Electrical Stimulation of Contractile Activity Accelerates Growth of Cultured Neonatal Cardiocytes

Thomas B. Johnson, Robert L. Kent, Beth A. Bubolz, Paul J. McDermott

Abstract An electrical stimulation system was designed to regulate synchronized contractile activity of neonatal rat cardiocytes and to examine the effects of mechanical contraction on cardiocyte growth. Continuous electrical stimulation at a pulse duration of 5 milliseconds and frequency of 3 Hz resulted in a time-dependent accumulation of cell protein that reached 34% above initial values, as measured by the protein-to-DNA ratio. The growth response did not occur using voltage amplitudes that were subthreshold for contraction and was independent of contraction frequencies set at ≥0.5 Hz. The RNA-to-DNA ratio increased in parallel to cell protein, indicating that the capacity for protein synthesis was enhanced by contraction. Rates of 28S rRNA synthesis were accelerated twofold in contracting cardiocytes. By comparison, protein and RNA accumulation did not occur in electrically stimulated cardiocytes in which contraction was blocked by either 10 μmol/L verapamil or by 5 mmol/L 2,3-butanedione monoxime, an inhibitor of actomyosin crossbridge cycling. Electrical stimulation of cardiocyte contraction did not enhance α-cardiac actin or myosin heavy chain (α+β) mRNA transcript levels relative to 28S rRNA during the period of rapid growth that occurred over the first 48 hours. It is concluded that (1) electrical stimulation of contraction accelerates cardiocyte growth and RNA accumulation, (2) mechanical contraction is involved in regulating the growth of electrically stimulated cardiocytes, and (3) the levels of α-actin and myosin heavy chain mRNA increase in proportion to rRNA during the growth of contracting cardiocytes. (Circ Res. 1994;74:448-459.)

Keywords: • hypertrophy • cardiocytes • contraction • electrical stimulation

In primary cultures of neonatal rat cardiocytes, the onset of spontaneous contractile activity has been linked to hypertrophic growth as measured by total protein and RNA accumulation.1,2 Growth of contracting cardiocytes develops over several days in serum-free media independent of hormonal or neural stimuli, and this accumulation of cell protein is facilitated by acceleration of the rate of total protein synthesis.1 An increased capacity for protein synthesis, as reflected by a net increase in rRNA content, is the primary translational mechanism by which the rate of protein synthesis is accelerated in contracting cardiocytes.3 Furthermore, it has been demonstrated that the synthesis rate of rRNA is accelerated after the onset of contraction and is regulated at the level of rDNA transcription.3 In terms of specific proteins, changes in the synthesis rates of actin and myosin have been shown to occur in contracting cells, either by transcriptional mechanisms involving selective changes in mRNA levels or by a posttranscriptional process.4,5 Accordingly, contractile activity is coupled to growth by affecting transcriptional and translational processes that regulate rates of total and contractile protein synthesis.

The use of spontaneous contractile activity as a growth stimulus to investigate the mechanisms regulating cardiocyte protein synthesis is subject to the following limitations. First, precise physiological parameters of spontaneous contraction are difficult to measure and control. For example, contraction occurs at variable rates even between groupings of synchronously contracting cardiocytes on the same culture dish. Second, the process of contraction is obviously complex, consisting of the sequence of primary events including excitation-contraction coupling, myofilament interaction and crossbridge cycling, and the subsequent generation of active tension and mechanical shortening of the cardiocyte. The extent to which each of these individual events is relevant for eliciting an anabolic response has not been determined. Third, it has not been possible to separate the active and passive components of contraction in accelerating growth. Recent studies have shown that increased load in the form of passive stretch is a stimulus for the growth of spontaneously contracting neonatal cardiocytes in culture.6-8 Specifically, active tension development that would presumably occur during contraction of passively stretched cells was not necessary for growth stimulation, because the anabolic response to passive stretch was unaffected on inhibition of contractile activity.9 In contrast, increased active tension is a potent stimulus for accelerating protein synthesis rates in studies using papillary muscle preparations in vitro.9,10

Electrical field stimulation has provided a means of greater control over contraction of muscle cells in culture.11-15 In neonatal rat cardiocytes, electrically stimulated contraction enhanced the expression of mRNA for atrial natriuretic factor and myosin light chain 2 and augmented the development of the myofibrillar apparatus.12 Similarly, using skeletal myocytes...
in culture, qualitative changes in myosin gene expression occurred in response to electrically stimulated contractile activity.\textsuperscript{13,14} Anabolic changes were also elicited by electrically stimulated contraction in skeletal myocytes as the synthesis of myosin heavy chain (MHC) was accelerated, and this occurred to a greater extent than the synthesis of total cell protein.\textsuperscript{15} In related studies using electrical stimulation to induce contraction of adult feline cardiocytes in culture, we have observed an acute acceleration of total protein synthesis and MHC synthesis rates.\textsuperscript{16}

In the present studies, a system to electrically stimulate and synchronize contraction of neonatal rat cardiocytes in culture was designed. The first objective using this system was to develop a model in which electrically stimulated contraction could accelerate cardiocyte growth. The indexes of a growth response were the accumulation of cardiocyte protein and rRNA, absolute synthesis rates of rRNA, and alterations in the relative abundances of contractile protein mRNA pools. The second objective was to demonstrate that active tension development and mechanical contraction are involved in regulating the growth of electrically stimulated cardiocytes. These studies demonstrated that cardiocyte growth occurred in response to electrically stimulated contractile activity. By use of inhibitors of contractility in concert with electrical stimulation, it was further demonstrated that cardiocyte growth was dependent on mechanical contraction.

\section*{Materials and Methods}

\subsection*{Preparation of Neonatal Rat Cardiocytes}

Primary cultures of neonatal ventricular rat cardiocytes were prepared by combined enzymatic and mechanical dissociation as described previously.\textsuperscript{1} The heart cell preparations were enriched for cardiocytes by differential adhesion and plated in MEM (GIBCO) containing 10\% newborn calf serum at a concentration of 1.5 x 10\(^6\) cells per square centimeter in four-well culture trays (Nunc). After an overnight incubation to allow for adherence to the culture dish, the cells were rinsed and maintained in chemically defined serum-free media containing 10 \(\mu\)mol/L verapamil.\textsuperscript{1} The media were subsequently changed after 48 hours and every 24 hours thereafter throughout the duration of each experiment. To stimulate and synchronize cardiocyte contraction in culture, electrical field stimulation was used; the system is illustrated in Fig 1A. A physiological stimulator (Multistim) was used to generate rectangular-wave electrical pulses at constant voltage through the culture medium via platinum wires that were submersed at opposite ends of each well in four-well culture trays. The area of each well was 16 cm\(^2\), and the distance between opposing electrodes was 6.5 cm. The voltage for most experiments was set at 50 V, a value that was approximately twice the threshold for synchronous contraction at 3 Hz using a pulse duration of 5 milliseconds (Fig 1B). The generation of radicals by oxidation was minimized by a custom-built polarity alternator that changed the direction of each electrical pulse across the electrodes, by having 250 \(\mu\)mol/L ascorbic acid in the serum-free media as a reducing agent, and by changing the media every 24 hours. There were no changes in the pH or temperature of the media during electrical stimulation.

\subsection*{Measurements of Cardiocyte Protein and rRNA Content}

The accumulation of cardiocyte protein and RNA was used to establish a hypertrophic response. Cardiocytes were rinsed three times in ice-cold phosphate-buffered saline (PBS), scraped from each well in 1.2 mL of 1 x standard sodium citrate buffer (SSC) containing 0.25\% (wt/vol) sodium dodecyl sulfate (SDS), and frozen at \(-20^\circ\)C. Before use, the extracts were thawed and vortexed extensively. Aliquots were used for measurements of total cell protein (BCA, Pierce Chemical Co) using bovine serum albumin as a standard. Measurements of RNA content were determined as described previously.\textsuperscript{2} DNA was measured fluorometrically with Hoechst dye 33258; calf thymus DNA was used as a standard.\textsuperscript{17} Protein-to-DNA and RNA-to-DNA ratios were calculated in the extracts obtained from individual wells of each culture tray.

\subsection*{Measurements of rRNA Synthesis}

The fractional rate of rRNA synthesis (\(K_s\)) was calculated from the incorporation of \([\textsuperscript{3}H]\)uridine into purified 28S rRNA and by dividing the corresponding specific radioactivity of 3'-UMP (disintegrations per minute per picomole) by the specific radioactivity of the cellular UTP pool (disintegrations per minute per picomole). The corresponding cellular rate of 28S rRNA synthesis was calculated by multiplying the \(K_s\) values by the corresponding rRNA-to-DNA ratios, assuming that \(\sim 62\%\) of total RNA is 28S rRNA.\textsuperscript{2}

\subsection*{Pulse Labeling of rRNA and Measurement of 5'-UTP–Specific Radioactivity}

Cardiocytes were pulse-labeled with 50 \(\mu\)mol/L \([\textsuperscript{3}H]\)uridine in the medium (25 \(\mu\)Ci/mL) for 6 hours. Thereafter, cardiocytes were rinsed with PBS, and two wells per tray were scraped into 0.5N HClO\(_4\). After centrifugation, the supernatant was neutralized by the addition of KHCO\(_3\) to a final concentration of 0.5N. The perchlorate was pelleted by cen-
trifugation, and the supernatant was filtered and taken to dryness in a vacuum centrifuge. UTP was purified by reverse-phase high-performance liquid chromatography (HPLC, Waters) as described previously. The specific radioactivity of the UTP pool (disintegrations per minute per picomole) was determined from the number of picomoles (calculated by integrating the area under the UTP peak using Waters BASELINE 810 HPLC software) and from the corresponding disintegrations per minute (measured with an on-line isotope detector [Beckman Instruments]).

**Purification of 3'-UMP in Radiolabeled rRNA**

Total RNA was extracted from companion wells of each tray using the RNAzol method (Cinna Biotech Laboratories, Inc.). The RNA was resuspended in a buffer containing 20 mmol/L Tris, pH 7.6, 10 mmol/L EDTA, and 50 mmol/L KCl and fractionated on 15% to 68% (wt/vol) exponential sucrose gradients containing the same buffer for 16 hours at 100,000g. The peak fraction containing 28S RNA was collected by means of an ISCO gradient fractionator and precipitated with ethanol. The radiolabeled rRNA fraction was hybridized to a corresponding 28S rDNA clone immobilized on Hybond-N (Amersham) membranes by slot blotting. Hybridization proceeded for 66 hours at 42°C in a buffer containing 50% (vol/vol) deionized formamide, 0.02% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficoll, 5x SSC, 10 mmol/L MOPS, pH 7.0, 2 mmol/L EDTA, 100 μg yeast tRNA/mL, and 0.2% (wt/vol) SDS. The slots were washed three times in 2x SSC and 0.1% sodium dodecyl sulfate at 42°C for 1 hour, followed by three more washes in 0.1x SSC at 42°C for 1 hour. The hybridized rRNA was hydrolyzed in 0.3N NaOH for 3 hours at 37°C, neutralized by adding H₃PO₄ to a pH of 6.5, and dried in a vacuum centrifuge. The specific radioactivity of 3'-UMP was measured by reverse-phase HPLC.

**Quantitation of MHC and α-Cardiac Actin mRNA Pools**

The size of contractile protein mRNA pools was determined by quantitative slot blotting using cDNA probes complementary to MHC and α-cardiac actin. pMHC-5 is a 436-bp segment of the rat α-MHC mRNAs that encodes for α-MHC mRNA in the region encoding the untranslated region between nucleotides 3459 and 3895. Because of the high degree of sequence similarity with β-MHC mRNA in this region (89%), it hybridizes to both α-MHC and β-MHC mRNA equally. The α-cardiac actin clone is a 620-bp segment derived from the original human clone 5'HMCa-1. These clones were subcloned into pGEM (Promega) and Bluescript BS+ (Stratagene) transcription vectors. Complementary 32P-labeled cDNA probes of high specific radioactivity were generated by the polymerase chain reaction (Cetus) using oligonucleotide primers consisting of sequences for the T7, SP6, or T3 promoter regions flanking either side of the cDNA insert. The same recombinant DNA was used to generate radiolabeled sense cDNA transcripts by in vitro transcription with [3H]UTP and the appropriate RNA polymerase. The sense cDNA transcripts were quantitated by HPLC and used as standards in the hybridizations.

For quantitative slot blotting, RNA was extracted by the RNAzol method. Equivalent amounts of RNA from each sample were heated for 5 minutes at 65°C in a buffer containing 67% (vol/vol) formamide, 7% (vol/vol) formaldehyde, 25 mmol/L MOPS, pH 7.0, 3 mmol/L EDTA, and 0.8 mmol/L sodium acetate. The RNA was immobilized on Hybond-N by means of a slot-blotting apparatus and was UV-cross-linked. The blots were hybridized for 24 hours at 42°C in hybridization buffer containing 50% (vol/vol) deionized formamide, 10x Denhardt's solution, 50 mmol/L Tris, pH 7.5, 0.1% Na₂P₂O₇, 1% SDS, and 100 μg denatured salmon sperm DNA/mL. The slot blots were washed three times in 2x SSC and 0.1% SDS at 42°C for 1 hour, followed by three washes of 20 minutes each in 0.2x SSC and 0.1% SDS at 60°C. The hybridization membranes were processed for autoradiography, and the integrated optical densities of the hybridization signals were measured by computer-assisted digital image analysis. The specificity of the cDNA probes was verified by Northern blot analysis under the same hybridization and washing conditions. To correct for quantitative differences in the relative amounts of RNA applied to the slots, dilutions of each sample were slotted onto a companion membrane and hybridized to a clone of 28S rDNA that was 32P-radiolabeled by nick translation (Amersham). Standard curves of sense cRNA transcripts were included on each slot blot to ensure the linearity of the hybridization reactions and that the autoradiographic signals were in the linear range of the curve. By using the cRNA standards, linear regression analyses were performed by plotting integrated optical densities as a function of transcript number. The number of mRNA transcripts in each sample was calculated by extrapolation from the standard curves and normalized to the corresponding amount of 28S rRNA transcripts.

**Analyses of MHC mRNA Isoforms**

The relative expression of α-MHC and β-MHC mRNA was determined using oligonucleotide probes specific for the corresponding 3' untranslated regions. The α-MHC oligonucleotide probe (5' GGCGAGGCTCTTCTGCTGGACAGGT 3') was complementary to nucleotides 1288 to 1311 in the 3' untranslated region. The β-MHC oligonucleotide probe (5' TCACAGGCATCCTTAGGTGGGTGA 3') was complementary to nucleotides 1249 to 1273 of the 3' untranslated region. The oligonucleotides were end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase (Promega). Total RNA samples were prepared by the RNAzol method, and equivalent amounts were slot-blotted as described above. The blots were hybridized for 20 hours at 45°C in 6x SSC, 10x Denhardt's solution, 0.1% SDS, 0.05% Na₂P₂O₇, and 200 μg tRNA/mL and washed three times in 2x SSC and 0.1% SDS at 45°C for 1 hour, followed by two washes of 20 minutes each at 45°C in 1x SSC and 0.1% SDS. Dilutions of each RNA sample were slotted onto a companion membrane and hybridized to the 28S rDNA probe to correct for differences in the relative amounts of RNA applied to the slots. The blots were processed for autoradiography and analyzed as described above.

**Analysis of α-Skeletal Actin mRNA Expression**

The expression of α-skeletal actin mRNA was distinguished from α-cardiac actin mRNA by Northern blot analyses using a 38-base oligonucleotide specific for the 3' untranslated region of rat α-skeletal actin mRNA. The oligonucleotide was end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The conditions for hybridization and washing of the blots were carried out exactly as described for α-cardiac actin.

**Measurements of Calcium Transients**

Single-cell recordings of intracellular calcium concentrations were measured by microspectrofluorescence with the calcium indicator dye fluo-3 (Molecular Probes Inc). The dye was introduced into the cardiocytes by incubating for 30 minutes in media containing 20 μmol/L fluo-3 acetoxyethyl ester. Cardiocytes were washed and incubated for 30 minutes in fresh medium to allow for hydrolysis of the acetoxyethyl ester. The cardiocytes were excited by 490-nm light, and the fluorescence emitted at 520 nm was measured by a photomultiplier tube coupled to an inverted phase-contrast microscope. The fluorescent image of each cardiocyte was stored digitally and processed by computer-assisted image analysis to determine the summed fluorescent intensity across the cardiocyte relative to the cell surface area.

In a separate set of experiments, intracellular calcium concentrations of individual cardiocytes were measured by ratio imaging using fura 2. Fura 2 was introduced into the cardiocytes by incubating for 30 minutes in media containing 20 μmol/L fura 2-AM (Calbiochem Corp). Cardiocytes were
washed and incubated for at least 30 minutes in fresh media without phenol red. Ratio imaging of fura 2–loaded cardiocytes was done at 1 Hz using alternating excitation wavelengths of 340 and 380 nm. Cytoplasmic calcium concentrations (excluding nuclei) were determined in individual cardiocytes by digital fluorescence microscopy (ATTOFLUOR, Alto Instruments, Inc). In each of three experiments, the cytosolic calcium concentrations of at least 20 individual cardiocytes were determined for both untreated and 2,3-butanedione monoxide (BDM)–treated groups.

Statistical Analyses

Fractional increases in protein or RNA accumulation of each experimental treatment group were calculated by dividing the net increase at each time point by the corresponding value at the start of the experiment (day 4 in culture). Values are expressed as the mean±SEM unless indicated otherwise. Statistical differences between multiple experimental treatment groups and the day 4 control values were compared by ANOVA. Differences between individual means of the treatment groups versus the day 4 value were tested for significance by the Bonferroni-Dunn test or Student-Newman-Keuls test (SuperANOVA, Abacus Concepts, Inc). Other tests for statistical significance are as indicated in the figure legends.

Results

Electrical Stimulation of Cardiocyte Contraction

To examine how mechanical contraction regulates cardiocyte growth, a system to electrically stimulate and synchronize contraction in an electrical field was developed. In Fig 1A, a schematic of the system is illustrated, and the details are described in “Materials and Methods.” By using this system, synchronous contraction of virtually the entire cardiocyte population at assigned frequencies and pulse durations was attained. In Fig 1B, the contractile response to electrical stimulation was tested. A strength-duration curve was generated in which the threshold voltage for synchronous contraction at a frequency of 3 Hz was plotted as a function of pulse duration. The cardiocytes behaved in a predictable manner as the threshold voltage for contraction decreased as a function of pulse duration. The threshold voltage for synchronous contraction at 5 milliseconds was ≈30 V, and the chronaxie value was ≈2.5 milliseconds. Taken together, these findings were consistent with previous studies using electrically stimulated cardiocytes and established the validity of the system as a physiological model of excitation-contraction coupling.11,24 For consistency, the cardiocytes were stimulated routinely at 50 V using a frequency of 3 Hz and pulse duration of 5 milliseconds.

Effects of Electrical Stimulation on Cardiocyte Growth

To determine whether synchronized contraction using electrical field stimulation could induce cardiocyte growth, the following experimental paradigm was used. Cardiocytes were maintained in serum-free media containing 10 μmol/L verapamil to minimize spontaneous contractions that occur as a result of relatively high plating densities. The cardiocytes were maintained under these conditions with several media changes until day 4 to allow for contraction-independent growth and subsequent development of a steady state with respect to cell protein.1 In agreement with previous studies, verapamil-treated cardiocytes maintained the differentiated phenotype and retained the ability to contract on washout of verapamil.1,4 In Fig 2A, the ability of contraction to stimulate cardiocyte growth is shown. Protein-to-DNA ratios were measured, and fractional changes in this ratio were calculated after 2 and 4 days of electrical stimulation. Electrical stimulation of cardiocytes in the presence of 10 μmol/L verapamil minimized synchronized contraction, and no significant accumulation of cardiocyte protein occurred. Furthermore, in the presence of verapamil, there were no differences in the protein-to-DNA ratios between nonstimulated and electrically stimulated cardiocytes. After verapamil washout, electrical stimulation of contractile activity resulted in a significant 19% increase in the protein-to-DNA ratio after 2 days. Cardiocyte protein increased cumulatively as reflected by a 34% increase in the protein-to-DNA ratio after 4 days. These increases in the protein-to-DNA ratio were the result of corresponding changes in cell protein, because DNA values at 2 and 4 days were not significantly different.
Fig. 3. Time course of protein accumulation in electrically stimulated cardiocytes. Cardiocytes were electrically stimulated to contract after verapamil washout on day 4 in culture using standard electrical parameters. For a steady-state comparison, companion trays were maintained in 10 μmol/L verapamil. Fractional changes in protein-to-DNA ratios were calculated as described in the legend for Fig 3. Values are the mean±SEM of 5, 16, 6, and 9 experiments for days 1, 2, 3, and 4, respectively. *P<.05 vs corresponding day 4 value as determined by a paired Student's t test.

In Fig 3, the protein-to-DNA ratio in electrically stimulated cardiocytes was characterized as a function of time. There was a time-dependent increase in the protein-to-DNA ratio of contracting cardiocytes. In contrast, there were no significant changes in the protein-to-DNA ratios of cardiocytes maintained in verapamil over the same time period. Further experiments were carried out to determine whether the fractional increase in the cardiocyte protein-to-DNA ratio was dependent on contraction frequency. In Fig 4, the protein-to-DNA ratios of cardiocytes contracting at frequencies of 0.5 and 3 Hz were compared after 2 days. At both frequencies, contraction increased cell protein as compared with cardiocytes maintained in verapamil. A similar result was observed on comparison of cardiocytes that were stimulated at 1 and 3 Hz (data not shown). These data suggest that contraction at a rate of ≥0.5 Hz was sufficient for accelerating cardiocyte growth and suggest that contraction, as such, is more relevant to growth than is frequency. However, the effects of contraction at frequency settings <0.5 Hz were not examined, because synchronization of cardiocytes did not occur at these lower frequencies.

As an alternative approach for examining the correlation between contractile activity and growth, cardiocytes plated at a relatively sparse density of 3.75×10^4/cm² were electrically stimulated at 3 Hz. At this density, intercellular contacts were minimal, and the standard electrical parameters were subthreshold for contraction. Thus, sparsely plated cardiocytes did not contract on electrical stimulation using the identical parameters that stimulated contraction of the cardiocytes plated at the normal density. The protein-to-DNA ratios of sparsely plated cardiocytes did not change over 3 days of electrical stimulation at 3 Hz (32.3±2.3 [mean±SEM] on day 4 versus 32.6±1.0 on day 7, n=3).

Fig. 4. Bar graph showing the effects of contraction frequency on protein accumulation in electrically stimulated cardiocytes. On day 4, cardiocytes were treated as indicated, and the protein-to-DNA ratios were measured after 2 days. Values are the mean±SEM of eight individual sample wells of a single experiment. *P<.05 vs day 4; #P<.05 vs verapamil and stimulation as determined by ANOVA followed by a Student-Newman-Keuls test.

Effects of Contraction on rRNA Content and 28S rRNA Synthesis

In addition to cardiocyte protein accumulation, the hypertrophic response of electrically stimulated cardiocytes was assessed by measuring changes in total RNA content. In Fig 2B, cardiocyte RNA content was assayed by measuring RNA-to-DNA ratios and calculating the fractional increase in RNA after 2 and 4 days. There was a significant 25% increase in RNA content of contracting cardiocytes over 2 days, and this increase was correlated with the accumulation of protein that occurred over the same time period. In contrast, RNA content did not increase in verapamil-treated cardiocytes whether they were electrically stimulated or not. Thus, significant accumulation of RNA occurred only in cardiocytes that were able to mechanically contract in response to electrical stimulation. The RNA-to-DNA ratio did not increase any further between 2 and 4 days of electrically induced contraction and thus reached a steady state with respect to RNA content. These data were consistent with the protein-to-DNA ratios of contracting cardiocytes in which the fractional increase was maximal by 3 days of contraction (Fig 3). The synthesis rate of rRNA is accelerated in response to hypertrophic stimuli, including spontaneous contraction.² Rates of 28S rRNA synthesis were measured to confirm that the increase in RNA-to-DNA ratios in contracting cardiocytes was attributable to an acceleration of the rRNA synthesis rate. The cellular rate of 28S rRNA synthesis was measured after 2 days of electrical stimulation in the presence and absence of verapamil. The rate of rRNA synthesis was accelerated signifi-
cantly as compared with verapamil-treated cardiocytes (Fig 5). The extent to which rRNA synthesis was accelerated was consistent with previous results.225

Effects of Contraction on Contractile Protein mRNA Pools

To determine whether contraction exerted an effect on cardiocyte-specific mRNA pools, total MHC mRNA and α-cardiac actin mRNA transcript levels were measured by quantitative slot blotting. In Fig 6, the sensitivity of the assay for detecting changes in mRNA transcript levels is shown. Total rat cardiocyte RNA was added in increasing amounts and hybridized to either the MHC or α-cardiac actin cDNA probe. The hybridization signal increased as a function of the amount of RNA applied to the slots and demonstrated the ability of the assay to detect relatively small changes in mRNA transcript levels per sample.

In the Table, total MHC mRNA-to-rRNA and α-cardiac actin mRNA-to-rRNA transcript ratios were measured in contracting cardiocytes and compared with cardiocytes maintained in verapamil over the same time period. The MHC mRNA-to-28S rRNA ratio actually decreased in the cardiocytes between days 4 and 6 in culture and reached a level that was 47% of the initial value on day 4. This decrease was not affected by electrically induced contraction after verapamil wash-out. Furthermore, the decline in MHC mRNA was not the result of chronic verapamil treatment over the first 4 days in culture, because a similar decline was observed in cardiocytes maintained in serum-free media without verapamil (data not shown).

The MHC probe used in these studies hybridized to both the α and β isoforms of MHC mRNA equally.18 To determine whether the decrease in the total MHC mRNA pool was due to a selective change in MHC isoforms, α-MHC and β-MHC mRNA expression was assessed using specific oligonucleotide probes complementary to the corresponding 3′ untranslated regions.20 A representative slot blot is shown in Fig 7. The α-MHC and β-MHC mRNA isoforms were expressed in a ratio of 58% to 42% (SEM, 7%; n=4) on day 4 in culture. Thus, there was significant constitutive expression of β-MHC mRNA after 3 days of verapamil treatment.

Fig 5. Bar graph showing the effects of contraction on rRNA synthesis rates. Rates of 28S rRNA synthesis were measured after 2 days of electrical stimulation at 3 Hz and pulse duration of 5 milliseconds. Values are the mean±SEM of four separate experiments. *P<.05 as determined by ANOVA followed by a Student-Newman-Keuls test.

Fig 6. Sensitivity of the quantitative slot-blotting method. Total cardiocyte RNA was slot-blotted using the amounts indicated and probed with either myosin heavy chain (MHC) or α-cardiac actin probes. The number of transcripts was calculated from standard curves (left). Shown on the right are the corresponding autoradiograms used for computer-assisted digital image analysis. R² was .95 for both α-cardiac actin and MHC.
Between days 4 and 6 in culture, α-MHC mRNA expression decreased by 84±5% in verapamil-treated cardiocytes and 87±3% after verapamil washout and electrical stimulation (Fig 7). In contrast, β-MHC mRNA expression was maintained so that it was the predominantly expressed isoform on day 6. Similar results were observed in each of four experiments. These experiments demonstrated that the decrease in the total MHC mRNA pool, as measured by the quantitative slot-blotting procedure, was attributable to a loss of α-MHC mRNA expression in cardiocytes maintained in serum-free media. Thus, the slot-blotting data for total MHC mRNA on day 6 (Table) reflect primarily the corresponding levels of β-MHC mRNA and indicate that the β-MHC mRNA-to-28S rRNA ratio was not altered in contracting cardiocytes. Rather, MHC mRNA increased in proportion to RNA content.

In contrast to MHC mRNA, the α-cardiac actin mRNA-to-28S rRNA ratio was stable between days 4 and 6, and there was no significant increase in the α-cardiac actin mRNA-to-28S rRNA ratio over 2 days of electrical stimulation (Table). In a separate set of experiments, α-cardiac actin and MHC mRNA levels were measured after 4 days of continuous electrical stimulation of contractile activity and compared with verapamil-treated cardiocytes. The α-cardiac actin mRNA-to-28S rRNA ratio was increased by 105±32% in contracting cardiocytes (*P<.06 as determined by a paired Student's t test). There were no significant changes in MHC levels between contracting and verapamil-treated cardiocytes.

In neonatal rat cardiocytes, it has been demonstrated that the α-skeletal actin isoform is expressed at very low levels so that it constitutes a relatively small fraction of the total α-actin mRNA pool.4,26,27 The probe used in these studies recognizes both α-skeletal and α-cardiac actin mRNA. To determine whether any changes in α-actin expression occurred in contracting cardiocytes, Northern blots were probed with an oligonucleotide specific for the 3' untranslated region of rat α-skeletal mRNA. A single band that corresponded to the α-skeletal actin mRNA isolated from rat skeletal muscle was obtained. In agreement with previous studies, it was expressed at a low level and required an exposure time of 7 days. There was no detectable increase in α-skeletal actin mRNA after 4 days of continuous electrical stimulation.

### Role of Tension Development

The electrical stimulation system was used to determine the role of active tension in regulating growth in contracting cardiocytes. Cardiocytes were electrically stimulated in the presence and absence of 5 mmol/L BDM. BDM is a chemical agent whose primary effect in both skeletal and cardiac muscle is to prevent tension development by inhibiting actomyosin crossbridge formation.28-30 As shown in Fig 8, protein-to-DNA and RNA-to-DNA ratios were stable in BDM-treated cardiocytes at a concentration of 5 mmol/L for 2 days. As before, electrically stimulated contraction subsequent to verapamil washout resulted in significant increases in cardiocyte protein and RNA content. There was also an increase observed in nonstimulated cardiocytes as a result of spontaneous contractile activity after verapamil washout. BDM treatment blocked the ability of electrical stimulation to stimulate growth, because there were no significant increases in the protein-to-DNA and RNA-to-DNA ratios.

To determine whether BDM affected calcium transients during electrical stimulation, changes in intracellular calcium were measured with the calcium indicator dye fluo-3 (Fig 9). BDM at a concentration of 5 mmol/L had a small but insignificant effect on the myoplasmic calcium transient during electrical stimulation. In an additional set of experiments, these results were confirmed by determining absolute levels of cytosolic calcium in individual cardiocytes using fura 2. The cytosolic calcium concentration in normal quiescent cardiocytes was 86±12 nmol/L; this value was 100±12 nmol/L in quiescent cardiocytes treated with 5 mmol/L BDM.

<table>
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<tr>
<th>Treatment</th>
<th>Days in Culture</th>
<th>MHC mRNA (28S rRNA) ×10⁴</th>
<th>Actin mRNA (28S rRNA) ×10⁴</th>
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<td>Verapamil</td>
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<td>6.5±1.2</td>
<td>5.6±0.9</td>
</tr>
<tr>
<td>Verapamil</td>
<td>6</td>
<td>3.4±0.4*</td>
<td>5.2±1.1</td>
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<tr>
<td>Verapamil washout and stimulation</td>
<td>6</td>
<td>2.7±0.4*</td>
<td>6.5±0.9</td>
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</table>

MHC indicates myosin heavy chain. Values are the mean±SEM of five and six experiments for MHC and α-cardiac actin, respectively. *P<.05 vs the corresponding day 4 value as determined by unpaired Student’s t test.

**Fig 7.** Expression of myosin heavy chain (MHC) mRNA isoforms. Shown is a representative autoradiogram of total cardiocyte RNA samples (2 µg RNA per slot) that were slot-blotted and hybridized to either α-MHC or β-MHC oligonucleotide probes as indicated. Aliquots of each RNA sample (1 µg) were slot-blotted onto a companion membrane and probed for 28S rRNA. The exposure times were 40 hours for the MHC blots and 1 hour for 28S rRNA.
Values were versus stimulated cardiocytes were ensured by (BDM) monoxime of 2 nmol/L during contraction. Norepinephrine-Induced Hypertrophy of 2,3-butanedione monoxime (BDM), cardiocytes were loaded with the calcium indicator dye fluo-3. The percent change in fluorescence in systole vs diastole was measured by computer-assisted digital image analysis. Values are the mean±SEM of three separate experiments representing at least 10 cardiocytes per experiment. There was no statistically significant effect of BDM in these experiments.

BDM was the result of specific effects of these agents on contraction. Because it was observed previously that contraction was not required for norepinephrine-induced growth of neonatal cardiocytes,1 the ability of verapamil and BDM to suppress norepinephrine-induced hypertrophy was examined (Fig 10). Cardiocytes were maintained in media containing 10 μmol/L verapamil until day 4. After verapamil washout, spontaneous contractions were not observed in these experiments; thus, no significant growth occurred over the ensuing 48 hours. However, there was a significant increase in the protein-to-DNA ratio of cardiocytes treated with 2 μmol/L norepinephrine. The increase in cardiocyte growth in response to norepinephrine was unaffected by simultaneous treatment with either verapamil or BDM. These data demonstrate that the suppression of growth in electrically stimulated cardiocytes treated with either verapamil or BDM was exerted through changes in contractility rather than through nonspecific effects of these inhibitors on the ability of the cardiocytes to accelerate growth.

**Discussion**

In the present studies, electrical field stimulation was used to examine the role of mechanical contraction in regulating the growth of neonatal cardiocytes in primary culture. This system provided the ability to control and synchronize contraction under defined physiological parameters such as pulse amplitude, pulse duration, and frequency. Cardiocytes were plated at a relatively high density of 1.5x10^5 cells per square centimeter so that intercellular contacts could develop and thereby facilitate synchronized contraction using relatively low electrical field strengths. However, spontaneous contractile activity also occurred at this plating density and resulted in cardiocyte growth (Figs 4 and 8). Spontaneously contracting cardiocytes were, therefore, not a suitable steady-state control, because it was necessary

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**Fig 8.** Bar graphs showing the effects of 2,3-butanedione monoxime (BDM) on protein and RNA accumulation of electrically stimulated cardiocytes. Cardiocytes were maintained in 10 μmol/L verapamil until day 4 in culture as before, and the media were changed as indicated. A. Protein-to-DNA ratios were measured on day 4 (control) and in the indicated treatment groups on day 6. B, Corresponding RNA-to-DNA ratios are shown. Values are the mean±SEM. *P<.05 vs the day 4 value as determined by ANOVA followed by a Bonferroni-Dunn test.

**Fig 9.** Bar graph showing calcium transients during electrical field stimulation. After 2 days of electrical stimulation in the presence and absence of 5 mmol/L 2,3-butanedione monoxime (BDM), cardiocytes were loaded with the calcium indicator dye fluo-3. The percent change in fluorescence in systole vs diastole was measured by computer-assisted digital image analysis. Values are the mean±SEM of three separate experiments representing at least 10 cardiocytes per experiment. There was no statistically significant effect of BDM in these experiments.

(mean±SEM, n=3 experiments). When these cardiocytes were electrically stimulated to contract at a frequency of 2 Hz, the average calcium concentration during contraction was 197±8 nmol/L in untreated cells versus 176±17 nmol/L in BDM-treated cardiocytes.

**Effects of Verapamil and BDM on Norepinephrine-Induced Hypertrophy**

Control studies were done to ensure that the absence of growth in cardiocytes electrically stimulated in the presence of either 10 μmol/L verapamil or 5 mmol/L
to inhibit these contractions with the calcium channel blocker verapamil. After 3 days of verapamil treatment, cardiocytes reached a steady state with respect to cell protein and RNA content. Furthermore, they could be electrically stimulated to contract immediately after verapamil washout, and their fractional rates of protein accumulation were measured over several days. These findings are consistent with studies demonstrating that quiescent cardiocytes retain their differentiated state during verapamil treatment and have the utility of serving as a steady-state control.\textsuperscript{1,4} As an initial strategy for avoiding the use of an arresting agent such as verapamil, attempts were made to electrically stimulate and synchronize contraction of sparsely plated cardiocytes. At this plating density, intercellular contacts were minimized, and spontaneous contraction proceeded in a small percentage of the population at low, variable frequencies. It was not feasible to electrically stimulate sparsely plated cardiocytes for prolonged periods of time because they required a significantly higher voltage to stimulate contraction. The higher threshold probably reflects the paucity of electrical coupling between cardiocytes, resulting in a greatly diminished effective electrical length as compared with more densely plated cultures.\textsuperscript{24}

In related work, electrical stimulation of adult feline cardiocytes in primary culture was used to examine the effects of contraction on protein synthesis rates.\textsuperscript{16} In contrast to the neonatal model, these studies used protein synthesis rates as the only anabolic end point. It was demonstrated that contraction of adult cardiocytes resulted in an acute acceleration of protein synthesis rate that was dependent on sarcomere motion and the resultant cell shortening. Because the effects of contraction were evaluated over a relatively short time period of 4 hours, it was not established that there was any net accumulation of cell protein or that growth occurred. The role of sustained contractile activity in regulating growth of well-differentiated adult cardiocytes maintained in a serum-free medium is currently under investigation. By using neonatal cardiocytes, it was possible to demonstrate for the first time that electrical stimulation of contractile activity over several days resulted in net protein and RNA accumulation. Furthermore, the neonatal cardiocyte model provided the utility of determining the extent to which rRNA synthesis and contractile protein mRNA levels were modified by electrically stimulated contraction.

The time course and extent of protein accumulation that occurred in response to electrical stimulation were nearly identical to previous studies using spontaneously contracting cardiocytes.\textsuperscript{1,2} In contrast, when verapamil-treated cardiocytes were electrically stimulated, there was not a significant increase in protein accumulation. These findings suggest that calcium transients and/or mechanical contraction may be responsible for accelerating growth of electrically stimulated cardiocytes. This conclusion is supported by the data showing that sparsely plated cardiocytes, in which the standard pulse amplitude of 50 V was subthreshold for contraction, do not demonstrate any significant growth during electrical stimulation. A frequency setting of 0.5 Hz or greater was sufficient for protein accumulation in electrically stimulated cardiocytes. The data obtained using BDM suggest that the generation of tension during contraction is probably more relevant for stimulating growth than contraction frequency (Fig 8). Because contraction could not be synchronized at frequencies of <0.5 Hz, it is also possible that the frequency dependence occurs over a range of 0 to 0.5 Hz. In electrically stimulated adult feline cardiocytes, protein synthesis rates were accelerated as a function of contraction frequency and were maximal at frequencies of \( \geq 0.5 \) Hz.\textsuperscript{16}

In neonatal rat cardiocytes, it was demonstrated that the expression of atrial natriuretic factor during electrical stimulation was frequency dependent between 0 and 3 Hz.\textsuperscript{11} In contrast to the present studies, the frequency dependence of cardiocyte protein or RNA accumula-

![Bar graph showing the effects of verapamil and 2,3-butanedione monoxime (BDM) on norepinephrine (NE)-stimulated growth. Cardiocytes were maintained in 10 \( \mu \text{mol/L} \) verapamil until day 4 in culture as before, and the media were changed as indicated. Protein-to-DNA ratios were measured on day 4 (verapamil) and in the indicated treatment groups on day 6. Values are the mean±SEM. \(*P<0.05\) vs the day 4 value as determined by ANOVA followed by a Bonferroni-Dunn test.](image_url)
tion was not measured, electrical stimulation was initiated at a different time point in culture (day 1 versus day 4), and spontaneous contraction ostensibly did not occur in control cardiocytes. Spontaneous contractile activity was observed routinely in our preparations. In fact, reproducible increases in protein accumulation on electrical stimulation were not observed in cardiocytes that had not had prior treatment with an arresting agent such as verapamil. This reflected the fact that cardiocytes that spontaneously contracted over the first few days in culture have significantly higher protein-to-DNA ratios as compared with those in which contractile activity is arrested.\textsuperscript{1,4} Thus, there was a limited potential to stimulate growth of spontaneously contracting cardiocytes by synchronization of contractile activity.

The conditions and medium used during chronic electrical stimulation did not compromise cardiocyte viability or the ability to respond to contraction with growth. This conclusion is supported by several experimental observations. First, electrical stimulation did not adversely affect cardiocyte growth either in the presence or absence of verapamil. Increases in the protein-to-DNA and RNA-to-DNA ratios occurred over several days in an electrical field, proving that the conditions were satisfactory for both protein synthesis and contraction. Second, electrical stimulation using the same parameters that facilitated growth in contracting cardiocytes had no effect on the protein-to-DNA and RNA-to-DNA ratios of sparsely plated (noncontracting) cardiocytes or confluent fibroblast cultures (data not shown). Third, RNA synthesis and \(\alpha\)-actin mRNA levels were stable in verapamil-treated cardiocytes subjected to electrical field stimulation and actually increased in contracting cardiocytes. Fourth, the strength of the electrical field used in these studies was \(=2\) V/cm, a value that has been calculated to be at least an order of magnitude below the field strength required to damage the cardiocyte by electroporation of the sarcolemma.\textsuperscript{31} Therefore, the ability to electrically stimulate and precisely regulate contraction in viable cardiocytes provided a system to identify and examine the components of contraction that regulate cardiocyte growth.

A role for active tension in regulating cardiocyte growth was ascertained by direct comparison of electrical stimulation in the presence and absence of BDM, an inhibitor of actomyosin crossbridge cycling. Studies have demonstrated that the primary site of action for BDM at concentrations of \(\leq 7\) mmol/L is at the level of actomyosin crossbridge cycling and calcium sensitivity of the thin filaments, whereas BDM has minimal effects on calcium transients.\textsuperscript{28-30,32,33} These findings have been confirmed using adult rat cardiocytes in culture.\textsuperscript{34} In effect, BDM uncouples electrical depolarization and calcium influx from tension development, which facilitates mechanical contraction. In the present studies, calcium transients and cytosolic calcium concentrations were not significantly altered in BDM-treated cardiocytes, but the accumulation of protein and RNA was significantly inhibited. Similar effects were observed in the adult feline cardiocyte model as BDM blocked the ability of contraction to accelerate rates of total protein synthesis.\textsuperscript{10} Direct measurements of sarcomere motion and cell shortening were significantly decreased in the adult cardiocyte model, but calcium transients during electrically stimulated contraction were unaffected. These findings suggest that active tension development is a trigger for growth of contracting cardiocytes.

It is well established that increased load is of primary importance for regulating growth of the adult myocardium.\textsuperscript{35} Both the active tension and passive strain components of increased load may elicit an anabolic response.\textsuperscript{36,37} In superfused papillary muscle preparations, as active tension was increased along the ascending limb of the length-tension curve, the corresponding protein synthesis rates were accelerated markedly within 2 hours.\textsuperscript{10} The extent to which the rate of protein synthesis was accelerated at a fixed resting length was significantly greater in muscles generating active tension than that incurred by the imposition of passive tension through stretch alone.\textsuperscript{10,11} Thus, active tension is a much more potent stimulus for accelerating protein synthesis rates than is passive strain.

The differential effects of active versus passive load in regulating growth in culture have not been determined using cardiocytes in culture. The ability of cardiocytes to adhere to the surface of the culture dish may constitute a basal load and regulate protein synthesis.\textsuperscript{38} Our recent studies using an electrically stimulated adult feline cardiocyte model support the conclusion that active tension was involved in regulating growth, because the ability of contraction to accelerate protein synthesis was blocked by BDM treatment.\textsuperscript{16} Contractile activation has also been shown to increase protein content and promote myofibrillar development of adult cardiocytes in culture.\textsuperscript{39,40} Passive tension as exerted via static stretch to increase resting length by 10% to 20% increases protein synthesis in both neonatal and adult cardiocytes.\textsuperscript{7,41} The extent of protein accumulation (=35%) in passively stretched neonatal cardiocytes was essentially the same as that observed in response to electrically stimulated contraction, although it accumulated at a faster rate. Active tension was not required to stimulate protein synthesis in passively stretched neonatal cardiocytes, but most of the contraction-mediated increase observed in these studies was attributable to active tension development and mechanical contraction. Thus, active and passive load may be equally effective in stimulating growth of cardiocytes in culture. It remains to be determined whether a classic length-tension relation actually exists in cultured cardiocytes that are adherent to a culture dish. If so, the extent to which absolute rates of protein synthesis are accelerated should be significantly higher in contracting cells that are stretched to a length at which maximal tension is developed.

Specific induction of contractile protein mRNAs occurs in response to hypertrophic stimuli in neonatal cardiocytes.\textsuperscript{4,6,12,26,27,42} In the present studies, MHC and \(\alpha\)-cardiac actin mRNA pools in contracting cardiocytes remained unchanged when normalized to 28S rRNA. Thus, the absolute amount of contractile protein mRNAs increased in parallel with RNA content during the period of growth that occurred over 2 days of contraction. The \(\alpha\)-cardiac actin mRNA-to-rRNA ratio was increased significantly after 4 days of contraction, but the increase did not temporally coincide with the period of most rapid growth in contracting cardiocytes. The increase in \(\alpha\)-cardiac actin mRNA in contracting
cells was slower than that reported in response to norepinephrine or thyroid hormone treatment, although it was similar in magnitude.26,27 Taken together, these observations support the conclusion that contraction stimulates growth in neonatal cardiocytes through a different mechanism than those used by adrenergic agonists or thyroid hormone.

In contrast to α-cardiac actin mRNA, the total MHC mRNA pool (α+β) actually decreased relative to rRNA in both contracting and verapamil-treated cells, and this decrease was accounted for by a loss of α-MHC mRNA expression. Although transcription of MHC mRNA was not measured directly, the loss of α-MHC mRNA expression was probably the result of maintaining the cardiocytes in serum-free media for 3 days before electrical stimulation.26 Thyroid hormone, a potent inducer of the α-MHC isoform, was not added to the media used in these studies. Because constitutive expression of β-MHC mRNA was retained in verapamil-treated cardiocytes, the net result was that it became the predominant isoform in both verapamil-treated and contracting cardiocytes. An increase in β-MHC mRNA did not accompany the onset of contraction, a finding that was previously observed in cardiocytes maintained in serum-supplemented media.5 However, an increase in β-MHC mRNA expression has been shown to occur in response to spontaneous contraction, adrenergic stimulation, and passive stretch.4,7,42 The high degree of β-MHC mRNA expression after day 4 in culture may have accounted for the lack of a selective MHC mRNA increase in contracting cardiocytes. The MHC mRNA-to-rRNA ratio in contracting cardiocytes was unchanged, demonstrating that the MHC pool increased in proportion to rRNA content and that the overall capacity to synthesize MHC was enhanced. In addition, other mechanisms may be involved in regulating MHC levels during growth, such as posttranscriptional processes and modification of MHC degradation rates.43,44

In agreement with previous studies, the RNA-to-DNA ratio was increased in contracting cardiocytes and reflected an increased capacity for protein synthesis. RNA accumulation is a hallmark of cardiac growth and was the result of an accelerated rRNA synthesis rate (Fig 5). The primary regulatory site for rRNA synthesis in neonatal cardiocytes is at the level of rDNA transcription.3 It has been demonstrated in spontaneously contracting cardiocytes that there is an increase in the amount of UBF, an rDNA transcription factor, relative to noncontracting cardiocytes.45 Thus, it is quite possible that the increased rate of rRNA synthesis and subsequent accumulation of rRNA measured in response to electrical stimulation involve a mechanism by which UBF activity is enhanced by the active-tension component of contraction.

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