The mechanism by which phorbol esters induce hypertrophic growth of cardiomyocytes was investigated. Control and 4α-phorbol 12,13-didecanoate-treated myocytes demonstrated a slow rate of growth as measured by the protein/DNA ratio and cell area. In contrast, treatment with phorbol 12-myristate 13-acetate (PMA) stimulated protein accumulation by 34%, while cell area was increased by 68% over control myocytes after 72 h. RNA content in PMA-treated myocytes was 33% higher than in control cells and 4α-phorbol 12,13-didecanoate-treated cells after 72 h. Membrane-associated protein kinase C activity was transiently increased after PMA treatment but returned to normal by 48 h. Cytosolic protein kinase C activity was not significantly altered by PMA. Membrane-associated and cytosolic protein kinase C activities were not altered by 4α-phorbol 12,13-didecanoate. Protein kinase C activity, RNA polymerase I activity, and the transcriptional rate of ribosomal DNA (rDNA) were increased in nuclei isolated from PMA-treated cells. However, consistent with a high rate of processing of pre-ribosomal RNA (pre-rRNA), the pool size of pre-rRNA relative to the 28 S rRNA was unaltered by PMA treatment. These data demonstrated that PMA-induced hypertrophic growth of cardiomyocytes was due to an increase in the capacity for protein synthesis (rRNA), and suggest that this results from protein kinase C mediated increase in the rate of transcription of rDNA.

Myocardial hypertrophic growth, characterized by an increase in cell size rather than cell number, occurs during normal postnatal growth (Peterson et al., 1989; Camacho et al., 1990), and as a consequence of pathologies such as ischemia, hypertension, and thyrotoxicosis (Anversa et al., 1986; Chilian and Marcus, 1987; Koyanagi et al., 1982). Experimental hypertrophic growth has also been demonstrated in vivo and in isolated cardiomyocytes in response to several external applied stimuli including contraction (McDermott et al., 1989; Marino et al., 1987), hormones (Simpson, 1985; Zierhut and Zimmer, 1989; Fuller et al., 1990), pressure overload (Lorell et al., 1986; de la Bastie et al., 1990), phorbol esters (Henrich and Simpson, 1988), and passive stretch (Cooper, 1987; Konaura et al., 1991). In all of these models of hypertrophic growth, increases in total protein and RNA content were observed.

An increase in ribosome content, as measured by total RNA, is the primary mechanism by which protein synthesis is accelerated during hypertrophic growth (Morgan et al., 1987; McDermott et al., 1989; McDermott and Morgan, 1988; Camacho et al., 1990). Ribosome accumulation can be due to changes at the level of rDNA transcription, processing of the 45 S rRNA precursor, synthesis of ribosomal proteins, and assembly into mature subunits (Sollier-Webb and Tower, 1986; Mager, 1988). In a recent study, the contraction-induced increase in RNA content in cultured cardiomyocytes was shown to be due to an accelerated rate of 18 S and 28 S rRNA synthesis rather than a decrease in the rate of degradation (McDermott et al., 1989). Contraction appeared to increase rRNA synthesis by accelerating rDNA transcription. The rate of pre-rRNA processing kept pace with the faster rate of transcription, resulting in no change in the 45 S RNA pool (McDermott et al., 1991).

Since its discovery by Nishizuka and colleagues (Takai et al., 1977; Inoue et al., 1977), protein kinase C has been implicated as the intracellular mediator of some of the effects of α1-adrenergic agonists, angiotensin, tumor promoters, and growth factors (Nishizuka, 1984; Henrich and Simpson, 1988; Griendling et al., 1986; Rozengurt, 1986). Some of these growth stimuli activate protein kinase C through increased contents of inositol lipid or phosphatidylcholine derived diacylglycerol. Phorbol esters such as PMA, which are structural analogs of diacylglycerol, are known to directly activate protein kinase C (Castagna et al., 1982). However, an indirect mechanism of protein kinase C stimulation by PMA was suggested by its ability to stimulate phospholipid turnover leading to diacylglycerol accumulation (Lockney et al., 1984; Grove and Schimmel, 1982; Hii et al., 1990). Irrespective of the mechanism by which protein kinase C activity is increased, a critical role in various cellular processes is evident. First, some cellular responses linked to protein kinase C can be elicited by PMA but not by the inactive phorbol ester, 4α-phorbol 12,13-didecanoate. Second, a role for protein kinase C in several cellular responses has been demonstrated in studies using inhibitors of the enzyme (Hannun et al., 1987; Watson et al., 1988) or by attenuation of responses following previous exposure to PMA, resulting in down-regulation or depletion of protein kinase C (Adams and Gullick, 1989). Third, a significant role for protein kinase C in hypertrophic growth is suggested by the changes in its distribution and activity.

The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinopropanesulfonic acid; EGTA, [ethylenebis(oxyethyl)enitrilo]tetraacetic acid.

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activity induced by several growth stimuli (Henrich and Simp-son, 1988; Cooper et al., 1987). Fourth, activation of protein kinase C by PMA has been linked to increased expression of c-myc and c-fos, which may play a role in hypertrophy (Rabin et al., 1986; Angel et al., 1987; Lee et al., 1987).

Although several hypertrophic stimuli elicits changes in the activity and distribution of protein kinase C, the role of protein kinase C in hypertrophic growth of cardiomyocytes remains to be clearly defined. Therefore, the goals of this study were: 1) to characterize the temporal relationship between activation of PKC by phorbol esters and increased rRNA and protein accumulation, and 2) investigate the mechanisms by which phorbol esters stimulate rRNA synthesis during hypertrophic growth of cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Cardiomyocyte Culture**—Neonatal cardiomyocytes were isolated by enzymatic digestion as previously described (McDermott et al., 1989; McDermott and Morgan, 1989). Cell number was determined with a Coulter counter and cells resuspended in an appropriate medium containing 10% newborn calf serum and 0.1 mM 5-bromo-2'-deoxyuridine. The cells were plated at a density of 0.5 x 10^6 cells/60-mm dish precoated with 0.1% gelatin. Following an overnight incubation to permit attachment of viable cardiomyocytes, the cells were maintained in serum-substituted medium containing 10^-7 M PMA or 4a-phorbol 12,13-didecanoate (inactive phorbol ester) as indicated for each experiment. Stock solutions of phorbol esters in dimethyl sulfoxide were used and the final concentration of dimethyl sulfoxide in each dish was 0.001%. The culture medium was changed every 2 days.

**Measurement of Cardiomyocyte Growth**—Total cellular protein was used as one measure of cell growth. At the completion of the appropriate experimental protocol, duplicate dishes of cells were rinsed 3 times with phosphate-buffered saline, scraped with 1 ml of sodium citrate buffer containing 0.25% SDS (SSC/SDS), and immediately frozen and stored at -20°C. The samples were thawed and vortexed, and duplicate aliquots of 75-100 pl were assayed for protein by the method of Lowry et al. (1951), using bovine serum albumin as a standard. DNA concentration was determined fluorometrically in duplicate 100-ml aliquots as previously described (Cesarone et al., 1979), using calf thymus DNA as a standard.

Cell area was determined by digitized image analysis using an Image Master 2000 computer analysis system (Technology Resources, Inc., Cleveland, OH). These morphometric studies, cells plated at 0.5 x 10^6 cells/60-mm dish were sufficiently sparse to allow individual cells to be clearly outlined and analyzed. Five frames of video images/dish were recorded at 320 times magnification on a Zeiss phase-contrast microscope and a video camera (Dage-MTI Inc., Michigan City, IN). Myocytes (4-6) in each frame were outlined, and cell area was obtained by computer analysis of the digitized images. Duplicate dishes were analyzed at each experimental point. The instrument was calibrated with a micrometer.

**Determination of the Capacity for Protein Synthesis**—The capacity for protein synthesis was estimated by measuring the RNA/DNA ratio. Briefly, RNA in 500-µl aliquots of SSC/SDS homogenates of cells was precipitated and washed 3 times with 500 µl of 5 M perchloric acid. The pellet was hydrolyzed in 750 µl of 0.3 M NaOH at 37°C for 24 h. The sample was neutralized with 250 µl of 4 N HCl, and the absorbance at 260 nm (A260) and 232 nm (A232) of the supernatant measured. The RNA concentration was calculated as previously described (McDermott et al., 1989; McDermott and Morgan, 1989).

**Measurement of Protein Kinase C Activity**—Cardiomyocyte nuclear, membrane-associated, and cytosolic protein kinase C activities were assayed using modification of previously described methods (Allo and Schaffer, 1990; Henrich and Simpson, 1988). Duplicate dishes of myocytes were rinsed 3 times with ice-cold Hank's balanced salt solution and scraped into 1.0 ml of ice-cold sucrose buffer containing 250 mM sucrose, 10 mM MOPS, 2.5 mM EDTA, 2.0 mM EGTA, 0.2 mM leupeptin, and 0.2 mM fluorode. Nuclei were isolated as described below. For measurement of membrane associated and cytosolic protein kinase C activity, the cells were sonicated twice for 10 s each and centrifuged at 100,000 x g for 40 min. The supernatant was used to measure cytosolic protein kinase C activity. The pellet was resuspended in sucrose buffer, solubilized with 0.1% Triton X-100 for 30 min on ice, and used for measurement of membrane-bound protein kinase C activity.

Protein kinase C activity was measured in a reaction mixture containing 250 mM sucrose, 10 mM MOPS, 2.5 mM EGTA, 0.2 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 10 mM NaF, 2 mM CaCl2, 100 µg/ml phosphatidylserine, and 10 µg/ml diethylene in a final volume of 125 µl. The reaction was started by the addition of [γ-32P]ATP (1.5 x 10^6 cpm) at a concentration of 20 µM and incubated at 30°C. The reaction was terminated by spotting 100 µl of the reaction mixture onto a 2.5 x 1-cm Whatman 3MM filter paper which was immediately dropped into 10% trichloroacetic acid. Following 3-15 min washes with 10% trichloroacetic acid and 5-min washes with 66% ethanol and then ether, the filters were dried and counted for radioactivity. Protein concentration was determined with a Bio-Rad protein assay based on the procedure by Bradford (1976), using bovine serum albumin as a standard. Ca²⁺-phospholipid independent kinase activity was determined in the absence of CaC12 and phospholipid in a reaction mixture containing 2.5 mM EGTA. Protein kinase C activity was determined by subtracting the Ca²⁺-phospholipid independent 32P incorporation into Sigma histone III-S from 32P incorporation measured in the presence of Ca²⁺, diethylene, and phosphatidylserine. Ca²⁺-phospholipid independent 32P incorporation was approximately 30% of total 32P incorporation.

**Isolation of RNA**—Cells were rinsed three times with ice-cold phosphate-buffered saline and lysed by adding RNAzol (CINNA/Laboratories, Inc., New York, NY) and chloroform/isoamyl alcohol (24:1), and centrifuged at 1500 x g for 10 min. The RNA in the aqueous phase was precipitated overnight at -20°C with an equal volume of isopropl alcohol. The samples were centrifuged at 12,000 x g for 15 min at 4°C, and the pellet washed twice with ice-cold 70% ethanol. Following centrifugation in a microfuge for 15 min at 4°C, the pellet was dissolved in water and the RNA concentration estimated by measuring absorbance at 260 nm.

**Measurement of Pre-rRNA Pool Size**—Pre-rRNA pool size was determined with 32P-labeled rRNA probes to the external transcribed spacer (ETS). RNA was prepared by phenol extraction and ethanol precipitation methods (McDermott and Morgan, 1989; Resendez et al., 1986). The rRNA intermediates in 2 µg of total extracted RNA were separated by electrophoresis on a denaturing formaldehyde-formamide agarose gel (1%) at 40 V for 18 h. The RNA on the gel was subjected to partial alkaline hydrolysis by incubating in 50 mM NaOH, 10 mM NaCl for 45 min at room temperature. Following neutralization, the RNA was transferred onto HYBOND-N membrane. The RNA blot was dried, UV cross-linked, and prehybridized for 1 h at 52°C in 10 ml of solution containing 50% deionized formamide, 0.02% polyvinylpyrrolidone, 2 mg/ml sheared salmon sperm DNA, 2× SSC, and 0.1% SDS. The labeled probes were added and allowed to hybridize for 2 days. The blot was washed 3 times with 2× SSC, 0.1% SDS, and rinsed with 0.1× SSC. The blot was air dried and analyzed for radioactivity by autoradiography and with an AMBIS® radiodetector. The cross-hybridization of the rRNA probe to the 28 S rRNA was used as an internal standard for each experimental sample. Pre-rRNA pool size was estimated by the ratio of probe hybridized to 45 S pre-rRNA relative to 28 S rRNA. It was determined that the amount of p2.0 probe that hybridized to the 45 S and 28 S bands was linear up to 4 µg of RNA loaded. Therefore 2 µg of RNA were loaded in all gels.

**Isolation of Nuclei**—Nuclei from control and PMA-treated cells were isolated by modification of a previously described method (Bowman, 1987). Cells were rinsed twice with phosphate-buffered saline and once with Buffer I containing 10 mM Tris, pH 8.0, 10 mM NaCl, 2.5 mM MgCl₂, and 5 mM dithiothreitol. The dishes were scraped with Buffer I, samples pooled and placed on ice. An equal volume of Buffer I supplemented with 0.6 M sucrose and 0.6% Triton X-100 was added to the cell suspension and the cells homogenized by 6-8 strokes in a Dounce (A) homogenizer. The homogenate was layered onto a discontinuous sucrose gradient (2.5 ml 30% sucrose, 2.5 ml 40% sucrose, and 1 ml 50% sucrose) and centrifuged at 1500 x g for 10 min at 4°C. For transcription assays, the pellet was resuspended in buffer containing 50 mM Tris, pH 8.0, 5 mM MgCl₂, 0.1 mM EDTA, and 20% glycerol. For nuclear PKC assays, the pellet was suspended in sucrose buffer (250 mM sucrose, 25 mM Tris-Cl, 2.5 mM EDTA, 0.5 mM EGTA, 5 mM dithiothreitol, and 0.5% phenylmethylsulfonyl fluoride) containing 0.5% Triton X-100, sonicated 5 times, and centrifuged at 100,000 x g for 30 min. The supernatant was used to measure PKC activity.
Determination of Nuclei RNA Polymerase I Activity—RNA polymerase I activity was determined by measuring α-amanitin-insensitive RNA polymerase activity. The assay mixture (50 μl) containing 50 mM Tris, pH 8.0, 5 mM MgCl₂, 10 mM dithiotheritritol, 100 mM (NH₄)₂SO₄, 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.065 MCl/ml of [³H]UTP, 0.05 M UTP, and 0.4 mg/ml of α-amanitin was preincubated at 37 °C for 10 min. The reaction was started by the addition of 50 μl of nuclear suspension. After 5 min, the reaction was terminated by adding 100 μl of 20% trichloroacetic acid, 2 mM UTP, and 60 mM Na₂P₂O₇. The mixture was precipitated on ice for 15 min and centrifuged for 5 min. The pellet was washed 3 times with 10% trichloroacetic acid, 10 mM Na₂P₂O₇ and hydrolyzed overnight with 260 μl of 0.3 M NaOH at 37 °C. The samples were neutralized by adding 150 μl of 4 N perchloric acid, and centrifuged. Aliquots (100 μl) of the supernatant were counted for radioactivity. The specific activity of [³H]UTP in the assay mixture was determined by reverse phase high pressure liquid chromatography with an on-line radioactivity detector as previously described (McDermott et al., 1989). Aliquots of the nuclear suspension were dissolved in 1 × SSC, 0.25% SDS and assayed for DNA content as described above. 

Transcription in Isolated Nuclei—Run-on transcription assays were performed as described above for RNA polymerase I activity, except α-amanitin, [⁹⁷Tc]UTP, and 1 mM UTP were omitted and 66 μM α-amanitin [⁹⁷Tc]TP and 5 units/ml of RNasin (Promega) were added. After 15 min incubation, the assay mixture was digested with RNase-free DNase I (20 units/ml) for 10 min. Carrier tRNA (10 μg/ml) and [³H]p2.0 sense transcript (marker for recovery and hybridization efficiency) were added. The mixture was incubated for 60 min after the addition of 25 μl of Proteinase K (25 μg in 100 mM Tris, pH 7.5, 5 mM EDTA, 1% SDS). Following phenol/chloroform extraction, the aqueous phase was precipitated with an equal volume of 20% trichloroacetic acid, 60 mM Na₂P₂O₇, 2 mM UTP, and washed twice with ice-cold 70% ethanol. The rRNA in the pellet was hybridized to 1.5 μg of recombinant p2.0 or 5.1 E/E rDNA immobilized on a filter. p2.0 is a genomic clone of rat rDNA containing the sequence from −167 to base pairs upstream of the transcription start site to the first 2 kilobases encoding the external transcribed spacer of 45 S pre-rRNA (Cassidy et al., 1987), while the 5.1 E/E is a clone extending from −300 to +300 base pairs of the transcription start site. A filter containing pBR322 DNA was also added to monitor nonspecific hybridization. After hybridization for 3 days, the filters were washed 3 times with 2 × SSC, 0.1% SDS at 52 °C for 30 min each, followed by 2 washes with 0.1× SSC at 65 °C for 10 min. The DNA on the filters was eluted by hydrolysis with 0.3 N NaOH, neutralized with H₂PO₄, and the [²²P]/[³H] ratio determined by scintillation counting. The transcriptional activity was obtained by multiplying the [²²P]/[³H] ratio by the input counts/min of [³H] and expressed relative to input DNA per assay. The recovery and hybridization efficiency averaged 16%.

Statistical Methods—Data are expressed as the mean ± S.E. of the indicated number of separate determinations. Statistical analysis of multiple groups was performed by one-way ANOVA followed by the Student's t test, while comparison with concurrent controls was performed by the paired Student's t test. A value of p < 0.05 was taken to indicate statistically significant difference between the groups compared.

Results

Phorbol Ester-stimulated Cardiomyocyte Hypertrophy—Hypertrophic growth of cultured cardiomyocytes in response to phorbol ester treatment was characterized to determine the temporal relationship between protein kinase C activation, increased RNA content, and protein accumulation. As assayed by autoradiography, cardiomyocyte growth, both protein accumulation and cell area were measured. Similar to previous findings, myocytes maintained in serum-free medium grew rapidly during the first 3 days, primarily due to repair of cellular damage caused during isolation (McDermott and Morgan, 1989). However, by day 4 in culture, the rate of growth was very slow, providing a relatively stable base line (time 0) from which to evaluate the effects of PMA on protein and RNA accumulation. DNA content (~13 pg/cell) and cell number remained unchanged in control and treated myocytes during the course of the experiments. Therefore, the data were normalized to the DNA content per dish to correct for minor variations in cell number. After 72 h, protein/DNA ratios for control and 4α-phorbol 12,13-didecanoate-treated cells were 14.34 ± 1.11 and 14.02 ± 1.61, respectively (Fig. 1); these ratios were 26% higher than the basal value (11.26 ± 1.53) that was determined at the beginning of the experimental protocol (time 0). By comparison, incubation with 10⁻⁷ M PMA resulted in a significant increase in protein accumulation by 48 h, and reached a maximal 70% increase relative to the basal protein/DNA ratios. This change amounted to a 34% increase in protein content in PMA-treated myocytes relative to control cells after 72 h in culture. After 72 h, a steady state was achieved with respect to cell protein for both control and PMA-treated myocytes (Fig. 1).

Cell area, as measured by computer generated digitized image analysis, was increased by PMA treatment. However, in contrast to protein accumulation, the increase in cell area in response to PMA treatment was evident at 12 h and plateaued by 72 h (Fig. 2). PMA treatment resulted in a 68% increase in cell area after 72 h, a significantly greater increase than that observed for protein accumulation. Treatment with the biochemically inactive phorbol, 4α-phorbol 12,13-didecanoate, did not alter cell area relative to control myocytes, demonstrating that hypertrophic growth was specific for the biochemical effects exerted by phorbol ester on the myocytes.

Capacity for Protein Synthesis—The primary mechanism by which protein synthesis is accelerated during hypertrophic

FIG. 1. Effect of phorbol ester on cardiomyocyte protein accumulation. Protein accumulation was measured in control (C), 10⁻⁷ M 4α-phorbol 12,13-didecanoate (Δ), and 10⁻⁷ M PMA (A) treated cardiomyocytes. Values are the means ± S.E. of 3–5 separate preparations. Asterisk (*) denotes significant difference from control cells (p < 0.05). Number sign (#) denotes significant difference from control and 4α-phorbol 12,13-didecanoate-treated cells (p < 0.05).

FIG. 2. Effect of phorbol ester on cardiomyocyte area. Cell area of control (C), 4α-phorbol 12,13-didecanoate (Δ), and PMA (A) treated cells was determined by computer generated digitized analysis of video images. Values are means ± S.E. of 3 separate preparations in which approximately 140 cells were analyzed per data point for each experimental group. Number sign (#) denotes significant difference from control and 4α-phorbol 12,13-didecanoate-treated cells (p < 0.05).
growth is through an increase in ribosome content (Morgan et al., 1987; McDermott et al., 1989). Changes in ribosome content during phorbol ester-induced hypertrophic growth were investigated by measuring the RNA/DNA ratio, because over 85% of total RNA is ribosomal RNA. The basal RNA/DNA ratio on day 4 in culture was 0.48 ± 0.006 (Fig. 3). Over the next 4 days, RNA content in control and 4α-phorbol 12,13-didecanoate-treated myocytes remained constant. In contrast, PMA treatment for 24 h resulted in a 16% increase in the RNA/DNA ratio (Fig. 3). After 72 h of exposure to PMA, RNA content was 34% higher than control and 4α-phorbol 12,13-didecanoate-treated myocytes. These data demonstrated that phorbol ester-accelerated growth occurred through an increase in the capacity for protein synthesis. When cells were incubated with PMA and 0.5-5 nM staurosporine, a PKC inhibitor, the PMA-induced increase in RNA content was progressively inhibited. Complete inhibition was obtained with 5 nM staurosporine (data not shown).

Phorbol Ester-induced Changes in Protein Kinase C Activity—The intracellular receptor and cellular mediator of phorbol ester-induced effects is PKC (Castagna et al., 1982; Nishizuka, 1984). The effect of PMA on both membrane-associated and cytosolic PKC activity was examined. Cellular response of PKC to PMA treatment was rapid and transient. The specific activity of membrane-associated PKC was increased 5-fold after 1 min of PMA treatment (Figs. 4 and 5A). Similar rapid increases in membrane-associated PKC specific activity have been observed in other tissues treated with agents that activate PKC (Pelech et al., 1990). The specific activity of membrane-associated PKC remained elevated 20 min after PMA administration, but was not significantly different from control values after 24 h (Fig. 4). The specific activity of cytosolic protein kinase C was unaltered by PMA and 4α-phorbol 12,13-didecanoate treatment and averaged 384.6 ± 62.1 pmol/mg/min. For comparison of the effect of PMA and 4α-phorbol 12,13-didecanoate treatment on PKC distribution, the percent of total activity present in each fraction was calculated using the specific activity reported in Fig. 4 and the milligrams of protein in the cell fraction. This comparison is particularly important in view of the demonstration that the specific activity of membrane-associated and cytosolic PKC can be enhanced independent of translocation (Pelech et al., 1990). In control cells about 17% of the protein kinase

![Fig. 3. Effect of phorbol ester on the capacity for protein synthesis.](image)

![Fig. 4. Effect of PMA on cardiomyocyte protein kinase C activity.](image)
in PMA-induced growth, the inactive phorbol, 4α-phorbol 12,13-didecanoate, did not alter membrane-associated PKC activity (Figure 5A).

Nuclear PKC activity was increased by 51% after 1 min of PMA treatment (Fig. 5B). Longer exposure to PMA (48 h) resulted in a decrease in nuclear PKC activity to values at or below those for control and 4α-phorbol 12,13-didecanoate-treated cells (Fig. 5C). These results were consistent with earlier studies that demonstrated that exposure of many cell types to phorbol esters resulted in increased membrane protein kinase C activity, followed by depletion or down-regulation after prolonged exposure (Adams and Gullick, 1989; Cooper et al., 1987).

Transcriptional Activity in Isolated Nuclei—Run-on transcription in isolated nuclei was measured by hybridization of transcripts radiolabeled with [32P]UTP. The amount of run-on transcript hybridized to a recombinant p2.0 rDNA clone/μg of nuclear DNA served as an indicator of the rate of initiation of rDNA gene transcription. Transcription of rDNA was increased by 48 ± 10% in nuclei isolated from PMAtreated relative to control myocytes (Table I). This increase was consistent with a 59 ± 14% increase in RNA polymerase I activity, measured as α-amanitin insensitive activity, in PMAtreated as compared to control myocytes. (Control 1.63 ± 0.24 pmol of UTP incorporated per μg of DNA/5 min; PMAtreated, 2.60 ± 0.42; values are mean ± S.E. of 5 different preparations, p < 0.05 using a paired t test.)

Pre-rRNA Pool Size—Pre-rRNA pool size was estimated as described under “Experimental Procedures,” using a 32P-cRNA probe made from the p2.0 clone of rDNA. Equal amounts of RNA (2 μg) from control and PMAtreated myocytes were subjected to Northern blotting (Fig. 6). Quantitative analysis of the hybridized 32P-cRNA is shown in Table II. The ratio of 45 S pre-rRNA/28 S rRNA was unaltered in cells treated with PMA. After 24 and 48 h of PMA treatment, the relative size of pre-rRNA pool was unaltered. Similar results were also obtained with the 5.1 E/E probe of rDNA. Using this probe, the 45 S/28 S ratios that were calculated from the AMBIS blot were 1.58 ± 0.14 and 1.53 ± 0.19 for control and PMAtreated cells, respectively. Thus, the relative size of the pre-rRNA pool remained constant during PMA-stimulated growth, despite the increased rate of transcription of rDNA.

TABLE I

Effect of PMA on tRNA transcription in cardiomyocytes

Nuclei were isolated from cardiomyocytes treated with PMA for 48 h and run-on transcription measured as described under “Experimental Procedures.” Recovery and hybridization efficiency were monitored by adding 1910 cpm of [3H]p2.0 sense RNA transcript. Values for each experiment are the mean ± S.E. of three run-on transcription assays.

| Transcriptional activity | DNA | Difference |
|--------------------------|-----|------------|
|                          | cpm/μg | %          |
| Experiment 1            |       |            |
| Control                 | 539 ± 22 |           |
| PMAtreated              | 776 ± 54 * | 44         |
| Experiment 2            |       |            |
| Control                 | 824 ± 13 |           |
| PMAtreated              | 1305 ± 86 * | 58        |
| Experiment 3            |       |            |
| Control                 | 633 ± 20 |           |
| PMAtreated              | 1040 ± 130 * | 64       |
| Experiment 4            |       |            |
| Control                 | 1103 ± 112 |         |
| PMAtreated              | 1376 ± 94 | 25        |

* Significant difference from control (p < 0.05).

DISCUSSION

This study characterized the phorbol ester-induced hypertrophic growth of cultured cardiomyocytes, particularly the mechanisms by which growth was stimulated. Similar to results obtained with other models of hypertrophic growth, phorbol ester treatment resulted in an increase in protein accumulation in cardiomyocytes. The extent of hypertrophic growth was greater when assessed by measurements of cell surface area. By this method, a 68% increase in surface area was observed after 72 h of PMA treatment. Thus, phorbol esters exert a spreading effect on cardiomyocytes independent of protein accumulation, the hallmark of hypotrophy. Therefore, measurements of surface area do not reflect absolute changes in cell protein during growth.

A common feature of hypertrophic growth is increased RNA content. This study demonstrated that the capacity for protein synthesis, as measured by the RNA/DNA ratio was increased following treatment with PMA. These data are consistent with previous findings in which hypertrophic
The inactive phorbol ester, which lacked a hypertrophic effect. Treatment of isolated nuclei with protein kinase NII to mediate transcription. Therefore, a role for regulation by the down-regulation of membrane-associated protein kinase polymerase I activity and transcription remains to be defined. Phosphorylation-dephosphorylation in the control of RNA polymerase I activity and transcription is facilitated through an increase in the capacity for protein synthesis, a mechanism common to most, if not all, models of cardiac hypertrophy.

A number of steps in ribosome biogenesis could be involved in regulating ribosome accumulation during myocyte growth. These include transcription of rDNA, processing of pre-rRNA, and assembly with r-proteins into ribosomes. Previous studies have reported changes at the level of transcription during myocardial hypertrophy (Kako et al., 1972; Nair et al., 1968). Transcription of rDNA is facilitated by RNA polymerase I, which is identified by its insensitivity to inhibition by α-amanitin (Sollner-Webb and Tower, 1986). Therefore, changes in RNA polymerase I activity result in altered transcriptional activity (Hossenlopp et al., 1975; Roeder, 1974). Consistent with previous studies in which increased RNA polymerase I activity was linked to hypertrophic growth (Cutilletta et al., 1978; McDermott et al., 1991), an increase in RNA polymerase I activity was observed in nuclei isolated from PMA-induced hypertrophic cardiomyocytes.

The mechanism by which phorbol esters affect rDNA transcription in myocytes has not been investigated. It is known that eukaryotic RNA polymerase binds to the DNA only after the binding of sequence specific transcription factors to form a functional promoter-polynucleosome complex. A potential mechanism by which RNA polymerase I activity may be increased in phorbol ester-induced hypertrophic growth is through protein kinase C-mediated phosphorylation of either the enzyme itself or an associated rDNA transcription factor (Sollner-Webb and Tower, 1986; Zahradka and Sells, 1987). Consistent with this hypothesis, Belenguer et al., (1989) recently showed that treatment of isolated nuclei with protein kinase NII resulted in phosphorylation of specific nuclear proteins and enhanced transcription of rDNA. We observed that despite the down-regulation of membrane-associated protein kinase C activity by 24 h, RNA polymerase I activity was still elevated after 48 h of PMA treatment. This fact suggested that the events initiated by membrane-associated protein kinase C activity, including nuclear events, must be long-lived to mediate transcription. Therefore, a role for regulation by phosphorylation-dephosphorylation in the control of RNA polymerase I activity and transcription remains to be defined.

Numerous studies suggest an important role for protein kinase C as an intracellular mediator of the effects of some hypertrophic growth stimuli (Nishizuka, 1984; Henrich and Simpson, 1988; Rozenburg, 1986). This is supported by our observation that 4α-phorbol 12,13-didecanoate did not stimulate growth and RNA accumulation. A role for PKC in contraction-induced hypertrophic growth is suggested by studies demonstrating that contraction resulted in translocation of PKC in skeletal muscle (Richter et al., 1987). We observed translocation of PKC from the cytosol to the membrane in cardiomyocytes following PMA treatment. Significantly, the inactive phorbol ester (4α-phorbol 12,13-didecanoate) did not alter membrane PKC activity. 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 lacked a hypertrophic effect. Inhibition of the PMA-induced increase in RNA content by staurosporine also suggests an important role for PKC. The translocation of protein kinase C to the nucleus following activation, and the ability to directly activate protein kinase C in isolated nuclei (Buckley et al., 1990) support the hypothesis that protein kinase C may regulate nuclear events involved in transcription and protein synthesis. Several studies have demonstrated the ability of protein kinase C to alter nuclear transcription of specific genes (Jetten et al., 1985; Rabin et al., 1986; Angel et al., 1987). It has been proposed that phorbol ester induction of ornithine decarboxylase is due to PKC mediated enhancement of transcription of the ornithine decarboxylase gene and synthesis of the enzyme. Consistent with increased RNA polymerase I activity, an increase in the transcription of rDNA was observed in nuclei isolated from PMA-treated myocytes. Despite the stimulation of the rate of transcription of rDNA, the pre-rRNA pool size relative to the 28S rRNA was unaltered by PMA treatment. This finding is consistent with previous studies suggesting that the processing of pre-rRNA was very efficient and may be regulated only by the rate at which the precursor was formed. Thus, the fractional rate of processing of pre-rRNA is accelerated in parallel to the rate of transcription of rDNA. Therefore, similar to the synthesis of hemoglobin, c-myc and c-fos, the control of rRNA synthesis after PMA treatment occurs primarily at the level of transcription (Groudine et al., 1981; Kelly et al., 1983; Cochran et al., 1984).

In conclusion, treatment of cardiomyocytes with active phorbol ester resulted in increased RNA polymerase I activity and rDNA transcriptional rate. The resulting increase in rRNA content accounted for increased protein accumulation in phorbol ester-treated cardiomyocytes. This study suggests that these effects are mediated through activation of protein kinase C.

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REFERENCES

Allo, S. N., and Schaffer, S. W. (1990) Biochim. Biophys. Acta 1023, 206-212
Adams, J. C., and Guillick, W. J. (1988) Biochem. J. 257, 905-911
Angel, P., Imagawa, M., Chiu, R., Stein, P., Inbra, R., Rahmendorf, H., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729-739
Anversa, P., Beghi, C., Kikkawa, Y., and Olivetti, G. (1986) Circ. Res. 58, 26-37
Belenguer, P., Baldin, V., Mathieu, C., Prata, H., Bensaid, M., Bouche, G., and Amalaric, F. (1989) Nucleic Acids Res. 17, 6625-6636
Bowman, L. H. (1987) Dev. Biol. 119, 152-163
Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
Buckley, A. R., Crowe, P. D., and Russel, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8649-8652
Camacho, J. A., Peterson, C. J., White, G. J., and Morgan, H. E. (1990) Am. J. Physiol. 258, C66-C91
Cassidy, B. G., Yang-Yen, H., and Rothblum, L. I. (1987) Mol. Cell. Biol. 7, 2388-2396
Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851
Cesarone, C. F., Bolognesi, C., and Santi, L. (1979) Anal. Biochem. 100, 188-197
Chilian, W. M., and Marcus, M. L. (1987) Annu. Rev. Physiol. 49, 477-487
Cochran, B. H., Zullo, J., Verma, I. M., and Stiles, C. D. (1984) Science 226, 1080-1082
Cooper, D. R., Konda, T. S., Standaert, M. L., Davis, J. S., Pollet, R. J., and Farese, R. V. (1987) J. Biol. Chem. 262, 3633-3639
Cooper, G., IV (1987) Annu. Rev. Physiol. 49, 501-518
Cutilletta, A. F., Rudnik, M., and Zak, R. (1978) J. Mol. Cell. Cardiol. 10, 677-687

2 P. J. McDermott, unpublished results.
de la Bastie, D., Leivitsky, D., Rappaport, L., Mercardier, J.-J., Marotte, F., Wisnewsky, C., Brovovich, V., Schwartz, K., and Lompree, A.-M. (1990) *Circ. Res.* **66**, 554–564

Fuller, S. J., Gaitanakis, C. J., and Sugden, P. H. (1990) *Biochem. J.* **266**, 727–736

Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., Jr., and Alexander, R. W. (1986) *J. Biol. Chem.* **261**, 5901–5906

Groudine, M., Peretz, M., and Weintraub, M. (1981) *Mol. Cell. Biol.* **1**, 281–288

Grove, R. I., and Schimmel, S. D. (1982) *Biochim. Biophys. Acta* **711**, 279–290

Haneda, T., and McDermott, P. J. (1991) *Mol. Cell. Biochem.* **104**, 169–177

Hannun, Y. A., Greenberg, C. S., and Bell, R. M. (1987) *J. Biol. Chem.* **262**, 13620–13626

Henrich, C. J., and Simpson, P. C. (1988) *J. Mol. Cell. Cardiol.* **20**, 1081–1085

Hii, C. S. T., Kokke, Y. S., Clark, K. J., and Murray, A. W. (1990) *Biochim. Biophys. Acta* **1052**, 327–332

Hossenlopp, P., Wells, D., and Chambon, P. (1975) *Eur. J. Biochem.* **58**, 237–251

Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616

Jetten, A. M., Ganoor, B. R., Vanderbark, G. R., Shirley, J. E., and Bell, R. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1941–1945

Kako, K. J., Varini, K., and Beznak, M. (1972) *Cardiovasc. Res.* **6**, 57–66

Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. (1983) *Cell* **35**, 603–610

Komuro, I., Katoh, Y., Kaida, T., Shibasaki, Y., Kurabayashi, M., Hoh, E., Takaku, F., and Yazaki, Y. (1991) *J. Biol. Chem.* **266**, 1265–1268

Koyanagi, S., Eastham, C. L., Harrison, D. G., and Marcus, M. L. (1982) *Circ. Res.* **50**, 55–62

Lee, W., Mitchell, P., and Tjian, R. (1987) *Cell* **49**, 741–752

Lockney, W. W., Golomb, H. M., and Dawson, G. (1984) *Biochim. Biophys. Acta* **796**, 384–392

Lorell, B. H., Wexler, L. F., Momomura, S.-I., Weinberg, E., and Apstein, C. S. (1986) *Circ. Res.* **58**, 653–663

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mager, W. H. (1988) *Biochim. Biophys. Acta* **949**, 1–15

Marino, T. A., Keseryk, L., and Launa, I. K. (1987) *Am. J. Physiol.* **253**, H1391–H1396

McDermott, P. J., Carl, L. L, Conner, K. J., and Allo, S. N. (1991) *J. Biol. Chem.* **266**, 4409–4416

McDermott, P. J., and Morgan, H. E. (1989) *Circ. Res.* **64**, 542–553

McDermott, P. J., Rothblum, L. I., Smith, S. D., and Morgan, H. E. (1989) *J. Biol. Chem.* **264**, 18220–18227

Morgan, H. E., Gordon, E. E., Kira, Y., Chua, B. H. L., Russe, L. A., Peterson, C. J., McDermott, P. J., and Watson, P. A. (1987) *Annu. Rev. Physiol.* **49**, 533–543

Nair, K. G., Cutilletta, A. F., Zak, R., Koide, T., and Rabinowitz, M. (1968) *Circ. Res.* **23**, 451–462

Nishizuka, Y. (1984) *Nature* **308**, 693–698

Pelech, S. L., Charest, D. L., Howard, S. L., Paddon, H. B., and Salari, H. (1980) *Biochim. Biophys. Acta* **1051**, 100–107

Peterson, C. J., Whitman, V., Watson, P. A., Schuler, G., and Morgan, H. E. (1989) *Circ. Res.* **64**, 360–369

Rabin, M. S., Doherty, P. J., and Gottesman, M. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 357–360

Resende, E., Jr., Ting, J., Kim, K. S., Wooden, S. K., and Lee, A. S. (1986) *J. Cell Biol.* **103**, 2145–2152

Richter, E. A., Cleland, P. J. F., Rattigan, S., and Clark, M. G. (1987) *FEBS Lett.* **217**, 232–236

Roeder, R. G. (1974) *J. Biol. Chem.* **249**, 249–256

Rozengurt, E. (1986) *Science* **234**, 161–166

Simpson, P. (1986) *Circ. Res.* **56**, 884–894

Sollner-Webb, B., and Tower, J. (1989) *Annu. Rev. Biochem.* **55**, 801–830

Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7603–7609

Watson, S. F., McNally, J., Shipman, L. J., and Godfrey, P. P. (1988) *Biochem. J.* **249**, 245–250

Zahradka, P., and Sels, B. H. (1987) *Eur. J. Biochem.* **171**, 37–43

Zeilhub, W., and Zimmer, H. G. (1989) *Circ. Res.* **65**, 1417–1425