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Research Article

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α_1-Adrenergic Receptor Stimulation of Sarcomeric Actin Isogene Transcription in Hypertrophy of Cultured Rat Heart Muscle Cells

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Abstract

During pressure-load hypertrophy of the adult heart in vivo, there is up-regulation of the mRNA encoding skeletal α-actin, the sarcomeric actin iso-mRNA characteristic of mature skeletal muscle and the fetal/neonatal heart. We have shown previously that during α_1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes, the induction of skeletal α-actin mRNA is greater than that of the mRNA encoding cardiac α-actin, the sarcomeric actin iso-mRNA characteristic of the adult heart. To determine if this actin iso-mRNA switch during cardiac hypertrophy reflects changes in the transcriptional status of the myocyte nucleus, we quantified the rate of transcription of actin mRNAs and total RNA, using an in vitro run-on transcription assay with nuclei isolated from the cultured myocytes after stimulation with norepinephrine (NE). Transcription of skeletal α-actin was increased at 3 h after NE, reached a maximum 6.1-fold increase at 12 h, and returned to the control level at 24 h. The EC_{50} for NE was 200 nM, and pharmacologic studies indicated α_1-receptor specificity. Transcription of cardiac α-actin was also increased rapidly by NE (maximum 4.6-fold vs. control at 3 h). However, cardiac α-actin transcription had returned to the control level at 6 h, when NE-stimulated skeletal α-actin transcription was still increasing. Transcription of the cytoskeletal (β) actin gene was not changed significantly by NE treatment. Total RNA transcription was not increased until 6 h after NE (1.5-fold vs. control) and remained elevated through 24 h. Inhibition of protein synthesis did not attenuate NE-stimulated actin gene transcription. Thus the α_1-adrenoceptor mediates a rapid, transient, and selective increase in transcription of the sarcomeric actin isogenes during cardiac myocyte hypertrophy. Skeletal α-actin, the fetal/neonatal isogene, is induced preferentially to cardiac α-actin, the adult isogene. The different kinetics of actin isogene and total RNA transcription and the independence of transcription from protein synthesis suggest that transcriptional induction via the α_1 receptor is complex and may involve preexisting regulatory factors. These results are the first to demonstrate that the α_1-adrenergic receptor is a molecular mediator of transcriptional changes underlying an isogene switch that is known to be associated with cardiac myocyte hypertrophy.

Introduction

Sarcomeric (α) actin has two known isoforms, designated cardiac α-actin and skeletal α-actin because of their abundance in adult heart and adult skeletal muscle, respectively. Skeletal α-actin is expressed in the fetal and neonatal heart, but it is markedly down-regulated in the adult heart of birds and mammals, including humans (1, 2). It has been found recently that the skeletal α-actin iso-mRNA is reexpressed in the adult myocardium in response to pressure overload (3). Reexpression of isogenes characteristic of earlier developmental stages is a distinctive feature of certain forms of myocardial hypertrophy in vivo (4). Thus myocardial hypertrophy is not simply a quantitative change in protein mass, but also involves qualitative changes in myocyte mRNA and protein content. In other systems, such qualitative changes in gene expression reflect an altered differentiation state known to accompany many regenerative and neoplastic processes.

We have shown previously that the skeletal α-actin mRNA is reinduced during α_1-adrenoceptor-mediated hypertrophy of rat cardiac myocytes in culture (5). The control myocytes in this culture system, although derived from the neonatal heart, express predominantly cardiac α-actin, the adult sarcomeric actin isoform. Thus the culture system reproduces the sarcomeric actin iso-mRNA switching seen in overload-induced hypertrophy of the mature heart in vivo.

It is unknown whether iso-mRNA switching during cardiac hypertrophy in vivo and in culture simply reflects altered mRNA turnover rates, or whether there are more fundamental changes in the transcriptional status of the myocyte nucleus. Recently, it has been shown that α_1-adrenergic receptor stimulation leads to an increase in overall transcription and the rate of transcription of the gene encoding myosin light chain-2, a protein occurring as a single isoform (6).

The goal of the present series of experiments was to determine if the α_1-adrenergic receptor-induced actin iso-mRNA switch reflects fundamental changes in the transcriptional program of the myocyte nucleus. We used an in vitro run-on transcription assay with nuclei isolated from cultured cardiac myocytes after stimulation with norepinephrine (NE).[1] The

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1. *Abbreviation used in this paper:* NE, norepinephrine.

results show that α_1-adrenergic stimulation mediates a rapid and preferential increase in transcription of the skeletal α-actin gene as compared with total RNA. Moreover, the alteration in transcription of the skeletal α-actin gene is quantitatively and temporally distinct from that of the cardiac α-actin gene. These results are the first to demonstrate that the α_1-adrenergic receptor is a molecular mediator of transcriptional changes underlying an isogene switch that is known to be associated with cardiac myocyte hypertrophy.

Methods

Enzymes were obtained from Boerhinger Mannheim Diagnostics (Houston, TX) and New England Biolabs (Beverly, MA) and used according to the manufacturers' instructions. RNAsin was from Promega Biotec, (Madison, WI). [α-^{32}P]UTP and [α-^{32}P]GTP (400 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). α-Amanitin and cycloheximide were from Sigma Chemical Co. (St. Louis, MO). DNAse I (RNAse-free) was obtained from Cooper Biomedical Inc. (Malvern, PA). Sources of culture materials and adrenergic agents were as noted previously (7).

Cell culture system. Primary cultures of neonatal rat heart muscle cells were prepared as described previously (8). In brief, cells were obtained from the hearts of 1-d-old Sprague-Dawley rats by trypsinization and plated in MEM (Hanks' salts) with 5% calf serum. After 12 h in culture, cells were transferred to a serum-free medium supplemented with transferrin and insulin (each 10 μg/ml). Cells were maintained in 100-mm culture dishes at a density of 100–150 cells per mm^2. Contaminating nonmuscle cells were kept at < 10% by preplating and by the addition of bromodeoxyuridine (0.1 mM) through day 2 of culture. Under these control conditions, there is no change in cell size or number and no beating of the myocytes (7–9).

On day 4 of culture, freshly made adrenergic agents (agonists and antagonists) or their vehicle, ascorbic acid (100 μM final), were added to a final concentration of 2 μM (except as noted for dose-response measurements). Cycloheximide, 2.8 μg/ml (10 μM), was present in some experiments, a concentration which inhibits protein synthesis by > 95% (7). Cells were then harvested for nuclear isolation after 1–24 h of treatment.

Nuclear run-on assay. Nuclei were isolated by a modification (10) of the procedure of Mulvihill and Palmiter (11). Cells were washed in cold phosphate-buffered saline and scraped into homogenization buffer (300 mM ultrapure sucrose, 10 mM Tris [pH 8.0], 2.5 mM Mg acetate, 0.5 mM dithiothreitol, and 0.25% Triton X-100). After cell disruption in a glass ball homogenizer, nuclei were pelleted at 2,000 rpm (750 *g*) at 4°C, and resuspended in a buffer containing 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% sterile glycerol. Resuspended nuclei were used immediately or after storage at −70°C. Storage at −70°C had no effect on transcriptional activity. Recovery of nuclei averaged 75% of cell numbers and was the same for NE-treated and control cultures. Nuclei were also isolated from freshly excised adult and neonatal rat hearts by a similar protocol (10).

Run-on transcription assays were performed as described previously (10). Five million nuclei from control or treated cells were incubated at 30°C in a transcription reaction containing (final in 300 μl): 28% glycerol (vol/vol), 150 mM KCl, 40 mM Tris (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol, 1.0 mM each ATP, CTP, GTP, 0.1 mM EDTA, 25 U RNAsin/ml, and 300 μCi [α-^{32}P]UTP. Reaction time was 15 min, and incorporation of [^{32}P]UTP into purified RNA was linear over 30 min (data not shown). α-Amanitin (1 μg/ml) was included in some reactions to test specificity for RNA polymerase II and to determine the contribution of polymerases I and III (insensitive to α-amanitin at this dose) to total transcription in control and treated cultures (12). Reactions were terminated by the addition of 125 μg/ml of RNAse-free DNAse I for an additional 10 min. Proteinase K (200 mg/ml) was then added for 1 h at 37°C. The ^{32}P-labeled run-off RNA produced was purified by sequential phenol/chloroform extractions, precipitation with ice-cold 20% TCA, and two final ethanol precipitations. In order to increase the ^{32}P-RNA specific activity, several experiments were carried out using 300 μCi of [α-^{32}P]GTP in addition to the [α-^{32}P]UTP.

Purified ^{32}P-labeled RNA run-on product was counted as a index of total transcriptional activity. Then 10–15 × 10^6 cpm of RNA produced by equal numbers of nuclei (5 × 10^6) from control and treated cells was hybridized to a panel of actin DNA probes. In some experiments, equal counts of ^{32}P-labeled RNA from the different groups were used for hybridization; results were comparable to those in which equal numbers of nuclei were used. The hybridization panel consisted of (*a*) pAC 15.2, a 3.7-kb skeletal α-actin genomic fragment; (*b*) pc18, a 440-bp cardiac α-actin cDNA fragment containing 163 bp of the 3′ untranslated region; (*c*) pAC 18.1, a 4.4-kb β-actin genomic fragment; and (*d*) as control, plasmid vector pBR322 DNA. 5 μg of each plasmid probe was linearized and dotted onto nitrocellulose as described by Kafatos et al. (13). Hybridization of increasing amounts of ^{32}P-RNA run-on product resulted in a corresponding increase in specific counts bound, verifying that the DNA probes were in excess (data not shown). Filters were prehybridized for 14–24 h at 52°C in 30% deionized, freshly recrystallized formamide, 500 mM NaCl, 300 μg/ml salmon sperm DNA, 50 mM Hepes (pH 7.0), 0.4% SDS, and 2 mM EDTA. After hybridization for 72 h, blots were washed under stringent conditions by the method of Spindler et al. (14). The washing conditions prevented cross-hybridization between the actin probes, as described previously (15). Specificity of the probes was confirmed in two ways: (*a*) run-on experiments with nuclei isolated from neonatal and adult rat heart and skeletal muscle; and (*b*) hybridizations with control [^{32}P]- and [^{3}H]cRNAs transcribed in vitro from coding sequences for each actin isoform inserted into $pGEM_4$ vectors (data not shown). To quantify hybridization, labeled dots were excised and counted in a liquid scintillation counter to < 5% error. Gene-specific hybridization, in parts per million (ppm), was calculated by dividing the background-corrected counts per minute hybridized to each probe by the total counts per minute included in the hybridization reaction and correcting for efficiency of hybridization. Background was determined by hybridization to vector pBR322 DNA. Hybridization efficiency (average 30% under these conditions) was determined by hybridization of known amounts of labeled [^{3}H]cRNA synthesized from a cDNA insert for cardiac α-actin cloned into a $pGEM_4$ plasmid.

DNA synthesis. Cells were incubated with [^{3}H]thymidine (5 μCi/ml, 20 Ci/mmol) from the time of plating through culture day 6. Cultures were taken for autoradiography, and the proportion of myocytes with labeled nuclei was determined (8). In each of two experiments, 1,000 cells in randomly selected microscopic fields of two to three dishes were counted.

Statistics. Gene-specific transcription is presented as mean±standard error (SE) ppm hybridized per 5 × 10^6 nuclei per 15-min reaction. NE-treated values were compared to concurrent controls using the Student's *t* test. NE-treated/control ratios of mean ^{32}P incorporation into total purified run-off RNA were analyzed for their deviation from unity by calculation of confidence limits (16).

Results

Neonatal rat heart muscle cell cultures were prepared and maintained in serum-free culture for 3 d. On the fourth day after plating, cells were treated with either NE or vehicle (control) for 1–24 h. Myocyte nuclei were isolated and incubated with labeled RNA precursors to allow previously initiated RNA transcripts to elongate (run-on). The labeled RNA produced was purified, counted, and hybridized to gene-specific DNA fragments immobilized on nitrocellulose. The amount of gene-specific hybridization is an index of the polymerase II density on that gene at the time of nuclear isolation. Therefore, differences in hybridization to a particular gene between control and treated cells are indicative of changes in the rate of initiation of transcription of that gene (17).

In nuclei from control cultured myocytes, skeletal α-actin gene transcription was 2.5±0.5 ppm, whereas cardiac α-actin transcription was 6.0±1.6 ppm (n = 26, $P < 0.05$). The reduced level of transcription of the skeletal α-actin gene is consistent with the reduced level of skeletal α-actin mRNA in cultured neonatal cardiocytes (5). Transcription of skeletal α-actin and cardiac α-actin by control myocytes did not vary significantly over 24 h treatment with vehicle (Fig. 1 *A*).

Treatment of the cultured cells with 2 μM NE stimulated a transient increase in transcription of both the skeletal α-actin and cardiac α-actin genes (Figs. 1 *A* and 2). After 3 h, transcription of the skeletal α-actin gene was 3.2-fold higher in NE-treated cells than in control cells ($P < 0.05$). Skeletal α-actin gene transcription reached a maximum 6.1-fold increase vs. control at 12 h ($P < 0.01$) and returned to the control level at 24 h, despite the continued presence of NE (Fig. 1 *A*). Transcription of the cardiac α-actin gene was stimulated more rapidly than that of skeletal α-actin, with a 3.7-fold increase at 1 h after NE ($P < 0.01$) and a maximum 4.6-fold increase at 3 h ($P < 0.01$). However, NE-stimulated cardiac α-actin gene transcription was much more transient than that of the skeletal α-actin gene, returning to the control level before skeletal α-actin gene transcription reached its maximum increase (Fig. 1 *A*). β-actin gene transcription was unchanged by NE, except for a small and statistically insignificant increase at 3 h after NE (1.6-fold vs. control, $P > 0.05$). Inclusion of α-amanitin in the transcription reaction abolished specific hybridization to all actin probes, indicating specificity for RNA polymerase II (Fig. 2).

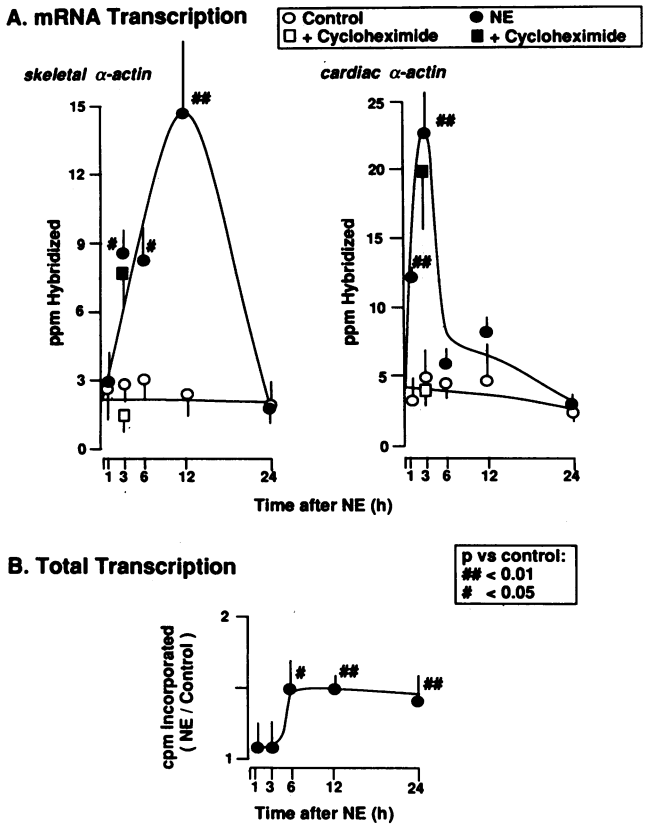

Figure 1. Time course of NE-stimulated actin mRNA and total RNA transcription. Cultured myocytes were treated with 2 μM NE or vehicle (control). At the times indicated, nuclei were isolated, and equal numbers of nuclei in the two groups were assayed for (*A*) gene-specific transcription and (*B*) total transcription, as described in Methods. (*A*) Mean±SE ppm run-on product hybridized to probes for (*left*) skeletal α-actin and (*right*) cardiac α-actin. For the time points at 1, 3, 6, 12, and 24 h, the number of separate experiments was 3, 4, 5, 10, and 4, respectively, with the exception of the 12-h cardiac α-actin value where n = 8. Cycloheximide was included at 2.8 μg/ml in several 3-h experiments with NE (n = 7) and vehicle (n = 4). (*B*) NE-treated/control ratio for total ^{32}P incorporation into purified run-on RNA at each time point. Each value is the mean±SE for the number of experiments indicated in *A*.

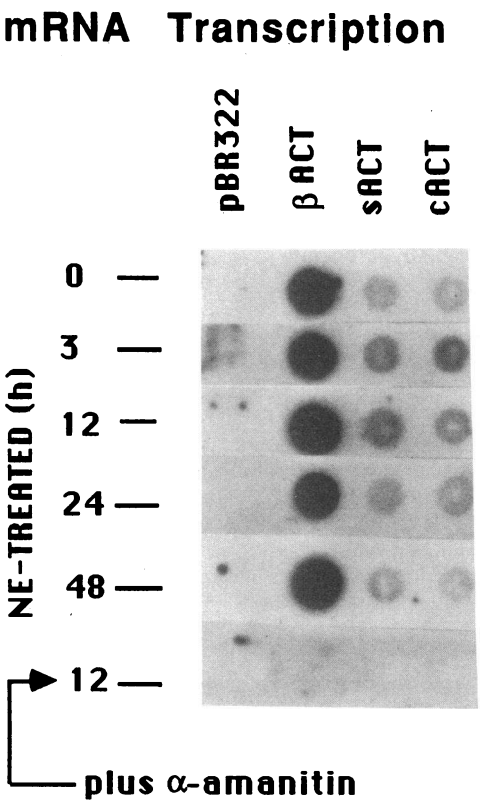

Figure 2. NE increases specific hybridization to actin probes. Cells were treated with 2 μM NE for the times indicated, and nuclei were harvested for the run-on transcription assay. The figure is an autoradiograph of run-on product hybridized to the probes indicated (*βACT*, β-actin; *sACT*, skeletal α-actin; *cACT*, cardiac α-actin). Note the increase in cardiac α-actin hybridization by run-on product from nuclei harvested at 3 h and the increase in skeletal α-actin hybridization after both 3 and 12 h of NE treatment. There is no detectable change in β-actin hybridization. Inclusion of α-amanitin (1 μg/ml) in the reaction done with nuclei harvested at 12 h abolishes all specific hybridization.

The total ^{32}P incorporation into purified run-on RNA by control nuclei during the 15-min in vitro reaction was 10.7±1.1 × 10^6 cpm per 5 × 10^6 nuclei (n = 26) and did not vary significantly over the times studied. Total incorporation was not changed by 2 μM NE at 1 or 3 h (Fig. 1 *B*), although transcription of the skeletal α-actin and cardiac α-actin genes was increased at these times (Fig. 1 *A*). At 6 h of NE treatment, NE-stimulated total transcription was increased 1.5±0.2-fold vs. control (n = 5, $P < 0.05$) and remained at this elevated level for 24 h (Fig. 1 *B*). Inclusion of α-amanitin in the in vitro reaction inhibited total transcription by 45–50%. Both α-

amanitin-sensitive and α-amanitin-resistant portions of total transcription were increased significantly by NE at 6 and 12 h (data not shown). At 24 h, the α-amanitin-resistant portion of total transcription (i.e., polymerases I and III activity) was increased in NE-treated cells (1.7±0.1-fold vs. control, $n = 4$, $P < 0.01$). However, at the same time (24 h), α-amanitin-sensitive transcription (i.e., polymerase II activity) was not increased significantly by NE (1.3±0.3-fold vs. control, $n = 4$, P = NS).

NE stimulation of skeletal α-actin transcription at 12 h was dose-dependent, with an EC_{50} of 200 nM (Fig. 3). The α_1-adrenergic antagonist terazosin (2 μM) reduced NE-stimulated skeletal α-actin transcription at 12 h to control levels (2.1±1.0 ppm for NE plus terazosin, vs. 2.2±3.7 ppm for control, $n = 3$ paired experiments, P = NS). Further, the β-adrenergic receptor agonist isoproterenol (2 μM) did not alter incorporation into skeletal α-actin transcripts at 12 h (1.6±1.7 ppm for isoproterenol, vs. 2.2±3.7 ppm for control, $n = 3$, P = NS). Similar α_1-adrenergic receptor specificity was seen for cardiac α-actin in cells treated with NE for 3 h (data not shown).

Cycloheximide (2.8 μg/ml, 10 μM) included in the culture medium inhibited protein synthesis by > 95% (7 and data not shown) but did not inhibit the NE-stimulated increase in skeletal α-actin or cardiac α-actin transcription at 3 h (Fig. 1).

NE did not stimulate DNA synthesis, as measured by autoradiography after incubation for 6 d in the presence of [^{3}H]thymidine. There were 5.8±0.4% control myocytes with labeled nuclei, vs. 4.0±1.0% of myocytes treated with 2 μM NE (mean ± range, $n = 2$). Cell numbers were the same in the two groups (7, 9 and data not shown). Thus changes in transcription were not produced by altered template availability. There were < 10% nonmuscle cells in all cultures, and these cells do not respond to NE (5, 7, 9).

Discussion

The critical new finding of this work is that stimulation of the α_1-adrenergic receptor is coupled to preferential transcriptional induction of the skeletal α-actin gene, the fetal/neonetal sarcomeric actin isogene characteristic of pressure-load myocardial hypertrophy in vivo. The increases in transcription of both sarcomeric actin isogenes exceeded the increases in transcription of cytoskeletal (β) actin and total RNA (Fig. 4), indicating that transcription of genes encoding contractile proteins is increased selectively in cardiac myocyte hypertrophy. However, the α_1-mediated increase in transcription of the skeletal α-actin gene was greater in magnitude and duration than the increase in transcription of the cardiac α-actin gene (Fig. 4). The greater increase in skeletal α-actin mRNA transcription is consistent with the disporportionate increase in skeletal α-actin mRNA content observed previously in this model of hypertrophy (Fig. 4; reference 5). Concomitant modifications of mRNA stability cannot be excluded but do not appear to be required to explain the observations. Therefore, this work provides the first evidence that an isogene switch characteristic of cardiac myocyte hypertrophy is due to an alteration in the transcriptional program of the cardiac myocyte, and that this program can be altered by stimulation of a specific cell surface receptor.

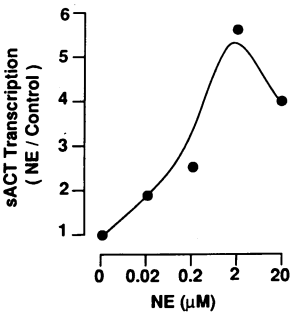

Figure 3. Response of skeletal α-actin transcription to NE concentration. Cells were treated for 12 h with the indicated concentrations of NE, and nuclei were isolated for determination of skeletal α-actin (*sACT*) transcription. Each point is the ratio of NE-treated/control hybridization. Control transcription was 2.3 ppm in this experiment.

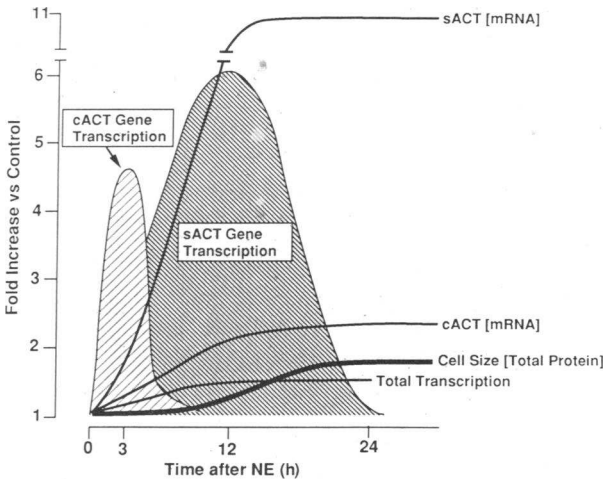

Figure 4. Transcriptional regulation in α_1-stimulated hypertrophy. The diagram summarizes the time course of activation of transcription and accumulation of specific mRNAs and total protein after stimulation of cultured neonatal rat heart muscle cells with NE. Results for cell protein and steady-state levels of actin iso-mRNAs are taken from references 5, 7, and 8. (*sACT*, skeletal α-actin; *cACT*, cardiac α-actin).

A second major finding in the current experiments was the resolution of distinct temporal stages in the transcriptional response after α_1-receptor stimulation (Fig. 4). Increased transcription of the skeletal α-actin and cardiac α-actin genes was separated in time, with delayed and prolonged activation of skeletal α-actin mRNA transcription. Asynchronous transcription of closely related genes has also been described in a cyclic AMP-dependent system (18). Thus a single receptor can mediate transcriptional regulation of specific genes according to different time courses.

Furthermore, the increase in actin isogene transcription was rapid and transient in comparison with total RNA transcription, which increased more slowly and was not transient (Fig. 4). In particular, amanitin-insensitive transcription of (presumably) rRNA and tRNA by polymerases I and III, respectively, was still increased at 24 h, when transcription of the actin isogenes had returned to control levels. In a previous report of altered transcription in a model for myocardial hypertrophy, the early and selective increase in contractile protein gene transcription seen in the present study was not observed, perhaps because specific gene transcription was measured at 24 h only (6). Transient activation of mRNA transcription has been seen in other systems, and attenuation of transcription has been attributed to synthesis of a transcriptional repressor (19, 20). Thus, rather than effecting a general, simultaneous increase in overall gene transcription, stimula-

tion of the α_1-adrenergic receptor leads to a distinctive temporal sequence of transcriptional activation. It will be interesting to see if conditions which induce myocardial hypertrophy in vivo also stimulate the same complex temporal sequence of gene transcription as does stimulation of the α_1-adrenergic receptor.

A major implication of this work is that the α_1 receptor transduces a rapid signal or signals to specific genes in the myocyte nucleus. The results with cycloheximide suggest that new protein synthesis is not required for signal generation. Thus the transcriptional changes may result from modification of preexisting molecules which interact with regulatory sequences of the specific genes (19, 21–23). A protein kinase C-mediated modification may be important in the present case, since protein kinase C is activated through the α_1 receptor in the cultured heart myocytes (24).

Finally, these results demonstrate a new dimension for the role of the α_1-adrenergic receptor in cardiac myocytes. In addition to its well-established role in mediating inotropic and chronotropic responses (25), we show here that the α_1-adrenergic receptor mediates changes in RNA transcription which underly a gene switch accompanying heart muscle cell hypertrophy. Regulation of transcription is independent of effects on contractility per se, since the myocytes in this culture system are quiescent with α_1 stimulation alone (7). It should now be possible to delineate the molecular mechanism(s) by which the α_1-adrenergic receptor mediates these vastly different effects.

Acknowledgments

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