**α1-Adrenergic Stimulation of Cardiac Gene Transcription in Neonatal Rat Myocardial Cells**

**EFFECTS ON MYOSIN LIGHT CHAIN-2 GENE EXPRESSION**

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Previous studies have demonstrated that α-adrenergic stimulation of cultured, neonatal rat myocardial cells results in an increase in intracellular volume and protein content of cultured neonatal rat myocardial cells. Utilizing this model of cardiac hypertrophy, we have examined the effects of α-adrenergic stimulation on the accumulation of sarcomeres and the expression of a rat cardiac myofibrillar gene, myosin light chain-2 (MLC-2). Following α-adrenergic stimulation, cultured myocardial cells displayed a severalfold increase in the number of sarcomeric units, as assessed by electron microscopy, an increase in cellular MLC-2 content, and a 2-3-fold increase in the steady state levels of MLC-2 mRNA. This effect of α-adrenergic stimulation was accompanied by a 2-3-fold increase in total transcriptional activity, which was dependent on the concentration and duration of exposure to the agonist, and displayed α1-adrenergic receptor specificity. The transcriptional response was not immediate, with a lag period of at least 1 h, and a maximal effect required continuous occupancy of the receptor. The increase in steady state levels of MLC-2 mRNA is regulated, in part, at the level of transcription of the cardiac MLC-2 gene. These results suggest that α1-adrenergic stimulation may be important in the growth of the neonatal heart through the activation of total transcriptional activity. In addition, increases in the levels of myofibrillar proteins during myocardial cell growth and hypertrophy, may be mediated in part by the stimulation of transcription of myofibrillar genes.

Myocardial α- and β-adrenergic agonists are of major importance in the regulation of cardiac metabolism and function (1-9). Stimulation of myocardial β-adrenergic receptors results in an increase in myocardial contractility (1, 2), glycolysis (4), and Ca2+ATPase activity of sarcoplasmic reticulum (5, 6). α1-Adrenergic stimulation produces chronotropic (7) and inotropic effects (8) on the heart, which are associated with an increase in phosphatidylinositol turnover (9), Ca2+ flux (10), and inhibition of cAMP phosphodiesterase activity (11). These effects of adrenergic stimulation are rapid in onset, occurring within minutes of the binding of the agonist to the adrenergic receptor (3, 12), and do not ordinarily require an increase in the synthesis of new or additional myocardial proteins.

In addition to these acute effects on cardiac metabolism, recent studies have elucidated a role for α1-adrenergic regulation of growth and hypertrophy in neonatal rat myocardial cells (13-15). α1-Adrenergic stimulation of cultured neonatal rat myocardial cells results in a 2-fold increase in intracellular volume (14), a 2-fold increase in cellular protein content (14, 15), and an increase in the fractional synthetic rates of contractile proteins (15). The molecular mechanisms which link the occupancy of the α1-adrenergic receptor with the increase in contractile protein synthesis are unknown. Conceptually, the increase in myofibrillar protein synthesis could be due to transcriptional, post-transcriptional, or translational effects of α1-adrenergic agonists. Currently, there are no available data on the effects of α1-adrenergic stimulation on the transcription of cardiac genes. In addition, it has not been directly demonstrated that the accumulation of myofibrillar protein during periods of cardiac growth or hypertrophy is due to a corresponding increase in transcription of individual myofibrillar genes (16).

Accordingly, we have utilized cultured neonatal rat myocardial cells to examine the effects of α-adrenergic stimulation on the expression of a contractile protein gene, myosin light chain-2 (MLC-2).1 The effects of α-adrenergic stimulation on the accumulation of sarcomeres was assessed by electron microscopy, and increases in MLC-2 content were measured by immunoblotting. To examine the effects of α-adrenergic stimulation on the transcription of cardiac genes, we have examined the relative rates of transcription, as well as steady state levels of MLC-2 mRNA, utilizing a rat cardiac MLC-2 cDNA probe, pRLC429 (17). These results suggest that α1-adrenergic stimulation may be important in the growth of the neonatal heart through the activation of total transcriptional activity. In addition, increases in the levels of an individual myofibrillar mRNA during myocardial cell growth and hypertrophy may be mediated in part by an increase in transcription of individual myofibrillar genes.

**EXPERIMENTAL PROCEDURES**

Cell Culture Procedure—Cultured neonatal rat myocardial cells were prepared as previously described (18) with minor modifications.

1 The abbreviations used are: MLC-2, myosin light chain-2; SDS, sodium dodecyl sulfate; SSC, 1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Myocytes were dispersed from the ventricles of 1–2-day-old Sprague-Dawley rats by digestion with collagenase II ( Worthington) and pancreatin ( Gibco) at 37 °C. To obtain primary myocardial cell cultures (15, 19), a differential plating period of 30 min was utilized to allow attachment of the nonmyocardial cells. The myocytes were plated in 10-cm dishes (Falcon) at a density of 4.5 × 10^5 cells in 5 ml of 4:1 Dulbecco’s modified Eagle’s medium/medium 199 ( Gibco), supplemented with 5% horse serum, 5% fetal calf serum, and antibiotics (ampicillin 34 μg/ml and gentamicin 3 μg/ml). Following incubation in serum for 24 h, the cultures were washed and incubated in medium 199 supplemented with antibodies for various time periods with platelet-derived growth factor β (PDGF β) and/or antago

_Immunoblotting Analyses—_Immunoblotting was performed by a modification of a previously described method (20). Myocardial cells were scraped into phosphate-buffered saline and collected by centrifugation for 5 min at 1,000 × g. The resulting cellular pellet was solubilized with 600 μl of 8 M urea and was centrifuged for 30 min at 10,000 rpm. The protein content of the cellular extracts was assayed by the method of Bradford (21), and 5-μg aliquots of cellular protein were taken for SDS-polyacrylamide gel electrophoresis. The samples were prepared for electrophoresis by mixing with an equal volume of 0.4 M Tris-HCl, pH 6.8, 20% sucrose, 2% SDS, and 0.1 M 2-mercaptoethanol, followed by boiling for 3 min. The resulting 30 μl of sample was then loaded onto a 10% acrylamide, and the running gel was 15% acrylamide. Following electrophoresis, the samples were transferred to nitrocellulose in a Trans blot cell (Bio-Rad) at 20 mA for 24 h. Nitrocellulose blots were stained for protein with Coomasie Blue, and developed utilizing an antisera overlay technique (20). The primary antibody was a rabbit anti-MLC polyclonal antibody which recognizes isoforms MLC-1 and MLC-2. The secondary antibody was a peroxidase-labeled goat anti-rabbit IgG. The peroxidase-conjugated IgG was reacted with 4-chloro-1-naphthol (Sigma), and quantitation was performed by densitometry of the samples in comparison to a purified myosin light chain standard (22).

_Isolation of RNA, Size Fractionation, and Hybridization—_The myocardial cells were detached from the plates with trypsin in a solution of 136 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4.1 mM NaHCO₃, 10 mM HEPES, and 1 mM EDTA at 37 °C, and harvested by gentle scraping into ice-cold serum. The cells were recovered by centrifugation at 1000 × g for 5 min at 4 °C. RNA was isolated by a hot phenol method (23), size fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters, as previously described (24). Alternatively, aliquots of RNA were immobilized directly on nitrocellulose filters for quantitative slot-blotting. Filters were baked for 2 h in vacuo at 80 °C and prehybridized at 50 °C in 5 × SSC, 50% formamide, 50% desoxyribonucleotide solution containing 50% formamide, 5 × Denhardt’s, 0.5-1 M NaCl, 20 mM EDTA, 0.2 M Tris, pH 8.0, and 0.1 M MgCl₂. The nuclei were pelleted by centrifugation at 2000 × g for 10 min at 4 °C. The nuclei were gently resuspended in cold buffer A and an aliquot was added to 0.4% trypan blue. To label the nascent RNA transcripts, the nuclei were incubated in 0.15–0.2 ml of buffer A supplemented with 0.1 mg/ml creatine kinase, 0.01 M MgCl₂, 14 mM 2-mercaptoethanol, 20% glycerol, 0.2 M Tris, pH 8.0) and the nuclei were pelleted by centrifugation at 2000 × g for 10 min at 4 °C. The nuclei were gently resuspended in cold buffer A and an aliquot was added to 0.4% trypan blue. To label the nascent RNA transcripts, the nuclei were incubated in 0.15–0.2 ml of buffer A supplemented with 0.1 mg/ml creatine kinase, 0.01 M phosphate buffer, 1 M each of ATP, GTP, and CTP, 0.03 M UTP, and 0.5 M of [α-32P]UTP (Du Pont-New England Nuclear, 3000 Ci/mmol) by nick translation (26) and added to the prehybridization solution. Following overnight hybridization at 50 °C, the filters were washed in 1 ml of sodium dodecyl sulfate at room temperature, followed by 0.1 × SSC, 0.1% SDS at 65 °C. 

_In Vivo Labeling of RNA—_Following various time periods of treatment, the myocardial cells were pulse labeled for 2 h with 0.01-0.05 mCi of (5, 6) [3H]uridine (Du Pont-New England Nuclear, 40 Ci/mmol). The incorporation of radioactivity into nuclear and cytosolic fractions was determined by precipitation with 10% trichloroacetic acid and assessment of acid-insoluble radioactivity by liquid scintillation spectroscopy.

_In Vitro Pulse Labeling of Nascent Transcripts—_"Run-on" transcription was performed by a modification of previously described methods (27, 28). The myocardial cell cultures were rinsed with ice-cold phosphate-buffered saline and lysed in 0.1% Nonidet P-40 for 1–2 min at 4 °C. Subsequently the plates were gently scraped with buffer A (0.14 M KCl, 0.01 M MgCl₂, 1 M MnCl₂, 14 M 2-mercaptoethanol, 20% glycerol, 0.2 M Tris, pH 8.0) and the nuclei were pelleted by centrifugation at 2000 × g for 10 min at 4 °C. The nuclei were gently resuspended in cold buffer A and an aliquot was added to 0.04% trypan blue. To label the nascent RNA transcripts, the nuclei were incubated in 0.15–0.2 ml of buffer A supplemented with 0.1 mg/ml catalase, 0.01 M phosphate buffer, 1 M each of ATP, GTP, and CTP, 0.03 M UTP, and 0.5 M of [α-32P]UTP (Du Pont-New England Nuclear, 3000 Ci/mmol) for 15 min at 30 °C. The nuclei were collected by centrifugation at 2500 × g for 45.1 min and lysed at 4 °C in 1 ml of buffer B (50 mm Tris, pH 7.2, 5 M MgCl₂, 10.5 mM LiCl, 5 mM vanadyl nuclease, and 0.5 mg/ml heparin). The nuclear extracts were digested for 2 min with 1–2 μg of RNase-free DNase ( Worthington), and the reaction was terminated by the addition of 2 ml of 0.05 M EDTA, 1% SDS, and 0.05 M Tris, pH 8.0. RNA was extracted with phenol/chloroform/isoamyl alcohol (3:1:0.05, vol/vol) at 65 °C, followed by chloroform/isoamyl alcohol (95:5) at room temperature, and the RNA was precipitated in ethanol. The pellet was dissolved in 0.01 M Tris, pH 7.4, 0.01 M EDTA, 0.2 M NaCl and partially hydrolyzed in 0.2 N NaOH for 10 min at 4 °C.

The radiolabeled RNA transcripts were diluted in 1 × Denhardt’s solution containing 50% formamide, 5 × SSC, 0.1 mg/ml herring sperm DNA, 30 μg/ml poly(A) and poly(C), and hybridized with 5 μg of pRLC429, and 5 μg of pBR322 immobilized on nitrocellulose filters. Typically, 3–15 × 10⁶ cpm were added to each set of filters and hybridization was performed for 3–5 days at 42 °C. Filters were washed at room temperature in 2 × SSC with 0.1% SDS followed by 0.1 × SSC for 2 h at 42 °C. Relative hybridization was quantitated by densitometric scanning of the corresponding autoradiograms and by direct counting of the radioactive bands by liquid scintillation spectroscopy.

_Morphological and Ultrastructural Analysis—_For morphologic analyses, the cells were plated in sterilized glass coverslips, as previously described (29). The cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The preparations were rinsed several times with 5% sucrose in 100 mM phosphate buffer, pH 7.4, and postfixed for 30 min to 1 h in 1% osmium tetroxide in 100 mM phosphate buffer. The cells were dehydrated in a graded series of alcohols and embedded in Poly-L. Molds were filled with epoxy and placed over the cells which were also covered with a layer of epoxy. After partial polymerization at 60 °C, the epoxy and attached cells were removed from the culture dishes. Thin sections were cut on a Du Pont-Sorvall MT 2B ultramicrotome with a diamond knife and mounted on copper grids. The grids were stained with uranyl acetate and lead citrate. The preparations were examined and photographed with a JEOL 100S transmission electron microscope.

**RESULTS**

_Accumulation of Sarcomeric Units—_Fig. 1 displays the effects of 48 h of treatment with the α-adrenergic agonist, phenylephrine, on the morphology of neonatal rat myocardial

**FIG. 1. Accumulation of contractile units in cultured neonatal rat myocardial cells following treatment with phenylephrine.** Myocytes were cultured in 10-cm dishes as described under "Experimental Procedures." After a 24-h-plating period, the cells were placed in serum-free M199 medium with ampicillin and gentamicin in the presence and absence of phenylephrine (0.1 mM). After 48 h the cells were processed for electron microscopy as described under "Experimental Procedures." Control (panel A): myofibrils (mf) were sparse and organized sarcomeres almost totally absent. Phenylephrine (panel B): myofibrils were numerous and frequently organized into columns of sarcomeres with prominent Z-bands. Arrows denote locations of contractile proteins. Magnification: × 8250 for panels A and B.
cells. Following 24 h in culture, myofibrils were absent or sparse in the control cells. When myofibrils were present in control cells, they were arranged in narrow, poorly organized bundles scattered throughout the cytoplasm with a tendency to be oriented toward the peripheral margins and cytoplasmic processes of the cells. Classic sarcomeres with typical banding patterns were not present. Treatment with the \( \alpha \)-adrenergic agonist, phenylephrine, resulted in a severalfold increase in the number of organized contractile units within the myocardial cells (Fig. 1). In the phenylephrine-treated cells, the myofibrils were frequently numerous and organized into broad bands typical of sarcomeres in intact cardiac muscle. The sarcomeres were arranged in rows and present in all areas of the cells, especially in the central cytoplasm and displayed prominent Z-bands. As assessed by light microscopy, treatment with phenylephrine had no significant effect on increasing the number of myocardial cells per dish, in agreement with previously published data (15).

**Accumulation of MLC-2**—The effect of phenylephrine on the accumulation of organized sarcomeres was paralleled by a similar effect on the accumulation of a cardiac contractile protein, myosin light chain-2. As assessed by immunoblotting techniques, there was a time-dependent increase in the MLC-2 content of both control and phenylephrine-treated cells (Fig. 2). At all time points examined, the MLC-2 content of the phenylephrine-treated cells was greater than the control cells. The difference in MLC-2 content between control and treated cells was maximal after 48 h, reaching a greater than 2-fold increase in MLC-2 content. These results suggest that phenylephrine increases the rate of accumulation of MLC-2, as well as the eventual amount of MLC-2 within the individual myocardial cells.

**Accumulation of MLC-2 mRNA**—To determine if the increase in MLC-2 was accompanied by an increase in steady state levels of MLC-2 mRNA, we utilized a rat cardiac MLC-2 cDNA probe, pRLC429. pRLC429 contains a 446 base pair insert consisting of 375 base pairs of the coding region and 71 base pairs of the 3′-untranslated region of the MLC-2 mRNA (17). As displayed in Fig. 3, pRLC429 hybridized to total neonatal rat myocardial cell RNA as a single band of approximately 750 bases, the expected length of MLC-2 mRNA. There was little hybridization to total RNA obtained from cultures of nonmyocardial cells obtained during differential plating. The small extent of hybridization was most likely due to a small number of contaminating myocytes in the differential attachment plates. This result is consistent with S1 nuclease hybridization studies which demonstrated tissue specific expression of the rat cardiac MLC-2 gene (17). Thus, the rat cardiac MLC-2 cDNA probe pRLC429 can be utilized to assess alterations in myofibrillar gene expression in this cultured neonatal rat myocardial cell model.

To determine if treatment with the \( \alpha \)-adrenergic agonist increased the content of the MLC-2 mRNA, quantitative slot-blot hybridization was performed using RNA from control and phenylephrine-treated cells. Hybridization studies with RNA isolated after 24 h of phenylephrine treatment, revealed a 2–3-fold increase in MLC-2 mRNA content following 24 h of phenylephrine treatment (Fig. 4). Slotting of increasing concentrations of total RNA resulted in a linear increase in the hybridization signal, verifying conditions of DNA excess hybridization.

**Increase in \([^{3}H]\)Uridine Labeling of Cytoplasmic and Nuclear Fractions**—The accumulation of myofibrillar protein and increases in myosin light chain-2 mRNA suggested the possibility that \( \alpha \)-adrenergic stimulation might have an effect on total RNA synthesis in cultured rat myocardial cells. Therefore, myocardial cells were treated in the presence and absence of phenylephrine for various time periods, and the cells were pulse labeled with \([^{3}H]\)uridine for 2 h. The cells were harvested by Nonidet P-40 lysis, fractionated into nuclear and cytoplasmic components by centrifugation, and incorporation of \([^{3}H]\)uridine into trichloroacetic acid insoluble material was assessed by precipitation (Fig. 5). Following treatment with phenylephrine, there was a time-dependent increase in \([^{3}H]\) uridine labeling of both the nuclear and cytoplasmic fractions.
phenylephrine.

Phenylephrine-treated groups was significant performed as described under "Experimental Procedures." Quantitation of the extent of hybridization was performed by densitometry of the corresponding autoradiogram. From triplicate experiments. The difference between control and phenylephrine was not immediate, with a lag period of at least 1 h. However, after 24 h of treatment, there was a 2-2.5-fold increase in total transcriptional activity. The increase in total transcriptional activity appeared to plateau after 24 h of phenylephrine treatment. Thus, enhancement of total transcriptional activity paralleled the time course of increases in MLC-2 mRNA content. This increase cannot be explained by an effect on the small number of nonmyocardial cells present in the cultures, since treatment of nonmyocardial cell cultures with phenylephrine did not result in an increase in total transcriptional activity. There was no significant difference in the recovery of intact nuclei between the treated and control groups. Thus, the effect of phenylephrine was not simply due to an increase in the number of intact nuclei. Normalization of the trichloroacetic acid insoluble counts per minute per myocardial nucleus resulted in a mean value of 2.5 and 6.1 for control and phenylephrine-treated groups, respectively, similar to previously reported values (30, 31).

Table I shows that 35% of the increase in total transcription was sensitive to α-amanitin at a concentration of 1 μg/ml, suggesting that the increase in transcription may be attributed to RNA polymerase I and RNA polymerase II transcripts.

![FIG. 4. MLC-2 mRNA content following treatment with phenylephrine.](image)

Neonatal cultured myocardial cells were incubated for 24 h in 100 medium supplemented with ampicillin (34 μg/ml) and gentamycin (3 μg/ml) in the presence and absence of phenylephrine (0.1 mM). Total RNA was isolated as described under "Experimental Procedures." Utilizing a slot-blot manifold, various concentrations of RNA were immobilized on nitrocellulose filters and were hybridized to [32P]pRLC429 radiolabeled by nick translation. The filters were washed with 0.1 M sodium phosphate, 0.1% SDS at 65 °C, and autoradiography was performed as described under "Experimental Procedures." Quantitation of the extent of hybridization was performed by densitometry of the corresponding autoradiogram. Inset, similar results were obtained from triplicate experiments. The difference between control and phenylephrine-treated groups was significant (P < 0.05) utilizing analysis of variance and Duncan's multiple range tests (41). Control ••• phenylephrine (0.1 mM) (PE) •••. Inset depicts original autoradiograms.

The possibility of changes in uridine nucleotide pools must be considered before concluding that an increase in RNA synthesis has occurred. Therefore, nuclear transcription studies were performed.

Increase in Total Transcriptional Activity of Isolated Nuclei—To directly measure the effects of α-adrenergic stimulation on transcription, nascent RNA transcripts were assayed by in vitro pulse labeling of nuclei isolated from control and treated cells. In this manner, the transcriptional activity of control and stimulated cells could be measured without influence of the pool size of RNA precursors, RNA transport rates, and/or RNA half-life (18). Following treatment with phenylephrine, there was a time-dependent increase in the total transcriptional activity of isolated nuclei from cultured myocardial cells (Fig. 6). The transcriptional effect of phenylephrine was not immediate, with a lag period of at least 1 h. However, after 24 h of treatment, there was a 2-2.5-fold increase in total transcriptional activity. The increase in total transcriptional activity appeared to plateau after 24 h of phenylephrine treatment. Thus, enhancement of total transcriptional activity paralleled the time course of increases in MLC-2 mRNA content. This increase cannot be explained by an effect on the small number of nonmyocardial cells present in the cultures, since treatment of nonmyocardial cell cultures with phenylephrine did not result in an increase in total transcriptional activity. There was no significant difference in the recovery of intact nuclei between the treated and control groups. Thus, the effect of phenylephrine was not simply due to an increase in the number of intact nuclei. Normalization of the trichloroacetic acid insoluble counts per minute per myocardial nucleus resulted in a mean value of 2.5 and 6.1 for control and phenylephrine-treated groups, respectively, similar to previously reported values (30, 31). Table I shows that 35% of the increase in total transcription was sensitive to α-amanitin at a concentration of 1 μg/ml, suggesting that the increase in transcription may be attributed to RNA polymerase I and RNA polymerase II transcripts.

Dose Response Effects and Receptor Specificity—To examine the receptor specificity of the transcriptional response, we utilized norepinephrine which has both an α- and β-adrenergic effect. As displayed in Fig. 7, the addition of increasing concentrations of norepinephrine resulted in a corresponding increase in the total transcriptional activity of cultured myocardial cells. Pretreatment of the cells with the α-adrenergic receptor antagonist, prazosin, abolished the norepinephrine-induced increase in total transcription (Fig. 7). However, the α2 receptor antagonist yohimbine or the B1 receptor antagonist propranolol, displayed minimal inhibitory effects (Fig. 7).

![FIG. 5. Incorporation of [3H]uridine into nuclear and cytosolic fractions following treatment with phenylephrine.](image)

Neonatal myocardial cells were incubated with 0.1 mM phenylephrine and pulse labeled with [3H]uridine for 2 h. The cells were harvested and separated into nuclear and cytosolic fractions as described under "Experimental Procedures." Aliquots were precipitated in 10% trichloroacetic acid and the [3H]uridine content was assessed by liquid scintillation counting. Panel A, cytosolic fraction; panel B, nuclear fraction; (−) control, (+) phenylephrine 0.1 mM. Data are normalized to equal number of myocardial cells obtained from 10-cm dishes. Results represent the mean of duplicate determinations.

![FIG. 6. Time course of increases in total transcription activity following treatment with phenylephrine.](image)

Cultured myocardial cells were incubated for various time periods in the presence and absence of phenylephrine (0.1 mM). The nuclei were isolated and incorporation of [32P]UTP into nascent RNA transcripts was assessed by a nuclear transcript elongation assay as described under "Experimental Procedures." Data were normalized to the number of nuclei in the treated and control samples. Results are expressed as the percent increase in incorporation of [32P]UTP into a trichloroacetic acid insoluble fraction in nuclei from treated versus control cells and represent the mean ± S.E. for n = 3 or greater. At 24 h, the difference between control and phenylephrine-treated groups was significant (P < 0.05; n = 8 separate experiments), utilizing analysis of variance and Duncan’s multiple range tests (41).
Table I

Effect of a-amanitin on total transcriptional activity following a-adrenergic stimulation

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33,691</td>
<td>23,679</td>
</tr>
<tr>
<td>Phenylephrine (0.1 mm)</td>
<td>53,984</td>
<td>48,670</td>
</tr>
<tr>
<td>Control + a-amanitin</td>
<td>19,883</td>
<td>14,787</td>
</tr>
<tr>
<td>Phenylephrine + a-amanitin</td>
<td>32,445</td>
<td>38,386</td>
</tr>
</tbody>
</table>

Fig. 7. Dose response and pharmacologic specificity of the norepinephrine effect on total transcriptional activity of cultured myocardial cells. Experimental details are similar to Fig. 6, with the exception that cultured myocardial cells were treated with various concentrations of norepinephrine and antagonists for 24 h. Cultured myocardial cells were either incubated with various concentrations of norepinephrine (NE) for 24 h, or with norepinephrine (10 μM) in the presence of various concentrations of adrenergic antagonists (prazosin, yohimbine, propranolol). Total transcriptional activity was measured as described under "Experimental Procedures." Results represent the mean of duplicate determinations and are expressed as a percent of the maximal percent increase (norepinephrine 10 μM versus control) in total transcriptional activity. Similar results were obtained from duplicate experiments.

Thus, the increase in total transcriptional activity appears to be dependent on stimulation of the α1-adrenergic receptor.

Maximal Effect Requires Continued Receptor Occupancy—The stimulation of total transcriptional activity could be due to a "trigger" effect, where only transient exposure to the α1-adrenergic agonist would result in a maximal effect. Alternatively, continuous occupancy of the α1-adrenergic receptor might be required to maintain maximal levels of transcription. To distinguish between these possibilities, cultured myocardial cells were exposed to phenylephrine for 1–3 h, followed by removal of the agonist and further incubation with an α1-adrenergic antagonist (prazosin) for an additional 21 h. In parallel, a set of cultures were treated continuously with phenylephrine. After 24 h the nuclei were harvested and total transcriptional activity was measured. As displayed in Table II, transient exposure to phenylephrine for 1–3 h produced only a marginal increase in total transcriptional activity compared to the increase found in cells treated continuously for 24 h. Thus, continuous occupancy of the α1-adrenergic recep-

Table II

Continuous occupancy of the α-adrenergic receptor and total transcriptional activity

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NE*</td>
<td>NE</td>
<td>23</td>
<td>13</td>
<td>243</td>
</tr>
<tr>
<td>3 28</td>
<td>14</td>
<td>85</td>
<td>NE*</td>
<td></td>
</tr>
<tr>
<td>24 106</td>
<td>120</td>
<td>155</td>
<td></td>
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</tr>
</tbody>
</table>

*NE = not evaluated.

α1-Adrenergic Stimulation Increases the Transcriptional Activity of Cultured Neonatal Rat Myocardial Cells—The role of α1-adrenergic stimuli in the regulation of cardiac function and intermediary metabolism has been a subject of extensive study. Stimulation with α-adrenergic agonists increases the activity of phosphofructokinase and thereby increases the rate of glycolysis in perfused rat hearts (32). In the newborn and adult heart, α1-adrenergic agonists produce important effects on contractility (8) and heart rate (7).

The current study documents an additional role for α1-adrenergic agonists in the regulation of transcription in neonatal rat myocardial cells. This effect is not a generalized
Adrenergic Stimulation of MLC-2 Gene Expression

**Fig. 8.** Hybridization of nascent [\(^{32}\)P]RNA transcripts with a MLC-2 cDNA probe, pRLC429. Nuclei were isolated from control myocardial cells and nascent nuclear transcripts were labeled from [\(\alpha\cdot^{32}\)P]UTP as described under "Experimental Procedures." Various amounts of radiolabeled transcripts were hybridized with 5 \(\mu\)g of pRLC429 and 5 \(\mu\)g of pBR322, immobilized on nitrocellulose filters by slot blotting. The filters were washed with 0.1 X SSC, 0.1% SDS at 50 °C, and the extent of hybridization was assessed by liquid scintillation counting of the corresponding filters. Data are expressed as the counts per minute hybridized per slot following subtraction of the hybridization signal obtained to the pBR322 vector alone.

**TABLE III**

Effect of phenylephrine on transcription of the cardiac MLC-2 gene

Cultured neonatal rat myocardial cells were treated in the presence and absence of phenylephrine (0.1 mM) for 24 h and nuclei were isolated for in uitro pulse labeling studies as described under "Materials and Methods." The total transcriptional activity is expressed as the total content of [\(^{32}\)P]RNA transcripts obtained during the in uitro labeling period (cpm X 10\(^{-6}\)) and as [\(^{32}\)P]RNA transcripts normalized to the number of nuclei in the treated and control samples. Specific transcription of the MLC-2 gene was assessed by hybridization of equal amounts of [\(^{32}\)P]RNA from control and treated samples with 5 \(\mu\)g of pRLC429 and 5 \(\mu\)g of pBR322 immobilized on nitrocellulose filters. Densitometry of the corresponding autoradiograms was performed and results are expressed as the percent increase in signal obtained with the [\(^{32}\)P]RNA derived from phenylephrine versus control samples.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Total [(^{32})P]RNA (cpm X 10(^{-6}))</th>
<th>Total [(^{32})P]RNA hybridized to pRLC429 (cpm/nucleus)</th>
<th>Total % increase in signal</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>14.2</td>
<td>2.4</td>
<td>+28%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>41.9</td>
<td>8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>13.6</td>
<td>1.5</td>
<td>+28%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>26.1</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>48.5</td>
<td>1.8</td>
<td>NC*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>78.8</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>10.0</td>
<td>1.4</td>
<td>+153%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>32.9</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>45.6</td>
<td>3.4</td>
<td>+39%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>92.9</td>
<td>7.2</td>
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<tr>
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<td>Control</td>
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<td>+21%</td>
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<tr>
<td></td>
<td>Phenylephrine</td>
<td>36.0</td>
<td>4.3</td>
<td></td>
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</tr>
</tbody>
</table>

* NC, no detectable change.

The response of all cells to \(\alpha\)-adrenergic stimulation, since phenylephrine does not produce a similar increase in the transcriptional activity of the nonmyocardial cells isolated from neonatal rat heart. These data suggest that a portion of the effects of \(\alpha\)-adrenergic stimuli on the structure and function of the neonatal heart may be mediated through alterations in the transcription of cardiac genes. Furthermore, these results indicate that the \(\alpha\)-adrenergic stimulus must generate a signal which is ultimately transmitted to the myocardial cell nucleus, in addition to previously described effects on the modulation of proteins located in the cytosol and sarcosomal membrane compartments (3, 12).

The biochemical mechanisms which link the occupancy of the \(\alpha\)-adrenergic receptor with the increase in transcriptional activity are unclear. Previous studies have suggested three potential mediators of \(\alpha\)-adrenergic signal transduction: 1) cAMP; 2) inositol triphosphates; and 3) diacylglycerol. Stimulation of adult rat myocardial cells with phenylephrine result in a decrease in phosphodiesterase activity which would result in a corresponding increase in intracellular levels of cAMP (11). In adult, neonatal, and embryonic myocardial cells, phenylephrine stimulates phosphatidylinositol hydrolysis resulting in the formation of diacylglycerol and inositol phosphates (9, 33). The present study does not discriminate between these three mechanisms. However, the fact that the addition of the \(\beta\)-adrenergic antagonist, propranolol, did not significantly blunt the effects of the combined \(\alpha\)– and \(\beta\)-adrenergic agonist, norepinephrine, argues against cAMP as the sole mediator of this effect. Interestingly, recent studies in our laboratory have demonstrated that tumor-promoting phorbol esters which activate protein kinase C (Ca\(^{2+}\)/phospholipid-dependent enzyme) can induce hypertrophy and can increase the transcriptional activity of cultured neonatal rat myocardial cells. Since phosphatidylinositol hydrolysis accompanies \(\alpha\)-adrenergic stimulation of cultured neonatal rat myocardial cells (34), these results suggest the possibility that the formation of diacylglycerol and the concomitant activation of protein kinase C may be important in the generation of the signal for increased transcriptional activity.

Transcriptional Mechanisms Contribute to the Accumulation of an Individual Contractile Protein during Myocardial Cell Hypertrophy—Myocardial hypertrophy is an important compensatory mechanism in many physiologic and pathophysiologic states in man. In response to a variety of hormonal and hemodynamic stimuli, the myocardium responds to the hypertrophic stimulus by increasing the myofibrillar protein content of individual myocardial cells, rather than by myocardial cell proliferation (35). Whether this bulk increase in contractile proteins during myocardial hypertrophy is due to selective increases in the transcription of individual contractile protein genes is unknown. However, this possibility has been supported by several studies which have examined the differential expression of myosin heavy chain genes during in uitro hypertrophy. Following conditions of pressure overload (36) or thyroid hormone administration (37, 38), the expression of \(\alpha\)– and \(\beta\)-myosin heavy chain genes switches at both the level of the individual proteins and their respective mRNAs. Similarly, in uitro hypertrophy is associated with the induction of \(\alpha\)-skeletal actin mRNA content of myocardial cells (39, 40). However, the inherent difficulty of measuring the transcription of individual cardiac genes in intact myocardium, and the predominance of nonmyocardial cells in intact heart, has made it difficult to determine if the changes in the levels of an individual mRNA are due to changes in the stability of the mRNA species, or rather due to alterations in the relative rate of transcription of the individual myofibrillar genes.

Previous studies have demonstrated that \(\alpha\)-adrenergic stim-
ulation of neonatal rat myocardial cells produces cellular hypertrophy, as assessed by an increase in the intracellular volume and protein content of myocardial cells without an associated increase in cell proliferation (13, 15). In the current study, we have utilized this cultured cell model to examine the expression of the rat cardiac MLC-2 gene during cellular hypertrophy. The MLC-2 gene was chosen as a model contractile protein gene for the following reasons: 1) as determined in this study by immunoblotting, the cellular content of MLC-2 is increased during hypertrophy of the cultured myocardial cells; 2) the rat cardiac MLC-2 gene is a single copy gene with a single isoform throughout development and thus a relative increase in expression is not secondary to the switching off of a related gene product (17); 3) post-transcriptional modification does not result in alternatively spliced MLC-2 transcripts, allowing simpler interpretation of the hybridization data; and 4) expression of the MLC-2 gene is tissue-specific (17).

Using a rat cardiac MLC-2 probe, we have demonstrated that the 2-3-fold increase in MLC-2 content during α-adrenergic stimulation of neonatal rat myocardial cells is accompanied by a 2-3-fold increase in the steady state levels of MLC-2 mRNA (normalized to total RNA) and a concomitant increase in transcription of the cardiac MLC-2 gene. However, these results do not exclude the possibility of a role for post-transcriptional mechanisms as contributing to the increase in the levels of MLC-2 mRNA during α1-adrenergic stimulation. Whether the increase in MLC-2 mRNA is due to an increase in mRNA stability is unknown but is currently under investigation. This cultured cell model offers several advantages in the study of the transcription of contractile protein genes during myocardial cell hypertrophy. 1) The effects of the stimulus on myocardial cells can be distinguished from effects on the interstitial or nonmyocardial cells; 2) the stimulus can be delivered quantitatively to the myocardial cells in a dose-dependent manner; 3) the uniform radiolabeling of myocardial cells can be readily achieved; and 4) the isolation of intact nuclei can be readily accomplished to facilitate in vitro pulse labeling studies of transcription. However, there are disadvantages to the study of in vivo cardiac hypertrophy using a neonatal rat cardiac myocyte culture model. 1) Neonatal hearts are inherently in a significant growth phase, which is not the case for mature adult animals; 2) the effects of enzymatic digestion during the isolation of cardiac myocytes may damage or alter various aspects of cell homeostasis, including cell surface receptors, energy production and use, and contractile function; 3) important factors in in vitro hypertrophy such as myocardial stretch are difficult to study in cell culture; 4) it is debatable whether “hypertrophy” in single heart cells can be defined by changes in cell volume and protein accumulation only, and whether this can be compared to in vivo hypertrophy.

Nevertheless, this myocyte culture system serves as a model to study molecular mechanisms of signal transduction during myocardial cell hypertrophy. Whether these findings in a cultured neonatal rat myocardial cell model are relevant to the more complex physiology of in vivo hypertrophy is unknown but clearly worthy of future study.

Acknowledgments—The purified myosin light chain standards and antisera were the generous gifts of Mary Nunnally and James T. Stull. We gratefully acknowledge the assistance of Anna Siler in the morphologic studies.

REFERENCES