding scrambled ODNs (Scr), or two other antisense ODNs (As2 and As3) targeted to different areas on PKCα cDNA. After liposomal transfection, cells were washed, incubated in serum-free medium for 24 h, and exposed to PE (100 μM) for 4 min (A) or 30 min (B). After cell lysis, protein extracts were subjected to kinase activity assay, which was accomplished by measuring the transfer of radioactive phosphor by active PKC. Each bar represents results of four to six separate experiments obtained from three independent cell cultures. *, $p < 0.05$ compared with untreated control cells; **, $p < 0.01$ compared with untreated control cells; #, $p < 0.05$ compared with scrambled ODN-treated cells.

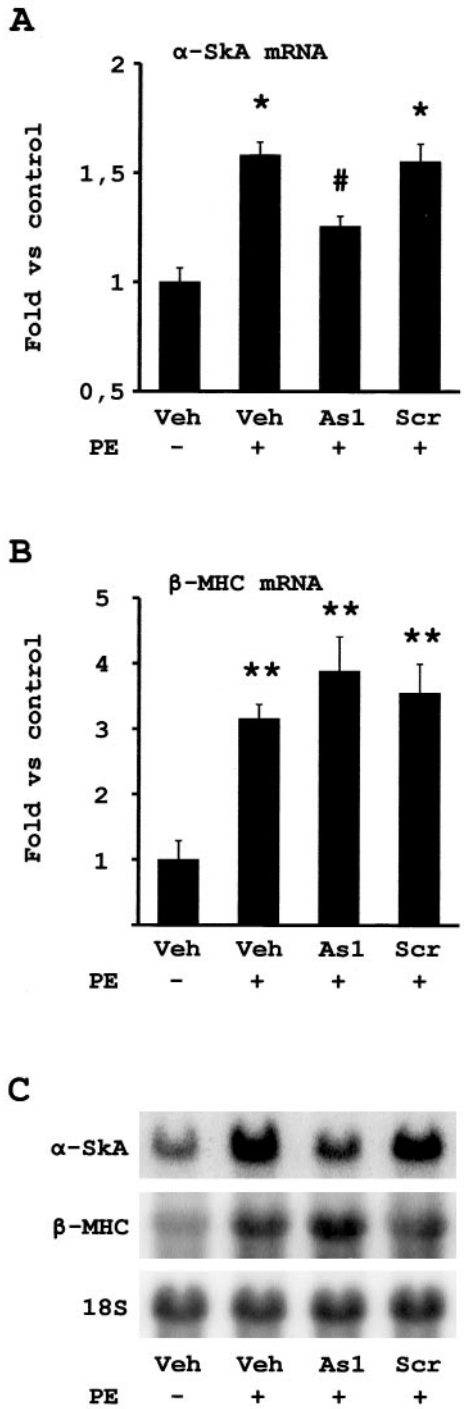

Fig. 5. Hypertrophic gene expression. Myocytes were transfected with PKCα antisense ODNs or corresponding scrambled ODNs. Cells were washed twice with DMEM and serum-free medium was added. When appropriate, cells were exposed to PE (100 μM) for 24 (C and D) or 48 (A and B) h. A, Northern blot analysis showing the effect of antisense and scrambled ODN treatments on α-SkA mRNA levels. B, Northern blot analysis showing the effect of antisense and scrambled ODN treatments on β-MHC mRNA levels. Results are expressed as ratio to 18S, as determined by Northern blot analysis. Values represent the mean ± S.E.M. from four to six separate experiments. Similar results were obtained in three independent cultures. **, $p < 0.001$ compared with untreated control cells; *, $p < 0.01$ compared with untreated control cells; #, $p < 0.05$ compared with scrambled ODN-treated cells. C, representative Northern blots illustrating the effect of antisense PKCα or scrambled ODN treatment on *α-SkA* and *β-MHC* gene expression.

gene expression by 30% ($p < 0.05$), whereas *β-MHC* gene expression was not affected.

In a previous study, calcineurin has been suggested to regulate *α-SkA* gene expression (Bueno et al., 2002). To examine whether PKCα is an upstream regulator of calcineurin, we next determined the effect of PKCα inhibition on PE-induced calcineurin activity. In agreement with previous findings (Taigen et al., 2000), PE evoked a 4-fold induction in calcineurin activity (1.18 ± 0.12 versus 0.29 ± 0.08 μM PO_4 released, $p < 0.001$). Transfecting the cells with PKCα antisense ODNs had no significant effect on calcineurin activity (data not shown).

Increased ERK activity is associated with hypertrophic growth of cardiomyocyte, and several studies have found ERK as a downstream target of PKC (Goldberg et al., 1997; Ho et al., 1998; Sugden and Clerk, 1998; Molkentin and Dorn, 2001). To examine the role of α subunit of PKC on PE-induced ERK activity, an assay measuring transfer of phosphate by active ERK was used. We found that PE was a potent activator of ERK. Antisense PKCα treatment did not have significant effect on ERK activity at 4 min (Fig. 6A) or at 30 min (data not shown). Interestingly, at 24 h, the PE-induced increase in ERK activity was partially inhibited with antisense PKCα ODN treatment (Fig. 6B).

PE and ET-1 have been shown to increase intracellular calcium concentration transients in cultured neonatal cardiac myocytes (Furukawa et al., 1992; Eble et al., 1998). Using single-cell imaging of Fluo-3–loaded (10 μM; Molecular Probes) cultured cardiac myocytes, we found that PE induced a significant increase in intracellular calcium levels (218 ± 37%, $p < 0.001$). Antisense PKCα ODN treatment had no effect on PE- or ET-1–induced calcium concentration transients or spontaneous myocyte beating rate (data not shown).

To study the effect of PKCα inhibition on protein synthesis, another major hallmark of cardiac hypertrophy, we measured [^{3}H]leucine incorporation into myocytes and total protein content of the myocytes. Treatment of myocytes with PE for 24 h induced a 2.7-fold increase in leucine incorporation and a 1.5-fold increase in total protein content. As shown in Fig. 7, treatment of cells with antisense PKCα ODNs had no effect on PE-induced increase in protein synthesis or total protein content. In agreement with previous studies, ET-1 induced a 2.5-fold increase in [^{3}H]leucine incorporation that was not affected by antisense PKCα ODN treatment (Kerkelä et al., 2002). Antisense PKCα ODN treatment had no effect on PE- or ET-1–induced increase in myocyte size and myofilament organization (data not shown).

Effect of Antisense PKCα Treatment on Hypertrophic Agonist-Induced Natriuretic Peptide Secretion and Gene Expression. Under basal conditions, natriuretic peptide secretion from cultured neonatal ventricular myocytes is low compared with atrial myocytes and gradually decreases over time (Tokola et al., 1994). Treatment of myocytes with antisense PKCα ODNs had no effect on basal ANP or BNP secretion (data not shown). PE (100 μM) treatment increased ANP and BNP release by up to 32- and 15-fold, respectively, associated with 3.1- and 1.8-fold increase in ANP and BNP mRNA levels, respectively (Figs. 8 and 9). Inhibition of PKCα with antisense ODNs significantly inhibited PE-induced ANP release (Fig. 8A), whereas it had no effect on PE-induced BNP release (Fig. 8B). Antisense treatment had no effect on PE-induced increase in ANP (Fig. 9A) or BNP (Fig. 9B) mRNA levels.

To further characterize the significance of PKCα on hypertrophic signaling, we also studied effects of PKCα inhibition on ET-1–induced natriuretic peptide release and gene expression. ET-1 (100 nM) increased ANP and BNP secretion up to 3- and 10-fold, respectively. Interestingly, antisense inhibition of PKCα resulted in a marked decrease in both ET-1–induced ANP and BNP secretion (Fig. 8, C and D). Similar to PE stimulus, inhibition of PKCα had no effect on ET-induced increase in ANP or BNP mRNA levels (data not shown).

Discussion

Several studies have previously shown that hypertrophic phenotype of cardiomyocytes can be regulated by PKC (Shubeita et al., 1992; Clerk et al., 1994; Sugden and Clerk, 1998;

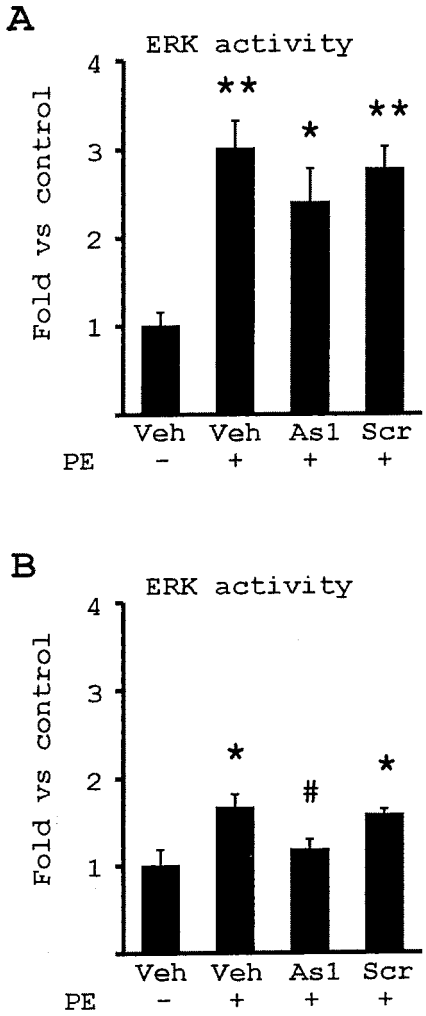

Fig. 6. ERK activity. Myocytes were transfected with PKCα antisense ODNs (As1) or corresponding scrambled ODNs (Scr). After liposomal transfection cells were washed, incubated in serum free medium for 24 h, and exposed to PE (100 μM) for 4 min (A) or 24 h (B). After cell lysis, proteins extracts were subjected to kinase activity assay, which was accomplished by measuring the transfer of radioactive phosphor by active p42/44 MAP kinase. Each bar represents results of 4–6 separate experiments obtained from three independent cell cultures. *, $p < 0.05$ compared with untreated control cells. **, $p < 0.01$ compared with untreated control cells. #, $p < 0.05$ compared with scrambled ODN-treated cells.

Braz et al., 2002), yet only limited data exist concerning the roles of different PKC isozymes in hypertrophic signaling and in the development of hypertrophic phenotype. Because of the lack of specific pharmacological inhibitors, studies concerning the role of different isozymes of PKC in development of cardiac hypertrophy have been accomplished by using antisense strategy or dominant negative constructs (Sugden and Clerk, 1998; Molkentin and Dorn, 2001; Strait et al., 2001). Indirect evidence of PKC participation in hypertrophic signaling has been obtained by studying translocation of PKC isozymes. In two studies using an aortic banding-induced pressure overload model, increased PKCα, PKCϵ, and PKCγ translocation and up-regulation of total PKCβ were observed (Gu and Bishop, 1994; Jalili et al., 1999). In a genetic $G_{\alpha q}$-mediated hypertrophy model, up-regulation of PKCα and PKCϵ activation were also noted (D'Angelo et al., 1997; Dorn et al., 2000). In transgenic animals, overexpression of *PKCβII* under truncated α-myosin heavy chain promoter was sufficient to produce hypertrophic phenotype (Wakasaki et al., 1997). A recent study using wild-type and dominant-negative mutant of PKC isozymes also implied a necessary role for PKCα in hypertrophic cardiomyocyte growth (Braz et al., 2002). In contrast, results of the current study suggest that PKCα plays a highly selective role in the induction of cardiomyocyte hypertrophy.

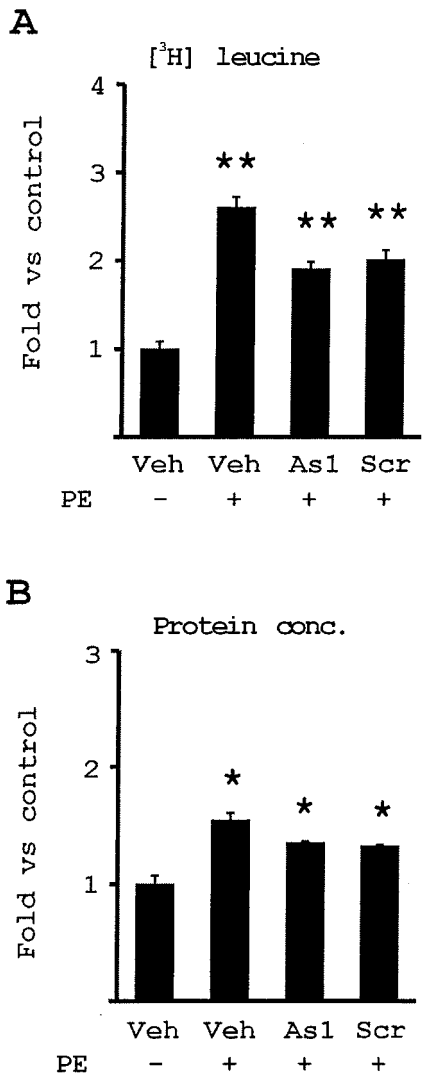

Fig. 7. Protein synthesis. Cardiac myocytes were plated onto 24-well Falcon plates and transfected with antisense PKCα or scrambled oligonucleotides according to protocols. On the third day of the culture, medium was replaced with complete serum-free medium supplemented with [^{3}H]leucine (5 μCi/ml) and cells were treated with PE (100 μM) After 24 h, cells were lysed and processed for total radioactivity of incorporated [^{3}H]leucine as determined by liquid scintillation counting (A) and total protein content (B); protein concentrations were obtained by spectrophotometric quantification. **, $p < 0.001$ compared with untreated control cells; *, $p < 0.01$ compared with untreated control cells; #, $p < 0.05$ compared with scrambled ODN-treated cells.

Antisense oligonucleotides hybridizing to different sites of mRNA have been shown to effectively block gene expression (Dean et al., 1994). In the current study, antisense oligonucleotide targeting 3′-untranslated sequence on the rat PKCα cDNA was most effective in inhibiting PKCα protein synthesis, which is similar to findings in previous studies using antisense PKCα oligonucleotides (Dean et al., 1994; Benimetskaya et al., 2001). The specificity of antisense treatment was also shown, because other cardiac isozymes of PKC were not affected by antisense PKCα treatment. The PKC activity assay also revealed a significant decrease in agonist-induced PKC activity in PKCα antisense ODN-treated cells. Antisense PKCα treatment had no effect on PKC activity in resting cells, probably because of low basal PKC activity.

Increased protein synthesis is a major hallmark of hypertrophic phenotype in cardiac myocytes. In the current study, inhibition of PKCα had no effect on PE- or ET-1–induced increase in protein synthesis or total protein content of the cells. Previously, PE-induced protein synthesis was not affected by pretreatment of cultured myocytes with TPA, also suggesting that conventional PKC activation might not be essential for mediating hypertrophic growth (Kondo et al., 1999). On the other hand, in myotropin-induced hypertrophic model both PKCα and PKCϵ isoforms were shown to participate in cardiomyocyte growth (Sil et al., 1998). In a recent study, overexpression of dominant-negative PKCα attenuated PE-induced protein synthesis, favoring the role of PKCα in hypertrophic cardiomyocyte growth (Braz et al., 2002). The reasons for these contradictory results are not clear, but may be attributable to different cell culture conditions and unequal duration of experiments (6 h versus 24 h). Treatment of myocytes with dominant-negative PKCα may also produce a longer lasting effect than antisense treatment, although the effect of dominant-negative PKCα treatment on PKC activity was not measured in the previous study (Braz et al., 2002). The reduction in PKCα achieved in the current study was approximately 60%, which may also explain the failure to block the PE-induced protein synthesis as well as PE-induced increases in *β-MHC*, *ANP*. and *BNP* gene expression.

The expression of several sarcomeric protein encoding genes is switched to expression of fetal isoforms in response to hypertrophic stimuli [e.g., transition from cardiac α-actin to *α-SkA* and from the α-form of myosin heavy chain to the *β-MHC* form (Sugden and Clerk, 1998)]. In our studies, PE induced a significant increase in both α-SkA and β-MHC mRNA levels. Antisense ODN inhibition of PKCα was sufficient to decrease PE-induced increase in α-SkA mRNA levels, but it had no effect on β-MHC gene expression. Previous studies have suggested a role for calcineurin in the regulation of α-SkA gene expression, but not in the regulation of β-MHC gene expression, implying a difference in mechanisms regulating these genes (Bueno et al., 2002). Our re-

sults suggest that the regulation of α-SkA gene expression involves PKCα, whereas calcineurin is not a downstream mediator of PKCα. The PKCα signaling pathway regulating *α-SkA* gene expression is likely to involve other mechanisms, possibly transcription factors regulated by ERK.

The ability of PKC to induce Ras and Raf, upstream activators of ERK, has previously been demonstrated (Sugden and Clerk, 1998), but the physiological significance remains unclear. In the current study, inhibition of PKCα with antisense PKCα ODNs also resulted in a modest but significant decrease in PE-induced ERK activity. Role of ERK in the development of hypertrophic phenotype remains a controversy (Yue et al., 2000; Kerkelä et al., 2002). Current data shows that antisense PKCα inhibition of ERK pathway is not sufficient to prevent hypertrophic agonist-induced protein synthesis. A previous study using a pharmacological inhibitor of ERK, PD98059 (50 and 20 μM, respectively), agrees with these findings (Kerkelä et al., 2002).

Treatment of cells with PKC-activating phorbol esters, such as PMA, has revealed a pivotal role for PKC in the regulation of ANP and BNP gene expression and secretion (Ruskoaho, 1992; LaPointe and Sitkins, 1993). Studies using pharmacological inhibitors of PKC, staurosporine, and GF109203X have further supported a role for PKC in regulation of the natriuretic peptide system (Uusimaa et al., 1992; Hanford and Glembotski, 1996). Previously, transient overexpression of constitutively active PKCα and PKCβ in neonatal ventricular myocytes has led to increased activity of ANP and myosin light chain-2 promoter constructs (Shubeita et al., 1992). Recently, overexpression of dominant-negative mutants of PKCα was sufficient to block PE-induced ANP protein expression (Braz et al., 2002). Surprisingly, we found that transient inhibition of PKCα only led to the reduction in peptide levels, but it was not associated with decrease in natriuretic peptide mRNA levels. The result cannot have been caused by poor transfection efficiency, because the samples for radioimmunoassay and Northern blot analysis were from the same cells. Cellular mechanisms regulating secretion of natriuretic peptides are poorly understood. However, previous findings suggest that ANP secretion from atrial

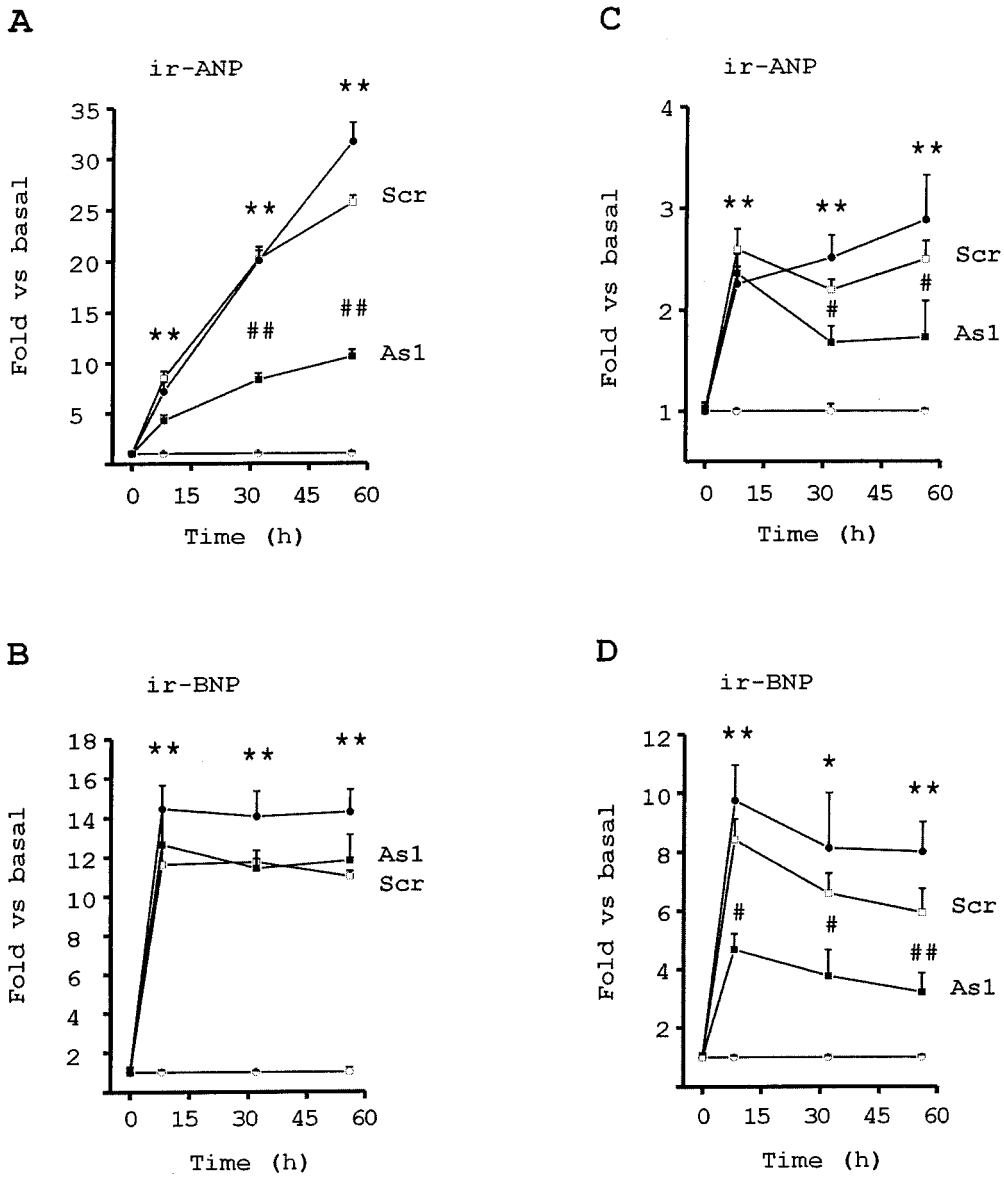

Fig. 8. ANP and BNP secretion. Myocytes were transfected with PKCα antisense ODNs or corresponding scrambled ODNs. Medium samples for ANP (A and C) and BNP (B and D) measurements were taken before the transfection, and 8, 32, and 56 h after start of PE (A and B) or ET-1 (C and D) treatments. ○, control; ●, PE; □, scrambled ODN and PE; ■, antisense ODN and PE. The results are indicated as mean ± S.E.M. for six independent experiments. Concentrations are expressed relative to those measured before transfection for each group. Similar results were obtained in three independent cultures. **, $p < 0.001$ compared with untreated control cells; *, $p < 0.01$ compared with untreated control cells; ##, $p < 0.001$ compared with scrambled ODN-treated cells; #, $p < 0.05$ compared with scrambled ODN-treated cells.

cells is stimulated both by the increase in calcium levels and by the activation of PKC, whereas ANP synthesis is mainly stimulated by the activation of PKC (Suzuki et al., 1992). Our results indicate that PKCα, the major calcium-dependent isozyme in neonatal ventricular myocytes, plays a pivotal role in the regulation of ANP and BNP secretion. Recent studies on exocytosis and membrane fusion give further support for the role of calcium in this process (Hu et al., 2002; for review, see Tavi et al., 2001). Because PE and ET-1 both increase intracellular calcium concentration transients, it is intriguing to speculate whether antisense PKCα treatment has effect on intracellular calcium oscillations (Furukawa et al., 1992; Eble et al., 1998). Using confocal microscopy imaging of Fluo-3–loaded myocytes, we did not find a difference in intracellular calcium transients between antisense PKCα and scrambled ODN-treated myocytes.

Treatment of cells with the PKCα antisense ODN led to marked reduction in PE-induced ANP secretion and had no effect on BNP secretion. On the other hand, ET-1–induced ANP and BNP secretion were inhibited with PKCα antisense treatment. Factors contributing to this differential regulation of ANP and BNP secretion are not clear. ET-1 and PE induced activation of downstream kinases are both known to involve G_q, whereas G_i signaling is only associated with ET-1. Treatment with high concentration of PE (100 μM) may also stimulate G_s via β-adrenergic receptors and thus result in the observed difference in the regulation of ANP and BNP secretion (Ruskoaho, 1992). G protein-coupled receptors are known to activate MAP kinases via Ras-dependent pathway, although there is also data suggesting that G_q-mediated activation of ERK pathway may be fully or partially PKC-dependent (Gutkind, 1998). Another mechanism involved may be differential phosphorylation of PKC isozymes by PE and ET-1. Whereas PE activates PKCϵ and ET-1 activates PKCϵ and PKCδ, membrane-bound diacylglycerol and PMA are known to activate PKCα and PKCϵ (Clerk et al., 1994; Puceat et al., 1994). Further studies are required to elucidate differences in downstream signaling mechanisms of ET-1 and PE, responsible for differing PKCα-dependent regulation of ANP and BNP secretion.

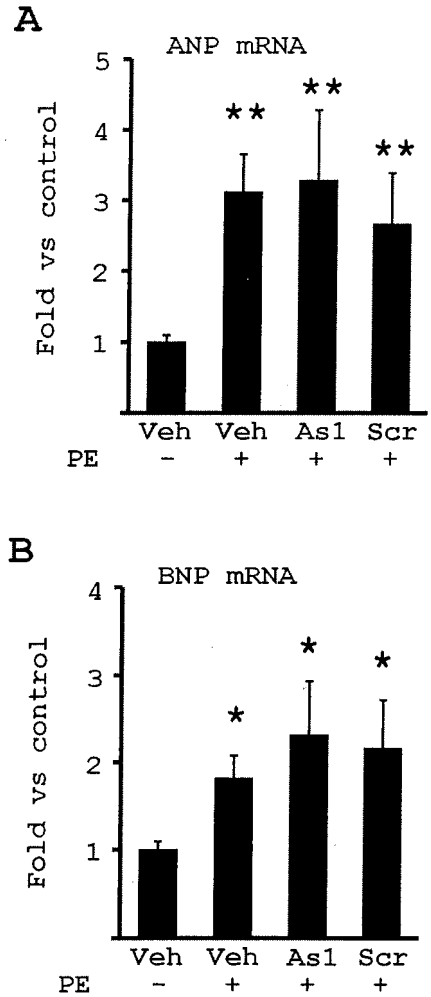

Fig. 9. ANP and BNP gene expression. Myocytes were transfected with PKCα antisense ODNs or corresponding scrambled ODNs. Cells were washed twice with DMEM and, when appropriate, exposed to PE (100 μM). After 56 h, cells were washed and RNA was extracted. mRNA results are expressed as ratio of ANP or BNP mRNA to 18S, as determined by Northern blot analysis. Data are expressed as mean ± S.E.M. for four independent experiments. Similar results were obtained in three independent cultures. **, $p < 0.001$ compared with untreated control cells; *, $p < 0.01$ compared with untreated control cells.

Taken together, these data indicate that PKCα plays a highly selective role in the regulation of hypertrophic cardiomyocyte growth. PKCα is involved in α-*SkA* gene expression, possibly by mechanisms involving ERK, but not in β-*MHC* gene expression. In addition, PKCα is required for PE- and ET-1–induced natriuretic peptide secretion, but antisense inhibition of PKCα is not sufficient to block hypertrophic agonist-induced *ANP* or *BNP* gene expression. We conclude that PKCα participates in the regulation of hypertrophic agonist-induced α-*SkA* gene expression and natriuretic peptide secretion but has a minor role in the development of hypertrophic phenotype. Although the role of all isozymes of PKC in the hypertrophic response in neonatal rat cardiac myocytes is not clear, these data provide novel strategies for future investigations, which are likely to identify other cellular targets controlled selectively by PKC isozymes.

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