Increased Protein Kinase C Activity in Myotrophin-Induced Myocyte Growth

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Abstract—Myotrophin, a novel protein that has been shown to stimulate myocyte growth, has been isolated, purified, and sequenced from the hearts of spontaneously hypertensive rats and dilated cardiomyopathic human tissue. Recently, the cDNA clones encoding myotrophin have been isolated and expressed in Escherichia coli, and the recombinant myotrophin was found to be as biologically and immunologically active as natural myotrophin. The mechanism by which myotrophin stimulates protein synthesis and initiates myocardial hypertrophy is not known. To evaluate the involvement of protein kinase C (PKC) in myotrophin-induced hypertrophy, PKC activity and its distribution in the subcellular fraction were determined in cultured neonatal and adult myocytes. PKC activity was determined by measuring the incorporation of 32P into histone type III-S and PKCζ substrate peptide (ε-pep) from [γ-32P]ATP in neonatal myocytes. Myotrophin significantly stimulated PKC activity in neonatal myocytes and was associated with a significant increase in protein synthesis. The effect of myotrophin on the stimulation of PKC activity and [3H]leucine incorporation was abolished by pretreatment with either staurosporine or H-7, two selective, pharmacological PKC inhibitors. Pretreatment of myocytes with staurosporine also reduced the myotrophin-induced mRNA levels of c-fos and β-myosin heavy chain. To evaluate the subcellular events whose occurrence was due to myotrophin and translocation of PKC, we studied the effect of genistein, a tyrosine kinase inhibitor, on myotrophin-induced neonatal myocyte growth. Genistein attenuated the [3H]leucine incorporation induced by myotrophin. To define the specificity of the PKC isofrom(s) involved in myotrophin-stimulated myocyte growth, both neonatal and adult myocytes were treated with myotrophin, and Western blot analyses were performed by using the antibodies of different PKC isoforms. Results showed that both PKCα and PKCε isoforms participated in the myotrophin-induced neonatal myocyte growth, whereas only the PKCε isoform was involved in myotrophin-induced adult myocyte hypertrophy. PKCδ and PKCζ do not seem to participate in either neonatal or adult myocyte growth induced by myotrophin. Treatment with antisense oligonucleotides specific for PKCα and PKCε isoforms further supported this result. PKCα is the major PKC isoform in neonatal myocytes and needs Ca2+ and phospholipids for its activation, and PKCε (the Ca2+-independent PKC isoform) is present in both neonatal and adult myocytes; the 15-mer antisense oligodeoxynucleotides of each were used for this study. Treatment of neonatal myocytes with the PKCα and PKCε antisense oligodeoxynucleotides for 5 days significantly reduced Ca2+-dependent and Ca2+-independent PKC activity, respectively, as well as the [3H]leucine incorporation induced by myotrophin. Furthermore, myotrophin-induced PKC activity was primarily located in the particulate fraction and did not result in a concomitant decrease in the cytosolic fraction. Myotrophin does not change PKC isoform expression (both Ca2+-dependent and independent PKC isoforms used in this study) in rat neonatal cardiac fibroblasts. Our data suggest that myotrophin exerts its action on protein synthesis, possibly through a tyrosine kinase–coupled pathway and translocation of PKC from the cytosol to the cell membrane. (Circ Res. 1998;82:1173-1188.)

Key Words: myotrophin ■ PKC ■ β-myosin heavy chain ■ myocytes ■ hypertrophy

Left ventricular hypertrophy is an important complication of hypertension and may precede heart failure or myocardial infarction.1,2 In response to hormonal and mechanical stimuli, the myocardium adapts to increased workloads through the hypertrophy of individual muscle cells. Cardiac hypertrophy is associated with substantial alteration in the composition of myocardial cells, including an alteration and increase in contractile proteins and collagen, with increased expression of several embryonic markers such as ANF and β-myosin heavy chain, which appear to depend largely on the activation of transcription of the corresponding cardiac genes that encode these proteins. Evidence suggests the involvement of PKC in myocardial cellular hypertrophy.3 Stimulation with α1-adrenergic agonists and angiotensin II promotes endogenous PKC activity via diacylglyceride; this event leads to the activation of cardiac gene transcription;4 the accumulation of contractile proteins, and the induction of early-response genes such as c-fos and Egr-1.5 Activation of PKC has been shown to lead to phosphorylation of transcription factors and subsequent gene expression in many tis-

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PKC Activity in Myotrophin-Treated Myocytes

Selected Abbreviations and Acronyms

ANF = atrial natriuretic factor
DVF₁₂ = Dulbecco’s modified Eagle’s medium/F-12
NE = norepinephrine
PKC = protein kinase C
ε₅₀ = PKCe substrate peptide
PMA = phorbol 12-myristate 13-acetate
TCA = trichloroacetic acid

This evidence suggests that activation of PKC may signal the cell to increase protein synthesis during the initiation or development of cardiac hypertrophy. Activation of PKC in neonatal cultured cardiomyocytes has also been shown when cardiomyocytes are stimulated with NE.⁸

Many investigators⁹–¹⁵ have shown that the development of hypertrophy in hypertension cannot be explained by increased blood pressure alone. Sen et al⁹ have also shown that control of blood pressure alone does not necessarily either prevent or regress the development of hypertension in spontaneously hypertensive rats or humans.⁹–¹¹,¹⁶ These findings have suggested the existence of factor(s)¹⁷ responsible for the development or initiation of cardiac hypertrophy. Sen et al¹⁸ have isolated a factor called myotrophin from the hypertrophied hearts of spontaneously hypertensive rats and from human dilated cardiomyopathic hearts.¹⁹,²⁰ This group has shown that myotrophin stimulates myocyte growth and is associated with an increase in protein synthesis; increased expression of early-response genes such as c-myc, c-fos, and c-jun; and an increase in the transcript levels of hypertrophy marker genes such as ANF and β-myosin heavy chain.²¹

Recently, the cDNA clones encoding myotrophin have been isolated²² and expressed in Escherichia coli. The recombinant protein was purified and tested for biological and immunologic reactivity; recombinant myotrophin was fully biologically active and cross-reacted with the antibody raised against natural myotrophin. The mechanisms by which myotrophin increases myocyte growth are not known. Because PKC has been shown to be involved in cardiac hypertrophy and myocyte growth, it is logical to evaluate the involvement of PKC in myotrophin-induced myocyte growth, especially subcellular events that occur between the time of application of myotrophin and the translocation of PKC.

PKC was initially identified by its dependence on calcium, phospholipids, and diacylglycerol for enzymatic activity.²³ PKC is a family of closely related serine-threonine protein kinases, and its activity within the cell arises from the combined activities of at least 12 different isoforms.²⁴ The different PKC isoforms known to date can be classified into three major categories, based on the requirements for their activation.²⁵⁻²⁸ All PKC isoforms contain a highly conserved carboxyl-terminal kinase domain that includes an ATP-binding site. PKC isoforms differ in their aminoterminal regulatory regions. The classic PKC isoforms are characterized by their requirements for calcium. The novel PKC isoforms, on the other hand, do not require calcium for their activation.⁹⁻¹² and not much is known yet about the two other new PKC isoforms. Immunologic and molecular approaches provide evidence that at least 4 different PKC isoforms (PKCα, PKCβ, PKCε, and PKCζ) are present in neonatal ventricular myocytes.³¹ PKC may be an important signal in the hypertrophic process in cultured myocytes in response to exogenous stimuli. It would be important to elucidate PKC activity and the distribution of PKC isoforms in subcellular fractions of isolated myocytes in response to myotrophin. In this study, we measured the distribution of PKC activity in the cytosolic and particulate fractions of neonatal myocytes in culture after treatment with myotrophin.

This article describes the involvement of PKC in myotrophin-induced myocyte growth in both adult and neonatal myocytes in culture. The present study also describes (1) the effect of pharmacological inhibitors and antisense oligonucleotides of PKC on myotrophin-induced PKC activity and protein synthesis; (2) the effect of the tyrosine kinase inhibitor genistein on myotrophin-induced protein synthesis; (3) the effect of staurosporine on the mRNA levels of the early-response gene c-fos and the hypertrophy marker β-myosin heavy chain; and (4) the involvement of specific PKC isoforms in the signal-transduction mechanism of myotrophin in both neonatal and adult myocytes.

Materials and Methods

Timed pregnant rats were obtained from Hilltop Farms, Scottsdale, Pa. The rats were fed Purina rat chow, given water ad libitum, and housed under sanitary conditions. Sprague-Dawley rats weighing 250 to 300 g used for the preparation of adult myocytes were purchased from Harlan Sprague Dawley, Inc, Indianapolis, Ind. The experimental procedures for animals were in accordance with National Institutes of Health guidelines. All polyclonal antibodies used in this study were purchased from Sigma Chemical Co, which generated the antisera in rabbits against synthetic peptides corresponding to unique sequences in the carboxy-terminal variable region of each PKC isoform. DVF₁₂, nonessential amino acids, and antibiotic solution used for the culture of neonatal myocytes were purchased from Gibco Bioresearch Laboratories. All other media and reagents used for preparation and culture of the neonatal myocytes and for the preparation of adult myocytes (including Joklik’s minimum essential medium, fetal BSA, insulin, transferrin, fetuin, hydrocortisone, and laminin) were purchased from Sigma Chemical Co. Collagenase was purchased from Worthington Biochemicals. PMA was purchased from Research Biochemicals International. [H]Leucine was obtained from Amersham Corp. Heparin and pentobarbital were purchased from Elkins-Sinn, Inc, and Abbott Laboratories, respectively. Genistein (tyrosine kinase inhibitor), staurosporine, H-7, (both PKC inhibitors), and histone (type III-S) were purchased from Sigma Chemical Co. ¹²⁵I-Labeled goat anti-rabbit IgG F(ab)² fragment was purchased from Du Pont–NEN. [γ⁻³²P]ATP was purchased from ICN Biomedicals. Antisense oligonucleotides and ε₅₀ were obtained from the molecular biology and chemistry core facilities at The Cleveland Clinic Foundation.

Preparation of Recombinant Myotrophin

Recombinant myotrophin was expressed and purified as described previously.²⁵ In brief, myotrophin was expressed in E. coli by using the T7 promoter–based vector pET3a (Novagen Inc). The myotrophin recombinant pET3a-51 vector was introduced into the BL21(DE3) LysS strain, which harbors a T7 RNA polymerase–based vector pET3a (Novagen Inc). The myotrophin recombinant protein was purified and tested for biological and immunologic reactivity; recombinant myotrophin was fully biologically active and cross-reacted with the antibody raised against natural myotrophin. The mechanisms by which myotrophin increases myocyte growth are not known. Because PKC has been shown to be involved in cardiac hypertrophy and myocyte growth, it is logical to evaluate the involvement of PKC in myotrophin-induced myocyte growth, especially subcellular events that occur between the time of application of myotrophin and the translocation of PKC.

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cutoff) followed by Centriconprep-10 (10-kDa cutoff) Amicon cartridges. The pure recombinant myotrophin, which migrated as a single band at the 12-kDa molecular-weight region, was then tested for biological activity by using [3H]leucine incorporation into myocyte protein and for immunoreactivity with the antibody raised against natural myotrophin. Because recombinant myotrophin showed both biological and immunologic properties identical to those of natural myotrophin, we used recombinant myotrophin for its further characterization.

Preparation of Neonatal Myocytes and Fibroblasts

Neonatal myocytes were isolated and cultured on laminin-coated wells according to the procedure described by Sen et al. In brief, hearts from 2- to 3-day-old normal Wistar rat pups were aseptically taken in DVF2 medium, and the ventricles were separated, minced in DVF2 medium containing collagenase (80 U/mL), and incubated at 37°C for 10 minutes in a water bath. The supernatant was discarded. The residual tissue was minced and incubated as before. The supernatant was collected and centrifuged at 1000 rpm for 2 minutes. The residue was collected in a 50-mL sterile tube and kept on ice. The procedure was repeated 3 times and the myocyte fractions were combined. The cells were then suspended in DVF2 medium containing 5% fetal BSA and allowed to settle in a sterile tissue-culture flask for 1 hour. The supernatant was collected in a 50-mL sterile tube. Fibroblasts became attached to the surface of the flask and were allowed to grow to confluence in the presence of 10% FBS. The cells were then passed through the second passage by trypsinization and used for the experiments as required. Myocytes, on the other hand, remained in the supernatant and were plated on laminin-coated wells (20 μg/35-mm well) at a density of 104 cells/35-mm well. The myocytes were allowed to grow in an incubator in DVF2 medium containing 10% fetal BSA at 37°C in an atmosphere of 95% O2 and 5% CO2 in the presence of 100 μmol/L bromodeoxyuridine. On culture day 2, old medium was aspirated, and the myocytes were incubated in fresh DVF2 medium containing fetal (25 mg/mL), transferrin (1 mg/mL), hydrocortisone (25 mg/mL), and 100 μmol/L bromodeoxyuridine. On culture day 3 (or 4), myocytes were incubated in DVF2 medium alone and were used for the experiment as required.

Preparation of Adult Myocytes

Calcium-tolerant adult myocytes were prepared according to the procedure described by Sil et al. In brief, hearts from 200- to 280-g male Wistar rats (15 weeks old) were injected intraperitoneally with heparin (100 U/100 g body weight). After the animal had been fully anesthetized, its thorax was opened with a midline incision, the heart was aseptically removed, and the aorta was separated from surrounding adjacent tissues and cut with fine, sterile scissors. The heart was aseptically removed and washed with ice-cold Joklik’s medium containing Joklik’s minimal essential medium (nominally calcium-free), 25 mmol/L glutamic acid, 30 mmol/L taurine, and 1 mmol/L adenosine. A syringe containing Joklik’s medium was attached to a cannula, and the cannula was introduced into the lumen of the aorta. A knot was tied with a piece of silk thread, thus fixing the cannula into the aorta. The blood was removed through the Joklik’s medium cannula, and the heart was then retrogradely perfused without recirculation at 37°C with the same medium on a modified Langendorff apparatus for ~10 minutes. The perfusion was continued in the same medium containing collagenase type II (100 U/mL) with recirculation for 30 minutes at the same temperature. After perfusion, the heart was removed from the apparatus, the atria and vessels were removed, and the ventricles were cut into small pieces. Those pieces were placed into a sterile Erlenmeyer flask. Five milliliters of fresh Joklik’s medium containing 100 U/mL collagenase was added, and the flask was kept in a water bath at 37°C for 5 minutes with occasional shaking. The tissue was disaggregated by trituration with a sterile, disposable, transfer pipette, and the released cells were removed by filtration through a piece of sterile nylon net into a 15-mL sterile polystyrene tube. Five milliliters of Joklik’s medium containing 5% FBS was added, and the cells were allowed to settle under gravity (10 minutes). Fresh collagenase solution was added and the process was repeated twice more. The fractions obtained were combined and washed with Joklik’s medium without serum.

Effect of Myotrophin on PKC Activity

In Neonatal Myocytes

Recombinant myotrophin used in this study was prepared according to the procedure as described. Ca2+-dependent PKC activity in neonatal myocytes was measured by following the procedure described by Shearman et al and Henrich and Simpson, with some modifications. For the Ca2+-independent PKC activity, we followed the procedure as described by Rybin and Steinberg. In brief, on culture day 4, neonatal myocytes were treated with 20 nmol/L myotrophin (or buffer for control) for 24 hours. Cells were then suspended by scraping in 20 mmol/L Tris HCl buffer, pH 7.5, containing 3 mmol/L EGTA, 2 mmol/L EDTA, 25 μmol/L aprotinin, and 50 μg/mL leupeptin. The cell suspension was then homogenized by sonication, followed by incubation on ice for 30 minutes. The suspension was then centrifuged at 40,000g for 30 minutes. The supernatant (cytosolic fraction) was collected and brought to a final concentration of 0.1% Triton X-100 and 10 mM 2-mercaptoethanol for PKC assay. The residue, buffer A containing 0.1% Triton X-100 was added and dissolved by sonication, and the supernatant (particulate fraction) was collected again by brief centrifugation and brought to a final concentration of 10 mM 2-mercaptoethanol for PKC assay. PKC activity was assayed by measuring the incorporation of [32P] into histone type III-S or ε2, from [γ-32P]ATP with or without purification on DEAE cellulose. PKC activity of the lysate was the same whether or not it was passed through the DEAE cellulose. The standard reaction mixture (250 μL) contained 20 mmol/L Tris HCl, pH 7.5, 400 μmol/L histone (for Ca2+-dependent PKC activity) or 50 μmol/L ε2 (for Ca2+-independent PKC activity), 10 μmol/L ATP (containing ~100,000 counts per minute per assay tube), 5 mmol/L MgCl2, 10 mM 2-mercaptoethanol with or without 200 μg/mL phosphatidylserine, 25 μg/mL diolein, and 2 mmol/L CaCl2. The synthetic peptide ε2 corresponds to the pseudosubstrate site of PKCε but with the phosphorylatable serine-for-alanine substitution (ERMRKPRKGOSVRRRRY).

PKC Activity

For all Ca2+-dependent and -independent PKC assays, we used 2 μmol/L NE and 100 nmol/L PMA–treated neonatal myocytes, respectively, as the positive controls because NE is known to stimulate Ca2+-dependent PKC activity. Assay tubes were incubated at 30°C for 10 to 30 minutes depending on the concentration of the cell supernatant. For Ca2+-dependent PKC activity, the reaction was terminated by adding 2 mL of 25% TCA. The TCA-precipitable material was collected by filtration over nitrocellulose membrane disks (0.45 μm). The disks were rinsed 3 times with 25% TCA, and the amount of radioactivity incorporated was quantified in a beta counter. For Ca2+-independent PKC activity, assays were terminated by spotting 40 μL of the reaction mixture onto phosphocellulose filter papers (P-81), which were immediately dropped into water. The filters were then counted for radioactivity after being washed 4 times with water for 5 minutes each time.

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Effect of Stauroporine and H-7 on Myotrophin-Induced Ca2+-Dependent PKC Activity

Stauroporine and H-7 are two known PKC inhibitors. To determine whether or not these two inhibitors showed any inhibitory effect on...
myotrophin-induced stimulation of PKC activity (Ca²⁺ dependent), we preincubated neonatal myocytes in the presence of 5 μmol/L staurosporine and 10 μmol/L H-7 for 2 hours. Myotrophin was then added, and PKC activity was determined as described before. NE-treated myocytes were used as positive controls.

### Effect of Staurosporine

**On Myotrophin-Induced Ca²⁺-Independent PKC Activity**

To determine whether staurosporine (PKC inhibitor) showed any inhibitory effect on myotrophin-induced stimulation of PKC activity (Ca²⁺ independent), we preincubated neonatal myocytes in the presence of 5 μmol/L staurosporine for 2 hours. Myotrophin was then added, and PKC activity (Ca²⁺ independent) was determined by following the procedure described earlier. PMA-treated myocytes were used as positive controls.

**On Myotrophin-Induced Stimulation of Protein Synthesis in Neonatal Myocytes**

To study the effect of staurosporine on myotrophin-induced stimulation of protein synthesis in neonatal myocytes, we cultured neonatal myocytes by following the procedure described for the determination of PKC activity in neonatal myocytes. To culture day 3, we preincubated neonatal myocytes in DVF₁ medium containing 10 μmol/L staurosporine for 2 hours. Then we added myotrophin (final concentration, 20 nmol/L) and continued the incubation for 24 hours. Ten micromoles of [³H]leucine was added per well, and the incorporation of radioactive leucine was then continued for 2 hours. The cells were then lysed with 1 mL of 0.1% SDS solution. A 50-μL aliquot (in duplicate) was taken from each well for the measurement of DNA. The lysed samples were then brought to 1 N with addition of NaOH solution. The plates were incubated at room temperature for 1 hour. BSA solution (1 mL of 0.5% BSA) was then added to each well and incubated for 30 minutes. TCA solution (1 mL of 20% TCA) was then added per well and kept for 30 minutes. The protein precipitate from each well was then collected on individual filter papers and a Millipore filter. The collected protein was washed thoroughly with 5% TCA until it was free of unbound radioactivity. Each filter paper was air dried for 1 hour and then counted in a beta counter after scintillation fluid had been added. Data were expressed as disintegrations per minute per nanogram of DNA. For control wells, instead of staurosporine, buffer was added and the assay was performed by following the method described earlier.

**On Myotrophin-Induced Stimulation of the Early-Response Gene**

Neonatal cardiac myocytes were used to determine the effect of staurosporine on the myotrophin-induced c-fos mRNA level. Total RNA was extracted from the cells by the methods of Chomczynski and Sacchi. Yield yields were quantified by absorbance at 260 nm. The RNA yield was 10 μg/35-mm well, with each well containing ~10⁶ cells. Cardiac myocytes were pretreated with staurosporine for 2 hours, followed by myotrophin treatment for 30 minutes. Experiments were done with at least 2 different sets of myocyte cultures on different days. Transcript levels were then assessed by Northern blot analysis. To assess the levels of c-fos proto-oncogene, total RNA samples from control, myotrophin-treated, and staurosporine-pre-treated (followed by myotrophin treatment) myocytes were fractioned on 1% agarose-formaldehyde gels. An oligonucleotide probe for c-fos was labeled at the 5’ end to a specific activity of 10⁶ cpm/μg DNA with T4 polynucleotide kinase. All hybridizations were performed at 42°C. After hybridization to the β-myosin heavy-chain probe and autoradiographic exposure, the filters were stripped off and rehybridized to the GAPDH probe. The blots were exposed to x-ray films with intensifying screens for 1 to 3 days at –70°C. Autoradiograms were normalized to GAPDH.

**Effect of Genistein on Myotrophin-Induced Stimulation of Protein Synthesis and PKC Activity in Neonatal Myocytes**

To study the effect of genistein on myotrophin-induced stimulation of protein synthesis in neonatal myocytes, we cultured neonatal myocytes by following the procedure described for the determination of PKC activity in neonatal myocytes. To culture day 4, neonatal myocytes were preincubated in DVF₁ (serum-free) medium containing 20 μmol/L genistein for 30 minutes. Myotrophin (final concentration, 20 μmol/L) was then added and incubated for 2 hours in the presence of 10 μCi [³H]leucine per well to measure the incorporation of radioactive leucine into myocyte proteins. The cells were then lysed with 1 mL of 0.1% SDS solution. A 50-μL aliquot (in duplicate) was taken from each well for the measurement of DNA. The lysed samples were then brought to 1 N with addition of NaOH solution. The plates were incubated at room temperature for 1 hour. BSA solution (1 mL of 0.5% BSA) was then added to each well and incubated for 30 minutes. TCA solution (1 mL of 20% TCA) was then added per well and kept for 30 minutes. The protein precipitate from each well was then collected on individual filter papers and a Millipore filter. The collected protein was washed thoroughly with 5% TCA until it was free of unbound radioactivity. Each filter paper was air dried for 1 hour and then counted in a beta counter after scintillation fluid had been added. Data were expressed as dpm per nanogram of DNA. For control wells, instead of genistein, buffer was added and the assay was performed by following the procedure described earlier. To determine whether genistein showed any inhibitory effect on myotrophin-induced stimulation of PKC activity, we preincubated neonatal myocytes in the presence of 20 μmol/L genistein for 30 minutes. Myotrophin was then added and incubated for 5 minutes, and PKC activity was determined by following the procedure described earlier.

**Determination of PKC Isoforms Involved in Myotrophin-Induced Myocyte Growth**

Both cultured neonatal and isolated adult rat ventricular myocytes were used for this study. Total cell extracts from isolated adult ventricular myocytes and cultured neonatal myocytes were prepared by following the procedure described by Rybin and Steinberg, with some modifications. In brief, ventricular myocytes were treated with myotrophin (20 nmol/L) and washed with PBS. The cells were then lysed with preheated (95°C) homogenization buffer (20 mmol/L Tris HCl, pH 7.5, 2 mmol/L EDTA, 2 mmol/L EGTA, 6 mmol/L of 2-mercaptoethanol, 50 μg/mL aprotinin, 25 μg/mL leupeptin, 5 μmol/L pepstatin A, 1 mmol/L PMSF, 0.1 mmol/L sodium vanadate, and 50 mmol/L NaF) containing 1% SDS and homogenized by sonication. Protein content in each preparation was measured by the Bradford protein microassay method with the use of standard Bio-Rad reagents. Cell extracts for the controls were made by following the same procedure except that PBS instead of myotrophin was used.
Effect of Myotrophin on PKC Isoform Expression in Cardiac Fibroblasts

Neonatal rat cardiac fibroblasts (passage 2) were used for this study. Fibroblasts were treated with myotrophin as described above (for neonatal myocytes), and the same procedure for the sample preparation for the PKC isoform expression study and Western blot analysis as described above was used, except that neonatal rat cardiac fibroblasts replaced the neonatal rat cardiac myocytes.

Effect of PKC\(\alpha\) and PKC\(\epsilon\) Antisense Oligonucleotides on Myotrophin-Induced Stimulation of PKC Activity and Protein Synthesis in Neonatal Myocytes

To study the effect of PKC antisense oligodeoxynucleotides on myotrophin-induced stimulation of PKC activity, we synthesized two antisense 15-mer oligodeoxynucleotides on the basis of the sequences obtained from the GenBank database and as described by Baxter et al.\(^2\) Oligonucleotides based on rat PKCo and PKCe began at the start codon. The sequence of the PKC\(\alpha\) antisense oligonucleotides we used was 5'-GTAAACGTCAGCCAT-3' and that for PKC\(\epsilon\) was 5'-ATTGAACACTACCAT-3'. We synthesized these oligonucleotides on an automatic DNA synthesizer at The Cleveland Clinic Foundation. The sense and antisense oligonucleotides were purified by heating at 55°C overnight, followed by evaporation to a dry mass in a Labconco Centrivap Concentrator. The dry mass was then dissolved in Tris-EDTA buffer, pH 7.5. Concentration of the antisense nucleotides was determined by measuring the absorbance at 260 nm. On culture day 3, neonatal myocytes were treated with 5 \(\mu\)mol/L antisense nucleotide in TE buffer, pH 7.5. After every 24 hours the medium was changed, and fresh antisense nucleotide solution was added. This procedure was followed for 4 days. We continued the incubation in the presence of myotrophin at a final concentration of 20 \(\mu\)mol/L for the last 24 hours. At the end of the incubation period, the cells were lysed and PKC activity was determined by following the procedure described earlier. In a set of parallel experiments, we determined the effect of both PKCo and PKCe sense and antisense oligonucleotides on myotrophin-induced stimulation of protein synthesis in neonatal myocytes by using the procedure as described earlier. For the control experiments, we treated myocytes with myotrophin only and then separately with myotrophin in the presence of sense nucleotides.

Effect of the Combination of PKC\(\alpha\) and PKC\(\epsilon\) Antisense Oligonucleotides on Myotrophin-Induced Stimulation of Protein Synthesis in Neonatal Myocytes

Neonatal myocytes were treated with a mixture of PKCo and PKCe sense and antisense oligonucleotides for 4 days. Myotrophin was then added and protein synthesis was measured by following the usual procedure of \[^{1}H\]leucine incorporation into myocyte protein as described before.

Effect of PKC\(\alpha\) and PKC\(\epsilon\) Antisense Oligonucleotides on Myotrophin-Induced PKC Isoform Expression in Neonatal Myocytes

Neonatal ventricular myocytes were treated with PKCo and PKCe antisense oligonucleotides for 4 days. Myotrophin (20 \(\mu\)mol/L) was added and the cells were incubated as described before. Cells were then washed with PBS and lysed in preheated (95°C) homogenization buffer as described earlier. Protein content in each preparation was measured by the Bradford protein microassay method and standard Bio-Rad reagents.\(^3\) PKC isoform expression in the cell extracts was then determined with the use of PKC isoform-specific antibodies. In a set of parallel experiments, tubulin-specific antibody was used instead of PKC isoform-specific antibodies to study the expression of “housekeeping” proteins.

Figure 1. Preparation of recombinant myotrophin. Myotrophin was expressed in E coli with use of the T7 promoter–based vector pET3a. Myotrophin recombinant pET3a-51 vector was introduced into the BL21(DE3) and purified according to procedure described in Materials and Methods. M indicates molecular weight markers; BL21, total protein from E coli strain without myotrophin, #51, BL21 (LysS) harboring pET3a vector containing myotrophin gene; and PM, purified recombinant myotrophin.

Western Blot Analysis

Western blot analysis was performed according to the procedures described by Towbin et al.\(^4\) and Tsang et al.\(^5\) with some modifications. In brief, samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels for 90 minutes at a constant voltage of 100 V at room temperature. Prestained molecular-weight markers were also electrophoresed on the same gel. Before transfer of the different proteins to the GeneScreen, the gel was equilibrated for 90 minutes in 25 mmol/L Tris and 192 mmol/L glycine buffer, pH 8.3. A piece of GeneScreen slightly larger than the gel was also electrophoresed in the same buffer for 30 minutes. A “sandwich” was then made with the gel, the membrane, two pieces of filter paper, and two sheets of Scotch-Brite pad. Electrophoretic transfer was then continued for 16 hours (overnight) at 4°C at a constant voltage of 30 V and transferred onto the GeneScreen. The membrane was then immersed from the apparatus, rinsed with 10 mmol/L sodium phosphate buffer, pH 7.4, containing 1% NaCl and 1% Tween-20, and dried in air for 30 minutes. The dried membrane was then immersed in excess 10% nonfat dry milk (Carnation) in PBS and incubated for 90 minutes to block nonspecific binding. After removal of the blocking solution, the GeneScreen was probed with excess diluted (1:500 dilution) primary PKC isoform–specific antisera in the milk solution (mentioned above) for 90 minutes at room temperature. Four different PKC isoform–specific antisera (as mentioned earlier in this article) were used. The solution was removed and the membrane rinsed 3 times (5 minutes each) with excess 20 mmol/L PBS containing 1% Tween-20. To detect the bound primary antibody, the membrane was immersed in excess milk solution (previously mentioned) containing \(^{125}\)I-labeled goat anti-rabbit IgG F(ab')\(^2\), fragment at a final dilution of 0.25 \(\mu\)Ci/mL and incubated for 3 hours at room temperature. The membrane was then rinsed with excess PBS containing 1% Tween-20 until the unbound radioactivity was removed (usually 7 times for 5 minutes each). Finally, the membrane was air dried at room temperature and autoradiographed with Kodak XAR film with intensifying screens at -70°C. The specificity of all immunoreactive proteins was established by immunoblot analysis in the presence and absence of competing immunizing peptide.
Effect of Myotrophin on Time-Dependent Translocation of PKC Activity in the Particulate Fractions in Neonatal Myocytes

Neonatal myocytes were treated with myotrophin for various periods of time (up to 2 hours), and the cytosolic and particulate fractions were made as follows. Cells were collected by scraping with a cell scraper in ice-cold buffer A. The suspension was then sonicated, incubated on ice for 30 minutes, and centrifuged at 40,000 g for 30 minutes. Triton X-100 was added to the supernatant to make a final concentration of 0.1% and was designated the “cytosolic fraction.” To the residue, buffer A containing 0.1% Triton X-100 was added, the residue dissolved by sonication, and the supernatant collected again by brief centrifugation. This supernatant was designated the “particulate fraction.” Both cytosolic and particulate fractions were brought to a final concentration of 10 mmol/L 2-mercaptoethanol before the PKC assay, which was performed as described earlier.

Measurement of Protein
Protein measurements were performed by the Bradford protein microassay method and standard Bio-Rad reagents.

Measurement of DNA
DNA was measured as described previously by Sen et al.

Statistical Analysis
Statistical analysis was done by Student’s paired t test and ANOVA where appropriate. For protein synthesis studies, 4 to 6 culture plates (6 wells per plate) were used in each experiment. For the PKC-related experiments we used both 35-mm 6-well plates and 100-mm Petri dishes for myocyte and fibroblast cultures. Three 6-well plates for controls and 3 6-well plates for treatment with different factors, or 3 100-mm dishes for controls and 3 100-mm Petri dishes, were used for these purpose. Experimental values for the treated groups were normalized to control values (vehicle treated) in each experiment. Results were expressed as mean ± SEM. The difference between two groups was tested by unpaired Student’s t test. Differences among 2 groups were tested by ANOVA for multiple sample comparison.

Results
Purity of Recombinant Myotrophin
Figure 1 shows the SDS gel of recombinant myotrophin. Recombinant myotrophin appeared as a single protein band with an apparent molecular mass of 12 kDa. Pure recombinant myotrophin was used for all experiments described in this article.

Effect of Myotrophin on PKC Activity in Myotrophin-Treated Myocytes

PKC Activity in Myotrophin-Treated Myocytes

![Figure 2](http://circres.ahajournals.org/)

Figure 2, a. Effect of myotrophin on PKC activity in neonatal myocytes. Neonatal myocytes were treated with myotrophin and then lysed in Tris HCl buffer containing EDTA, EGTA, aprotinin, leupeptin, and Triton X-100. Cell suspension was homogenized by sonication and incubated on ice. Supernatant was collected and Ca\(^{2+}\)-phospholipid–dependent PKC activity was determined by measuring incorporation of \(^{32}\)P into histone type III-S from \([\gamma-^{32}\text{P}]\)ATP. Ca\(^{2+}\)-independent PKC activity was determined by measuring incorporation of \(^{32}\)P into epep from \([\gamma-^{32}\text{P}]\)ATP. Bar 1 represents controls; bar 2, myotrophin-induced Ca\(^{2+}\)-phospholipid–dependent activity; and bar 3, myotrophin-induced Ca\(^{2+}\)-independent PKC activity in neonatal myocytes. Data are expressed as mean ± SEM, and each bar represents the mean of at least 4 different experiments. b. Effect of NE on Ca\(^{2+}\)-dependent PKC activity in neonatal myocytes. Neonatal myocytes were treated with NE in ascorbic acid according to standard procedure. Cells were lysed and PKC activity was measured as described in the legend to Figure 1a. Bar 1 represents controls and bar 2, NE-induced Ca\(^{2+}\)-phospholipid–dependent PKC activity. Data are expressed as mean ± SEM, and each bar represents the mean of 5 different experiments. c. Effect of PMA on Ca\(^{2+}\)-independent PKC activity in neonatal myocyte. Neonatal myocytes were treated with PMA in DMSO for 30 minutes according to standard procedure. Cells were lysed and Ca\(^{2+}\)-independent PKC activity was measured as described in the legend to Figure 1a. Bar 1 represents controls and bar 2, PMA-induced Ca\(^{2+}\)-independent PKC activity. Data are expressed as mean ± SEM, and each bar represents the mean of 5 different experiments.

In Neonatal Myocytes

Figure 2a shows the result of the effect of myotrophin on PKC activity (determined by the translocation of PKC activity from the cytosolic to the particulate fraction) in neonatal myocytes.
myocytes. Myotrophin significantly stimulated both Ca\textsuperscript{2+}-phospholipid–dependent and –independent PKC activity in neonatal myocytes over controls (54 ± 3.7% over control for Ca\textsuperscript{2+}-phospholipid dependent, n = 6, and 39 ± 2.9% over control for Ca\textsuperscript{2+}-phospholipid independent, n = 5). In parallel sets of experiments, myotrophin stimulated incorporation of [\textsuperscript{3}H]leucine (48 ± 4.1% over controls, n = 6). To validate our Ca\textsuperscript{2+}-dependent PKC assay procedure, we measured PKC activity after treating the neonatal myocytes with 2 μmol/L NE. Data are summarized in Figure 2b. A 42 ± 5.2% (n = 5) increase in Ca\textsuperscript{2+}- and phospholipid-dependent PKC activity was seen over controls. To validate our Ca\textsuperscript{2+}-independent PKC assay procedure, we measured the PKC activity after treating the neonatal myocytes with 100 nmol/L PMA. Data are summarized in Figure 2c. A 49 ± 4.7% (n = 4) increase in Ca\textsuperscript{2+}-independent PKC activity was found over controls.

**In Adult Myocytes**

Figure 3 shows the effect of myotrophin on PKC activity in adult myocytes (determined by the translocation of PKC activity from the cytosolic to the particulate fraction). Myotrophin did not show any significant stimulatory effect on Ca\textsuperscript{2+}- and phospholipid-dependent PKC activity (7 ± 4.9% over control) in adult rat ventricular myocytes. Ca\textsuperscript{2+}- and phospholipid-independent PKC activity, on the other hand, was significantly stimulated by myotrophin (32 ± 4.2% over control, n = 5, P < 0.02).

**Effect of Staurosporine and H-7 on Myotrophin-Stimulated Ca\textsuperscript{2+}-Dependent PKC Activity in Neonatal Myocytes**

We studied the effect of staurosporine and H-7, two known PKC inhibitors, on Ca\textsuperscript{2+}- and phospholipid-dependent PKC activity stimulated by myotrophin. Staurosporine

![Figure 3. Effect of myotrophin on PKC activity in adult myocytes. Adult myocytes were treated with myotrophin and then lysed according to the procedure described in the legend to Figure 1. Ca\textsuperscript{2+}-phospholipid–dependent PKC activity was determined by measuring incorporation of \textsuperscript{32}P into histone type III-S from [\textsuperscript{γ-32}P]ATP. Ca\textsuperscript{2+}-independent PKC activity was determined by measuring incorporation of \textsuperscript{32}P into \textit{e}\textsubscript{max} from [\textsuperscript{γ-32}P]ATP. For experimental details, see text. Bar 1 represents controls; bar 2, myotrophin-induced, Ca\textsuperscript{2+}-dependent PKC activity; and bar 3, myotrophin-induced Ca\textsuperscript{2+}-independent PKC activity in adult myocytes. Data are expressed as mean ± SEM, and each bar represents the mean of at least 6 different experiments.](image)

![Figure 4. Effect of staurosporine and H-7 (two selective PKC inhibitors) on myotrophin-induced PKC activity (Ca\textsuperscript{2+} dependent) in neonatal myocytes. Neonatal myocytes were preincubated separately with staurosporine and H-7 for different sets of experiments. Myotrophin was then added and PKC activity determined as described in text. Bar 1 represents controls; bar 2, myotrophin-induced PKC activity; bar 3, effect of staurosporine on myotrophin-induced PKC activity; and bar 4, effect of H-7 on myotrophin-induced PKC activity in neonatal myocytes. Data are mean ± SEM and are the means obtained from at least 6 different experiments.](image)

\(-2.5 ± 0.41\% \text{ over control, } n = 6\) and \(8 ± 0.85\% \text{ over control, } n = 6\) separately abolished the PKC activity stimulated by myotrophin (52 ± 5.8% over control, n = 6). Data are shown in Figure 4. These results suggest that myotrophin-induced stimulation of protein synthesis is possibly mediated by the activation of PKC.

**Effect of Staurosporine**

**On Myotrophin-Stimulated Ca\textsuperscript{2+}-Independent PKC Activity in Neonatal Myocytes**

We studied the effect of staurosporine on Ca\textsuperscript{2+}-independent PKC activity stimulated by myotrophin. Staurosporine (3.11 ± 1.24% over control, n = 5) reduced the PKC activity stimulated by myotrophin (41 ± 4.3% over control, n = 5). Data are shown in Figure 5.

**On Myotrophin-Induced Stimulation of Protein Synthesis in Neonatal Myocytes**

Because myotrophin-induced stimulation of PKC activity was blocked by the PKC inhibitor staurosporine, we determined the effect of the same inhibitor on myotrophin-induced protein synthesis in neonatal myocytes. Myotrophin alone significantly stimulated [\textsuperscript{3}H]leucine incorporation over controls (46.8 ± 3.7% over controls, n = 5, P < 0.01) (Figure 6). Preincubation with staurosporine significantly reduced the stimulation induced by myotrophin (Figure 6). These results indicate that the stimulatory effect of myotrophin on protein synthesis is mediated (at least partially) via the activation of PKC.

**On Myotrophin-Induced Early-Response Gene Transcript Level**

Northern blot analysis showed that the oligomer probe for c-fos specifically hybridized to a 2.2-kb c-fos mRNA and that...
myotrophin markedly induced the transcript level of c-fos in neonatal cardiac myocytes (Figure 7). Pretreatment of myocytes with staurosporine significantly reduced the myotrophin-induced stimulation of c-fos mRNA to control levels.

**Figure 5.** Effect of staurosporine on myotrophin-induced, Ca\(^{2+}\)-independent PKC activity in neonatal myocytes. Neonatal myocytes were preincubated with staurosporine. Myotrophin was then added and Ca\(^{2+}\)-independent PKC activity determined as described in text. Bar 1 represents controls; lane 2, myotrophin-induced Ca\(^{2+}\)-independent PKC activity; and lane 3, effect of staurosporine on myotrophin-induced Ca\(^{2+}\)-independent PKC activity in neonatal myocytes. Data are mean±SEM and are means obtained from at least 6 different experiments.

**Figure 6.** Effect of staurosporine on myotrophin-induced stimulation of protein synthesis in neonatal myocytes. On culture day 3, myocytes were preincubated with staurosporine. Myotrophin was then added and incorporation of \(^{3}H\)leucine into myocyte protein was measured as described in text. In a parallel set of experiments, myocytes treated with neither staurosporine nor myotrophin were used as controls. Data are mean±SEM and are the means of 5 different sets of results. Myotrophin significantly stimulated \(^{3}H\)leucine incorporation into myocyte protein. Pretreatment with staurosporine almost completely blocked stimulation.

**Figure 7.** Effect of staurosporine on myotrophin-induced mRNA level of early-response c-fos gene in rat neonatal cardiac myocytes. Myocytes were pretreated with 5 \(\mu\)mol/L staurosporine for 2 hours and then treated with myotrophin for 30 minutes. Cells were then lysed and total RNA was extracted. Ten micrograms of total RNA was run on 1.0% formaldehyde-agarose gel and transferred to a nylon membrane. Blot was hybridized with the specific oligonucleotide probe of c-fos, which was then depurinated and reprobed with GAPDH. For experimental details, see text. A, RNA from control myocytes; B, RNA from myotrophin-treated myocytes; and C, RNA from staurosporine pretreatment followed by myotrophin treatment myocytes. Data are from 1 experiment and are representative of 4 different sets of neonatal myocyte preparations.

**On Myotrophin-Induced β-Myosin Heavy-Chain Transcript Level**

From Northern blot analysis of total RNA obtained from myotrophin-treated and untreated myocytes, we observed a significant increase in the level of β-myosin heavy-chain transcript by myotrophin. Pretreatment of myocytes with staurosporine partially reduced that increment of myotrophin-induced β-myosin heavy-chain transcript level (Figure 8).

**Effect of Genistein on Myotrophin-Induced Protein Synthesis and PKC Activity**

Figure 9 shows the effect of genistein (a tyrosine kinase inhibitor) on myotrophin-induced protein synthesis in neonatal myocytes. Myotrophin alone significantly stimulated \(^{3}H\)leucine incorporation over controls (49.6±4.3% over controls, n=4) (Figure 9). Preincubation with genistein partially inhibited myotrophin-induced stimulation of protein synthesis in neonatal myocytes (23±2.2% over control, n=4) (Figure 9). PKC activity, on the other hand, was not reduced significantly by genistein.
Effect of Myotrophin on PKC Isoform Expression in Myotrophin-Induced Myocyte Growth

To evaluate the role of various PKC isoforms in myotrophin-induced myocyte growth, we performed Western blot analyses with antibodies specific to different PKC isoforms after stimulation of cultured neonatal and adult ventricular myocytes with myotrophin. The effect of myotrophin on the expression of four different PKC isoforms (PKCα, PKCδ, PKCε, and PKCζ) in neonatal myocytes is summarized in Figure 10a. Neonatal myocytes showed immunoreactivity for each of the PKC isoforms studied. Figure 10b shows quantification of the different PKC isoforms in neonatal myocytes stimulated by myotrophin. Myotrophin did not show any stimulatory effect on the PKCδ and PKCζ isoforms, but it significantly stimulated both PKCα and PKCε isoforms. In contrast, there was abundant PKCε isoform immunoreactivity in adult ventricular myocytes, and myotrophin significantly stimulated PKCε isoform only (Figure 11a). Figure 11b shows quantification of the different PKC isoforms in adult ventricular myocytes stimulated by myotrophin. Using antibodies (obtained from 3 different sources) specific to PKCα isoforms, we were unable to detect PKCα in adult ventricular myocytes in our experiments. PKCδ and PKCζ isoforms were detectable in adult ventricular myocytes, but the levels of these PKC isoforms were not influenced by myotrophin.

Effect of Myotrophin on PKC Isoform Expression in Cardiac Fibroblasts

A minor population of contaminating nonmyocytes (mainly fibroblasts) is always present in neonatal myocytes used for experiments. To determine whether these nonmyocytes made any contribution to the effect of myocyte induction by myotrophin, we studied the effect of myotrophin on PKC isoform expression in neonatal rat cardiac fibroblasts. Neonatal fibroblasts expressed all 4 PKC isoforms (PKCα, PKCδ, PKCε, and PKCζ), but none of those isoforms was influenced by myotrophin (Figure 12).
Effect of PKCα Antisense Oligonucleotides on Myotrophin-Induced PKC Activity and Protein Synthesis in Neonatal Myocytes

Figure 13 shows the effect of PKCα antisense and sense oligonucleotides on myotrophin-induced PKC activity in neonatal myocytes. Pretreatment of neonatal myocytes with the antisense oligonucleotides significantly reduced PKC activity induced by myotrophin (determined by the translocation of PKC activity from the cytosolic to the particulate fraction of neonatal myocytes), whereas pretreatment of the myocytes with sense nucleotides showed no significant change on the same activity. Figure 14 shows the effect of the same sense and antisense oligonucleotides on myotrophin-induced protein synthesis. Myotrophin-induced stimulation of protein synthesis in neonatal myocytes was also inhibited (though not to the same extent as PKC activity) by pretreatment with antisense oligonucleotides (48±3.9% stimulation of [3H]leucine over control in myotrophin-treated myocytes versus 27±3.2% over control in antisense-pretreated myocytes). However, pretreatment of the myocytes with sense oligonucleotides did not show any significant effect on myotrophin-induced protein synthesis. The results suggest that the PKCα isoform is also at least partly responsible for myotrophin-induced PKC activity in neonatal myocytes.

Effect of PKCe Antisense Oligonucleotides on Myotrophin-Induced PKC Activity and Protein Synthesis in Neonatal Myocytes

Figure 15 shows the effect of PKCe (sense and antisense) oligonucleotides on myotrophin-induced Ca2+-independent PKC activity in neonatal myocytes. Pretreatment of neonatal myocytes with the antisense nucleotides significantly reduced PKC activity induced by myotrophin (as determined by the translocation of PKC activity from the cytosolic to the particulate fraction of neonatal myocytes), whereas pretreatment of the myocytes with sense oligonucleotides showed very little effect on the same activity. Figure 16 shows the result of a set of parallel experiments in which we determined the effect of the same sense and antisense oligonucleotides on myotrophin-induced protein synthesis. Myotrophin-induced stimulation of protein synthesis in neonatal myocytes was also reduced (though not to the same extent as PKC activity) by pretreatment with PKCe antisense oligonucleotides (47±3.45% stimulation of [3H]leucine over control in myotrophin-treated myocytes versus 25±2.2% over control in antisense-pretreated myocytes). However, pretreatment of
Effect of the Combination of PKCα and PKCε Antisense Oligonucleotides on Myotrophin-Induced Protein Synthesis in Neonatal Myocytes

Figure 17 shows the effect of the combination of PKCα and PKCε (sense and antisense) oligonucleotides on myotrophin-induced protein synthesis. Myotrophin-induced stimulation of protein synthesis in neonatal myocytes was reduced (not to control levels but to a level less than that of the individual antisense oligonucleotides) by pretreatment with a mixture of PKCα antisense oligonucleotides (50±4.4% stimulation of [3H]leucine over control in myotrophin-treated myocytes versus 16±2.8% over controls in the antisense mixture—pretreated myocytes). Pretreatment of myocytes with a mixture of sense oligonucleotides did not show any significant effect on myotrophin-induced protein synthesis. The results suggest that the PKCα and PKCε isoforms are at least partly responsible for myotrophin-induced PKC activity in neonatal myocytes.

Discussion

This study has demonstrated that myotrophin significantly stimulated Ca2+- and phospholipid-dependent PKC activity in...
PKC Activity in Myotrophin-Treated Myocytes

**Figure 15.** Effect of PKCe antisense oligonucleotides on myotrophin-induced PKC activity in neonatal myocytes. 15-mer PKCe sense and antisense oligonucleotides were synthesized and purified according to procedures described in text. Concentration of antisense and sense nucleotides in Tris-EDTA buffer was determined by measuring absorbance at 260 nm. On culture day 3, myocytes were incubated separately with 5 μmol/L antisense and 5 μmol/L sense oligonucleotides. Incubation was continued for 4 days according to procedures as described. Myocytes were then treated with myotrophin, and after lysis, Ca2+-phospholipid–independent PKC activity was determined as described in the legend to Figure 1. Bar 1 represents controls; bar 2, myotrophin-induced PKC activity; bar 3, effect of PKCe sense on myotrophin-induced PKC activity; and bar 4, effect of PKCe antisense on myotrophin-induced PKC activity. Data are mean±SEM and each bar represents the mean of 5 different sets of experiments (*P<0.01).

PKC Activity (% over control)

Control M e-Sense+M e-Antisense+M

**Figure 16.** Effect of PKCe antisense oligonucleotides on myotrophin-induced stimulation of protein synthesis in neonatal myocytes. Myocytes were treated with PKCe antisense nucleotides as described in the legend to Figure 13. Cells were then lysed in SDS solution as described in text. For control experiments, myocytes were treated separately with sense oligonucleotides and myotrophin alone, and experiments were performed at the same time under similar conditions. Bar 1 represents controls; bar 2, myotrophin-induced protein synthesis; bar 3, effect of PKCe sense on myotrophin-induced protein synthesis; and bar 4, effect of PKCe antisense on myotrophin-induced protein synthesis in neonatal myocytes. Data are dpm/ng DNA over controls and are the mean±SEM of 5 different experiments (*P<0.01).

Isozyme-specific antisense downregulation study of neonatal myocytes with PKCa and PKCe antisense oligonucleotides, which inhibited myotrophin-induced stimulation of PKC activity and protein synthesis. Treatment of neonatal myocytes with a mixture of PKCa and PKCe antisense oligonucleotides reduced myotrophin-induced protein synthesis further but not to control levels. Data suggest that in addition to PKC, perhaps 1 or more other signal-transduction pathways are involved in myotrophin-induced myocyte growth. We could not study the effect of PKC antisense oligonucleotides on myotrophin-induced adult myocyte growth because of the difficulty of maintaining adult myocytes for the longer time (5 days) needed for this study. We were unable to detect any PKCa isofrom in adult ventricular myocytes after using 3 different commercially available PKCa antibodies.

Neonatal myocytes in serum-free-medium culture contained nonmyocyte cells, primarily fibroblasts (usually <10% under our experimental conditions), which might have contributed to the observed effects on myocytes induced by myotrophin. To rule out this possibility, in addition to neonatal myocytes we also studied the effect of myotrophin on PKC isoform expression in neonatal fibroblasts in culture. Using similar experimental conditions and 4 different PKC isoform–specific antibodies (PKCa, PKCd, PKCe, and PKCI), we detected the presence of 3 of these 4 isoforms in neonatal fibroblasts; their expression, however, remained unaltered by myotrophin. This result clearly suggested that the effect of myotrophin on neonatal myocytes was not due to contaminating fibroblasts. Previously we had also shown that the stimulatory effect of myotrophin is myocyte specific.18
The result from the previous study is consistent with the current study.

Very recently, Rybin and Steinberg\(^3\) demonstrated that thyroid hormone influenced PKCe expression in neonatal and adult rat ventricular myocytes but that the effects of thyroid hormone on PKC\(\alpha\) expression were confined to neonatal myocytes. These authors were also unable to detect the PKC\(\alpha\) isofrom in adult ventricular myocytes with the use of 3 commercially available antisera and concluded that the ability of thyroid hormone to influence PKC in adult myocytes was confined to the PKC\(\epsilon\) isofrom. Recently, Steinberg et al\(^4\) conducted a study to understand the species-dependent differences in the regulation of PKC isofrom expression in the heart. They examined PKC isofrom expression in both rat and dog hearts. Their goal was to determine whether PKC isoform heterogeneity and the age-dependent changes in PKC isofrom expression were general phenomena shared by all species or whether this unusual pattern occurred only in rats. Using immunoblot analyses, they showed that PKCe and PKC\(\zeta\) were readily detectable in the dog heart. PKC\(\alpha\) and PKC\(\delta\) isofroms were not detectable in the dog heart, although they were detected in considerable abundance in extracts of neonatal rat hearts run simultaneously as positive controls. PKCe and PKC\(\zeta\) immunoreactivity was higher in neonatal than adult tissue and also greater in atrial than ventricular extracts. Very recently, using immunoblot analysis, Paul et al\(^5\) showed that angiotensin II translocated PKCe to the particulate fraction in isovolumic perfused guinea pig hearts. PMA also translocated PKCe to the particulate fraction and produced a decrease in myocardial contractile function. In addition, the authors showed that mechanical stretch also translocated PKCe to the particulate fraction; however, that was not abolished by losartan. They concluded that in the adult guinea pig heart, left ventricular dilation produced stretch-mediated activation of phospholipase C, which resulted in phosphatidylinositol hydrolysis and PKCe activation in part via the local renin-angiotensin system.

Numerous studies suggest an important role for PKC as an intracellular mediator for the effects of some hypertrophic growth stimuli.\(^6,7,8\) PKC has been implicated as a candidate second messenger in neurohumoral induction of myocardial hypertrophy. Myocardial hypertrophy provides an adaptive response to hormonal and mechanical stimuli that increase the demand for contractile work by increasing myofibrillar protein content and sarcomere assembly in individual myocytes. A role for PKC in contraction-induced hypertrophic growth is also suggested by studies demonstrating that contraction results in translocation of PKC in skeletal muscle.\(^9\) Morgan et al\(^10\) observed translocation of PKC from the cytosol to the membrane in cardiomyocytes after PMA treatment. Previous studies demonstrated that activation of PKC caused translocation of the enzyme to the particulate fraction, with concomitant decreased activity in the cytosol.\(^11,12\) Recently, Gu and Bishop\(^13\) showed that the increase in PKC activity was restricted to the particulate fractions without a decrease of activity in the cytosolic fraction in the pressure-overload hypertrophy model. A significant role for PKC is also suggested by the observation that nuclear PKC activity was increased by PMA treatment but not by the inactive phorbol ester, which exhibited no hypertrophic effect.

In the current study, we demonstrated that myotrophin significantly stimulated PKC activity in the particulate fraction of neonatal myocytes without any decrease of same in the cytosolic fraction for at least 2 hours. Currently, we do not know the reason why myotrophin caused a prolonged increase in particulate PKC activity. However, a similar, recent report by Johnson and Mochly-Rosen\(^14\) examined the trans-
location of α, β, δ, ε, and ξ isozymes after a 0- to 60-minute exposure to 3 nmol/L 4-β-PMA. They observed that treatment of neonatal myocytes with 3 nmol/L PMA significantly caused a prolonged (at least 60-minute) increase of α and ε isoforms of PKC in the particulate fraction. They also showed that translocation of PKCα in that particular experiment was not detectable until 20 minutes after exposure to 3 nmol/L PMA, although the PKCe isozyme appeared after only 5 minutes of exposure to PMA.

Recent studies have taken a molecular approach to explore the role of distinct PKC isoforms in the hypertrophic response. By cotransfecting vectors that direct the expression of a mutant, constitutively active PKC with the ANF/luciferase and myosin light-chain 2/luciferase fusion genes, Shubeita et al52 showed that the conventional Ca$^{2+}$-dependent PKCα and PKCβ isoforms effectively coregulated transcription of the embryonic gene ANF and the contractile protein gene myosin light-chain 2. Kariya et al53 showed that both PKCα and PKCβ stimulated an activator protein-1 element. However, evidence that different isoforms of PKC have specific roles in the regulation of gene transcription during the hypertrophic response has also been presented, although neither of the studies cited explored the role of novel, Ca$^{2+}$-independent isoforms of PKC as potential intracellular mediators of the response to hypertrophic growth stimuli.

Cardiac growth due to hypertrophy is primarily caused by an increase in the protein content of myocytes. Chien et al 5 have shown that activation of PKC may represent one of the most proximal common events in the signaling cascade. Activation of cardiac target genes induces a program of embryonic gene expression, including expression of the ANF gene. The sequences that mediate cardiac-specific and inducible expression of an embryonic marker gene can be segregated by studies in both cultured cell models and in vivo models of hypertrophy in transgenic mice, suggesting that specific sets of regulatory elements may exist in inducible expression of the class of cardiac gene responses. Myocardial hypertrophy is also associated with qualitative changes in contractile protein composition, including induction of contractile protein genes that are normally expressed during embryonic development, eg, reactivation of skeletal α-actin and β-myosin heavy-chain gene expression in rodent and rabbit models of cardiac hypertrophy.54–56 Myotrophin has been shown to increase the levels of the proto-oncogenes c-myc, c-fos, and c-jun, as well as the transcript levels of hypertrophic markers such as ANF and β-myosin heavy chain.21 Therefore, the hypertrophic response caused by myotrophin appears to be mediated through PKC and is associated with an increase in the signaling of hypertrophy marker genes such as ANF and β-myosin heavy chain.

Recently, Gu and Bishop50 showed that PKC activity in left ventricular hypertrophy was increased significantly compared with control values in the cytosol, membrane, and nuclear cytoskeletal fractions in aorta-banded hypertrophied rat hearts. Immunoblot analyses using PKC isoform–specific antibodies have shown that both Ca$^{2+}$-dependent (α and β) and -independent (ε and ξ) isoforms were present in left ventricular cells. Compared with control values, increased concentrations of the membrane and nuclear cytoskeletal fraction for β and ε in the cytosol for α were found. PKCe was detected in the nuclear cytoskeletal fraction only and was not changed in left ventricular hypertrophy. Their
data suggested that PKC activity and concentration increased during development of left ventricular hypertrophy induced by pressure overload. The increased isozymes involved were PKCβ and PKCε, and the increase was present mainly in the membrane and nuclear cytoskeletal fraction. The mechanism of action in aorta-banded hypertrophy and myotrophin-induced increase in protein synthesis appeared to be very similar.

We have shown that myotrophin-stimulated protein synthesis is mediated by PKC, specifically by PKCα and PKCε in neonatal and PKCε in adult myocytes. As suggested by other investigators, as a result of external stimulation in the signal-transduction pathway, first different proto-oncogenes (eg, c-myc, c-fos, and c-jun) are turned on and then the transcript levels of hypertrophic markers such as ANF and β-myosin heavy chain are increased; this cascade eventually results in increased protein synthesis. When myotrophin was added to neonatal myocytes, a similar signal-transduction pathway was observed, suggesting that the myotrophin-induced increase in protein synthesis is possibly due to an increase in PKC activity. We have shown that in addition to neonatal myocytes, myotrophin also stimulates protein synthesis in adult myocytes.18,20 The types of PKC isozymes present in adult myocytes are not identical to those in neonatal myocytes, and it is also known that PKC isozymes differ intrinsically in their substrate specificity.57 For example, PKCε phosphorylates myelin basic protein only and therefore was not measured in the analysis for activity of PKC Ca2+-independent forms with histone as the substrate.58 In the current study, we also evaluated other Ca2+-independent isozymes of PKC (PKCδ, PKCε, and PKCζ) in both neonatal and adult myocytes and found that PKCε was involved in the signal-transduction mechanism of myotrophin for the stimulation of protein synthesis in both neonatal and adult myocytes. We have also shown that the tyrosine kinase inhibitor genistein attenuated the stimulatory effect of myotrophin on protein synthesis in neonatal myocytes.

Involvement of multiple protein kinase pathways has been suggested by several investigators for the functions of different biologically active molecules. Recently, Xu et al59 showed that phosphatic acid stimulated protein synthesis in adult rat cardiomyocytes and that an increase in protein synthesis by phosphatic acid was attenuated or abolished by preincubation of cardiomyocytes with the tyrosine kinase inhibitor genistein, the phospholipase C inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, the PKC inhibitor staurosporine, and the chelators of extracellular or intracellular free Ca2+, EGTA or BAPTA/AM, respectively. Watson and Gold60 showed that lysophosphatidylcholine, a naturally occurring intracellular phospholipid metabolite, modulates Na+ current in cardiac myocytes by a pathway that involves both PKC-dependent and tyrosine kinase–dependent phosphorylation. Data from our studies also suggest that myotrophin probably uses multiple signal-transduction pathways for its mechanism of action. Whether or not myotrophin also affects diacylglycerol and inositol triphosphate production has yet to be determined. In addition, the pathways leading from PKC activation to increased protein synthesis are still not fully known. Further studies are needed to define individual steps that eventually lead to hypertrophy of myocytes.

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References

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