Contractile Activity Modulates Actin Synthesis and Turnover in Cultured Neonatal Rat Heart Cells

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In this study, the role that active tension development plays in the formation and maintenance of cardiac myocyte myofibrillar structure and cellular shape was investigated. By use of the calcium channel blocker verapamil, spontaneous contractile activity of neonatal rat heart myocytes was inhibited for 24 to 96 hours. Confocal microscopy of rhodamine phalloidin–stained cells revealed that, within 24 hours of contractile arrest, actin filaments of myofibrils were no longer aligned with one another at their I bands and Z lines. Cellular shape was also affected, with the cells developing a less stellate appearance while remaining attached to the substrate as well as to one another. By 48 hours, actin filaments were largely absent from these cells. The disappearance of actin was confirmed by measurements of actin synthesis and accumulation rates and by pulse-chase biosynthetic labeling experiments. It was revealed that, although actin synthesis was significantly reduced in arrested myocytes, the rapid disappearance of total cellular actin was largely due to increased rates of actin degradation. Contractile arrest produced by L-type calcium channel blockade with verapamil (or other calcium channel blockers) accelerated actin degradation to a greater extent than K⁺ depolarization. Chloroquine partially suppressed the accelerated rate of actin degradation, indicating that lysosomal proteolysis may be involved in actin degradative processing. Protein kinase C activation also partially inhibited the accelerated rate of actin degradation but did not restore actin filaments in arrested myocytes. The reformation of actin filaments and their reassembly into striated myofibrils occurred when contractile activity was restored by removal of verapamil from the culture medium. The period of time required for myocytes to reassemble actin filaments and to regain their elongated morphology was proportional to the period of time that the cells were inhibited from contracting. Data are presented to indicate that active tension development by neonatal cardiac myocytes in culture is critical to the maintenance of filamentous actin structure via mechanisms involving actin assembly, disassembly, and degradation. (Circulation Research 1993;73:172-183)

KEY WORDS • heart • muscle • hypertrophy • actin • cytoskeleton • protein kinase C

Many cellular functions such as motility,1 chemotaxis,2 phagocytosis,3 and cytoskeletal contraction4–6 have been demonstrated to be highly dependent on the existence of a complex network of polymerized actin. This network of actin filaments is capable of rapid reorganization in order to adapt to changes in the extracellular milieu. The signals required for restructuring this actin network and for the corresponding changes in cellular shape are poorly understood. The hormonal, chemical, and mechanical forces surrounding the cell are thought to be primary initiators for such events. These initiators are thought to trigger second-messenger systems involving protein tyrosine kinases,7 inositol 1,3,5-trisphosphate,8 and calcium fluxes,9 as well as the direct interactions of actin filaments with the plasma membrane,10 all of which have been postulated as components of the signal transduction pathways used by the cell to generate these signals.

Changes in the microfilamentous networks of osteoblasts,11 fibroblasts,12 and endothelial,13 epithelial,14 and muscle cells15–19 in response to mechanical load have been well documented. Each of these cell types provides a model with which to study possible systems for transducing mechanical force into cytoskeletal and cell-shape changes. Cytoskeletal responses to changes in mechanical forces have been particularly well documented in cardiac muscle. Hemodynamic overload during postnatal development accelerates the accumulation of contractile proteins within cardiac muscle cells, resulting in myocyte cellular hypertrophy.16 Conversely, reductions in hemodynamic load in the adult heart decrease contractile protein mass and cause muscle fiber atrophy. Thompson et al17 showed that cutting the chordae tendineae of a papillary muscle (and thereby unloading it) caused a rapid loss of myofibrils within muscle cells. This pattern of atrophy was reversed when the muscle was "reloaded" by reconnecting the muscle to its former
attachments. Alterations in the myofibrillar network are dependent not only on mechanically induced changes in rates of contractile protein synthesis but also on changes in rates of protein degradation. Mechanical unloading of the rodent left ventricle by heterotopic transplantation produced cardiac atrophy by augmenting cardiac protein degradation as well as inhibiting protein synthesis as compared with the in situ growing left ventricle (Klein et al.\textsuperscript{20} in 1990). Similarly, studies of gastrocnemius muscle after denervation or tenotomy demonstrated that accelerated rates of protein degradation led to the atrophy of muscle fibers while fractional rates of protein synthesis remained normal.\textsuperscript{21} Bandman and Strohman\textsuperscript{22} have suggested that the rate of myofibrillar protein degradation is dependent on the rate at which these proteins are incorporated into filaments. They further postulated that the rates at which these proteins are incorporated into filaments are dependent on the degree of mechanical tension placed on the cell. However, experiments defining the relation between cardiac myofibrillar assembly and mechanical tension have yet to be performed.

In the present study, the relation between active tension development by isolated neonatal rat cardiac myocytes and the incorporation of actin into myofilaments has been examined. \textit{L}-type calcium channel blockade and membrane depolarization with KCl were used to block the spontaneous contractile activity of these cells maintained under serum-free conditions.\textsuperscript{23} The effects of contractile inhibition on the actin cytoskeleton were then observed via confocal microscopy of rhodamine phalloidin-stained cells. Changes in this microfilament network were then related to changes in actin synthesis and degradation using biosynthetic labeling techniques. Data are presented to indicate that active tension development by neonatal cardiac myocytes in culture is critical to the maintenance of filamentous actin structure via mechanisms involving actin assembly, disassembly, and degradation.

**Materials and Methods**

**Reagents**

Minimum essential medium Eagle (MEM, Joklik modification), Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (D-MEM/F-12, 1:1) and Hanks' balanced salts (HBSS, modified) were obtained from Sigma Chemical Co, St Louis, Mo. Ventrex PC-1 tissue culture medium was obtained from Endotronics, Inc, Minneapolis, Minn. Acid-soluble calf skin collagen and antibiotic/antimycotic solution were also purchased from Sigma. Collagenase (type CLS II) was obtained from Worthington Biochemical Corp, Freehold, NJ. Tissue culture plates were obtained from Costar Corp, Cambridge, Mass. [\textit{L}-\textit{H}]Leucine and [\textit{S}]methionine were purchased from Amersham, Arlington Heights, Ill. [\textit{N}-methyl-\textsuperscript{14}C]Dansyl chloride was obtained from Research Products International Corp, Mount Prospect, Ill. Rhodamine phalloidin was obtained from Molecular Probes, Inc, Eugene, Ore. Laminin was purified from the Engelbreth-Holm-Swarm (EHS) tumor maintained in mice as previously described.\textsuperscript{24} Nisoldipine was a generous gift from Stephen Lipsius, PhD, Loyola University of Chicago. All other reagents were of the highest grade commercially available and were obtained from Sigma and Baxter S/P, McGraw Park, Ill.

**Ventricular Dissociation and Cardiac Myocyte Isolation**

Ventricular myocytes were isolated from 2- to 3-day-old rat pups by collagenase digestion.\textsuperscript{25} Released cells were collected by centrifugation and resuspended in PC-1 medium at a density of 7.5 x 10^5 cells/mL. The cells were then plated onto collagen-coated 35-mm dishes (1.5 x 10^5 cells per well), 60-mm dishes (3.5 x 10^6 cells per dish), or laminin-coated glass coverslips within 35-mm wells and were allowed to attach (14 to 18 hours) in a 5% CO\textsubscript{2} incubator. Unattached cells were then removed by aspiration, the wells were washed with HBSS, and the myocytes were maintained in a mixture of DMEM/F-12 and PC-1 (2:1). To inhibit spontaneous contractile activity, (+)verapamil (10 \textmu M), other calcium channel blockers, or KCl was added to the culture medium, which was replenished daily.

**Staining and Confocal Microscopy**

Cells on laminin-coated coverslips were fixed for 10 minutes with 2% (wt/vol) paraformaldehyde in sodium phosphate-buffered saline (pH 7.2) and stained with rhodamine phalloidin to visualize F-actin filaments, as previously described.\textsuperscript{26} The phalloidin-stained cells were viewed using a scanning laser confocal microscope (model MRC-600, Bio-Rad, Cambridge, Mass). Confocal microscopy was used to examine the cells because it allowed for higher resolution observations of cytoskeletal staining than conventional fluorescence microscopy. Multiple optical sections approximately 1 \mu m thick were taken of each sample to eliminate out of focus fluorescence, and the images were stacked to show F-actin patterns through the entire cell.

**Actin Accumulation in Contracting and Arrested Neonatal Myocytes**

Total actin content in contracting and arrested myocytes was quantitatively analyzed at 0, 24, 48, 72, and 96 hours in maintenance culture by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), staining with Coomassie brilliant blue, and laser densitometry.\textsuperscript{27} Data were the means of duplicate wells from the same cell isolation and were expressed as micrograms of actin per dish. Weighted results of 8 to 14 isolations were used to construct actin accumulation curves spanning the entire 96 hours in culture. The data were fit to a variety of empirical (linear, second-degree polynomial and exponential) functions, with adjustments of parameters performed by \textit{MATLAB} (Division of Computer Research and Technology, National Institutes of Health [NIH]) using a Marquardt-Levenberg method and accessed via \textsc{Prophet} (version 3.1, Division of Research Resources, NIH) running on a Sun 3/60 graphics workstation. The best-fitting functions were then transformed to yield the instantaneous actin fractional accumulation rate (K\textsubscript{a} [percentage per hour]) at each time point in culture.\textsuperscript{26,27}
actin synthesis ($K_a$ [percentage per hour]) in contracting and verapamil-arrested myocytes. For equilibrium labeling experiments, contracting and arrested myocytes in 60-mm dishes were continuously labeled with $[{}^{3}H]$leucine ($4 \mu$Ci/mL of culture medium; final leucine-specific radioactivity, 25,000 dpm/nmol). At 4, 24, and 48 hours, cells were scraped from the dishes into 10 mM sodium phosphate and 0.1% (wt/vol) Triton X-100, pH 7.4, and centrifuged (10,000 g, 20 minutes), and the sedimented myofibrillar proteins were dissolved in 100 µL of 62.5 mM Tris-HCl, pH 6.8, containing 8% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 10% (wt/vol) glycerol (SDS sample buffer). Actin was electrophoretically separated from other cellular proteins and hydrolyzed in 6N HCl as previously described. $[{}^{3}H]$Leucine-specific radioactivity in the actin myosatines was analyzed by dual-isotope radioassay using $[{}^{14}C]$dansyl chloride. Average $K_a$ values in contracting and arrested cells over the entire 48-hour period were then estimated by fitting the data to the following formula (Clark and Zak 27 in 1983):

$$P^* = F^*(1 - e^{-kt}) \times 100$$

where $P^*$ is the leucine-specific radioactivity in actin, $F^*$ is the leucine-specific radioactivity in the culture medium, and $t$ is the labeling time in hours. Best-fitting actin $K_a$ values from four isolations were subsequently compared.

Pulse biosynthetic labeling experiments were also performed to indirectly determine the fractional rate of actin degradation ($K_d$ [percentage per hour]) in contracting and verapamil-arrested myocytes. Cells in 60-mm dishes were pulse-labeled (4 hours) at 0, 24, and 48 hours in culture with $[{}^{3}H]$leucine ($4 \mu$Ci/mL; final leucine-specific radioactivity, 25,000 dpm/nmol). Cells were scraped from the dishes, and actin was isolated as described above. $K_a$ values were estimated using the following formula (Mostow et al. 28 in 1988):

$$K_a = [P^*/(F^* \times t)] \times 100$$

The actual actin synthetic rate ($R_a$ [micrograms per hour]) was derived as the product of actin $K_a$ and the actin pool size (micrograms) at the beginning of each labeling period.

$K_d$ was then derived by subtracting $K_a$ at the beginning of each labeling period (as determined from the actin accumulation curves as in Fig 3) from the measured $K_a$ values at each time point. $K_a = K_d - K_a$.

Pulse-chase biosynthetic labeling experiments were also performed to directly estimate actin $K_a$ values in contracting and arrested myocytes, as previously described. Briefly, spontaneously contracting neonatal myocytes in 35-mm dishes were biosynthetically labeled (1 or 24 hours) with $[{}^{35}S]$methionine. The cells were washed with HBSS twice after labeling and were "chased" for 0 to 96 hours in medium supplemented with 2 nM of unlabelled methionine. Half of the cells were inhibited from contracting during the chase by addition of verapamil (10 µM) or other arresting agents to the culture medium. Chase media were replaced daily. Cells were harvested at various times during the chase by lysis in 500 µL of SDS sample buffer. The cellular proteins were then electrophoretically separated by SDS-PAGE. Fluorography and laser densitometry were then used to detect the amount of $[{}^{35}S]$-labeled actin remaining in the cells at various times during the chase. Densitometric intensity data were then fit to the mathematical model for first-order random decay:

$$P^* = P_{00}e^{-kt}$$

where $P_{00}$ is the amount of radioactivity (proportional to the densitometric intensity of the actin band) at time 0. $K_d$ values were then converted to actin apparent half-life ($T_{1/2}$) values by using the equation:

$$T_{1/2} = \ln 2/K_d$$

Best-fitting actin $K_a$ values from 15 individual pulse-chase labeling experiments were subsequently compared.

**Effects of Chloroquine and Protein Kinase C Activation on Actin Degradation in Arrested Neonatal Myocytes**

Contracting myocytes were labeled for 24 hours with $[{}^{35}S]$methionine (4 µCi/mL) and were then chased for 24 hours in unlabeled medium containing 2 mM methionine in the absence or presence of 10 µM verapamil. To determine whether the lysosomotropic amine chloroquine affected actin proteolysis in arrested myocytes, increasing concentrations (from 25 nM to 25 µM) of the agent were added to the verapamil-containing chase medium. SDS-mercaptoethanol lysis was used to harvest the cells, and the

**FIG 1.** Contracting neonatal rat ventricular myocytes plated onto laminin-coated coverslips for 96 hours (see "Materials and Methods"). Panel a: Photomicrograph of cells stained with rhodamine phalloidin and imaged with a confocal microscope. Panel b: Magnified region of panel A. Images were made as the average of several optical sections. Cells exhibit elongated morphology (panel A), parallel and tightly packed myofibers, and sarcomeric alignment (arrow in panel B). Bar, 25 µM in panel a and 10 µM in panel b.
Fig 2. Photomicrographs showing rhodamine phalloidin staining of myocytes plated on laminin-coated coverslips and treated with 10 μM verapamil (see "Materials and Methods"). After 24 hours of contractile inhibition (panel a), the cells begin to lose their elongated shape and show some loss of sarcomeric staining. Panel b shows a magnified view of the actin pattern seen in panel a and demonstrates this more clearly. The arrow indicates an area where the I band and Z lines are no longer closely aligned. This pattern of cell rounding and F-actin breakdown is furthered after 48 hours of contractile arrest (panels c and d). By 72 hours (panels e and f) and 96 hours (panels g and h) of contractile arrest, the cells are clearly rounded but remain attached to one another and to the substrate. Images in panels f and h show little sarcomeric actin with only punctate staining remaining. Bar, 25 μM in panels a, c, e, and g and 10 μM in panels b, d, f, and h.

Cellular proteins were separated by SDS-PAGE. The amount of remaining radioactivity in contracting, noncontracting, and noncontracting chloroquine-treated cells was quantified by fluorography and laser densitometry. To account for variation in labeling and fluorography conditions between individual pulse-chase experiments, data were presented as the percentage of actin densitometric intensity as found in contracting myocytes 24 hours after biosynthetic labeling. The results of four individual pulse-chase biosynthetic labeling experiments in the presence of verapamil and chloroquine were subsequently compared.
Similarly, the effects of phorbol 12-myristate 13-acetate (PMA, protein kinase C [PKC] activator) and 4α-phorbol (inactive phorbol) on actin degradation in verapamil-arrested myocytes were compared. Increasing concentrations of each agent (from 2 nM to 2 μM) were added to the chase medium of verapamil-arrested myocytes. The results of four individual pulse-chase biosynthetic labeling experiments in the presence of verapamil and PMA or 4α-phorbol were subsequently compared. To examine the effects of PMA on actin filament assembly in verapamil-arrested myocytes, cells that had been arrested with verapamil for 48 hours were treated with medium containing verapamil and 200 nM PMA for an additional 48 hours before fixation, rhodamine phalloidin staining, and imaging by laser confocal microscopy.

Data Analysis

All data were expressed as mean±SEM unless otherwise stated. The Wilk-Shapiro test was used to assess normality, and Levine’s test was used to determine the homogeneity of variance. One-way blocked analysis of variance followed by the Newman-Keuls multiple-range test was used for statistical comparisons between multiple groups. For statistical comparisons between paired data, the paired t test was used. Data were analyzed using the PROPHET computer system (Division of Research Resources, NIH).

Results

Verapamil Inhibits Spontaneous Contractile Activity of Neonatal Rat Ventricular Myocytes

High-density primary cultures of neonatal rat ventricular myocytes maintained in serum-free medium demonstrated spontaneous contractile activity (100 to 150 beats per minute) within 24 hours of isolation. As reported in previous studies, L-type calcium channel blockade or membrane depolarization produced sustained contractile arrest that lasted for the 24-hour period between media changes.21,29,30 Measurements of transmembrane potentials using intracellular microelectrodes showed that spontaneous action potentials were abolished by exposure to verapamil. Resting membrane potential was stable at −50 mV (data not shown). The agents used to induce contractile arrest did not appear to affect cellular viability at the concentration used, as assessed by visual inspection for cellular attachment and for loss of perchloric acid–precipitable DNA per well with time.29

Laser Confocal Microscopic Images of F-actin Filaments in Contracting and Verapamil-Arrested Myocytes

The structural appearance of the actin cytoskeleton of spontaneously contracting neonatal myocytes maintained in primary culture is depicted in Fig 1. Myofibrils within these cells were elongated, densely packed, and uniformly distributed throughout the thickness of the cell. Individual actin fibrils stained with rhodamine phalloidin demonstrated the typical striated registered pattern observed in neonatal myocytes in vivo. Changes in sarcomeric actin distribution as well as cell shape were observable within 24 hours of contractile arrest. As seen in Fig 2a, myocytes arrested for 24 hours had begun to lose their elongated shape as well as their organized sarcomeric appearance. Higher magnification and computer enhancement showed that individual myofibrils were no longer densely packed and appeared

**Figure 3.** Panel a: Graph showing response of myocytes maintained in growth medium in the presence (arrested, △) or absence (contracting, ▲) of 10 μM verapamil for 0 to 96 hours. The amount of total cellular actin was assessed by densitometric scanning of Coomassie brilliant blue–stained gels (see "Materials and Methods"). Data are the mean±SEM of 8 to 14 isolations. Total actin content of contracting myocytes increased over time in culture; whereas total actin content of verapamil-treated cells decreased. Panel b: Growth curves depicted in panel a transformed to yield the "instantaneous" fractional accumulation rate (Kg) for total cellular actin vs. time. Values above 0 indicate net accumulation ("hypertrophy"); values below 0 indicate net loss ("atrophy"). As is evident from both panels, neonatal myocytes maintained in growth medium accumulate actin and undergo hypertrophy if allowed to beat spontaneously in culture. In contrast, arrested myocytes undergo atrophy in response to L-type calcium channel blockade.
to be detaching from one another (Fig 2b). Furthermore, these separated myofilaments were no longer in register. After 48 hours, the observed changes in cellular shape were more pronounced, with the cells developing a less stellate appearance while retaining their attachments to the substrate as well as to one another (Fig 2c). The interior of these cells showed that only short segments of the once elongated sarcomeric actin remained (Fig 2d). In addition to these short segments were thin lightly stained actin filaments that did not display a sarcomeric pattern. By 72 to 96 hours, however, F-actin staining in either form was not observable. Only a punctate actin-staining pattern remained of the once extensive actin fibril network (Figs 2e through 2h). As in Fig 2c, the cells were rounded but remained attached to the substrate as well as to one another.

**Total Cellular Actin Accumulates in Contracting Myocytes but Is Reduced in Verapamil-Arrested Myocytes**

The time-dependent disappearance of F-actin from these noncontracting cells could be explained by depolymerization (i.e., the actin had shifted to a nonstaining form such as G-actin) or by a change in the total amount of actin within the myocytes. To examine these possibilities, total cellular actin in both contracting and arrested myocytes was quantitatively analyzed by SDS-PAGE and laser densitometry. As is evident in Fig 3a, the total actin content of spontaneously contracting myocytes increased over time in culture. In contrast, contractile arrest produced a time-dependent decrease in actin content. The extent of such decreases when expressed as percentage of actin in contracting cells as in Figs 4 and 5 was sometimes variable because of the inherent variability between different cell isolations. To minimize such variability, all experiments were performed as blocked experiments. In Fig 3, the rate of disappearance of total cellular actin from arrested myocytes appeared greatest within the first 24 hours after the addition of verapamil, with little further decrease in cellular actin content occurring at 72 to 96 hours. Thus, these results indicated that the disappearance of F-actin in Fig 2 was not merely due to a shift to the monomeric form but rather to an absolute decrease in total cellular actin content.
TABLE 1. Results of Pulse-Labeling Experiments

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<th>Time elapsed in maintenance culture</th>
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<tr>
<td></td>
<td>0 h</td>
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<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>$K_v$ (%/h)</td>
<td>1.9±0.10</td>
</tr>
<tr>
<td>$K_f$ (%/h)</td>
<td>+0.7</td>
</tr>
<tr>
<td>$K_d$ (%/h)</td>
<td>1.2</td>
</tr>
<tr>
<td>$R_c$ (ng/h per 35-mm dish)</td>
<td>320</td>
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C, contracting cells; V, verapamil-arrested cells; $K_v$, actin fractional synthetic rate; $K_f$, actin fractional accumulation rate; $K_d$, actin fractional degradative rate; $R_c$, actual actin synthetic rate. Values are mean±SEM for $K_v$ values.

C and V myocytes were pulse-labeled (4 hours) after 0, 24, and 48 hours in maintenance culture with $[^3]$H]leucine (4 μCi/mL, 25,000 dpm/nmol). $K_v$, $K_f$, and $K_d$ values were determined as described in "Materials and Methods." Data presented were fit to the empirical function of the form $y=a_0 e^{-b_1 t}+c$. $R_c$ values were also determined as described. No major differences in $K_v$ values of C and V cells were seen at the time points examined, although $R_c$ values were reduced. $K_v$ values were twofold to threefold greater in V myocytes when compared with C myocytes.

Total Actin Synthesis in Contracting and Verapamil-Arrested Neonatal Myocytes

The effect of contractile activity and contractile arrest on actin synthesis was examined using two different labeling techniques. An equilibrium labeling method was used to determine average actin $K_v$ values over a 48-hour labeling period in both contracting and verapamil-arrested cells. Using this technique, we found that average actin $K_v$ values were significantly decreased in arrested as compared with spontaneously contracting cells (1.9±0.1% versus 1.3±0.1% per hour in contracting versus arrested cells, respectively; n=4 experiments; P=0.004, paired t test). However, it appeared that the modest reduction in actin $K_v$ values could not fully explain the rapid disappearance of actin in arrested myocytes.

Indirect Assessment of Actin Degradation in Contracting and Arrested Neonatal Myocytes

To further analyze the relative contributions of protein synthesis and protein degradation to alterations in total actin content in these cells, we used a pulse-labeling technique to estimate actin $K_v$ values at 0, 24, and 48 hours in maintenance culture. $K_v$ values were then derived as the difference between $K_v$ and $K_f$ in both contracting and arrested cells. $K_v$ values at 0, 24, and 48 hours were estimated from the actin accumulation curves as depicted in Fig 3. As seen in Table 1, pulse biosynthetic labeling revealed no major differences in actin $K_v$ values at the time points examined. However, $R_c$ values (in nanograms per hour per 35-mm dish), which were obtained as the product of $K_v$ and the actin pool size at each time point, were indeed reduced in arrested cells. By use of the indirect approach, actin $K_v$ values appeared to be increased twofold to threefold at each time point. In other words, inhibition of actin synthesis alone could not explain the rapid disappearance of sarcomeric actin in contractile-arrested cells, indicating that a major increase in the susceptibility of actin to intracellular degradation must have occurred.

Pulse-Chase Biosynthetic Labeling Experiments Confirm That Prolabeled Total Cellular Actin Disappears Two to Three Times More Rapidly in Verapamil-Arrested Myocytes

To directly analyze the disappearance of actin from contracting and arrested cells, spontaneously contracting myocytes were biosynthetically labeled and chased with nonradioactive medium in the absence and presence of the calcium channel blocker verapamil. As seen in Table 2, $K_v$ values were significantly increased in arrested cells, confirming the results of the pulse-labeling experiments. $T_{1/2}$ in noncontracting cells was approximately 22 hours as compared with 67 hours in spontaneously beating cultures. These data further support the notion that the rapid disappearance of F-actin filaments in arrested cells was mediated in large part by an increased susceptibility of actin to intracellular proteolysis. This rapid disappearance of labeled total cellular actin was also observed when the cells were treated with other calcium channel blockers (Fig 4). However, when the cells were inhibited from contracting by membrane depolarization with KCl (final medium concentration, 50 mM), the disappearance of actin was less rapid. These data suggest that calcium channel blockade has a greater effect on actin degradation than does membrane depolarization.

Accelerated Actin Degradation in Verapamil-Arrested Myocytes Is Partially Suppressed by Chloroquine

Pulse-chase biosynthetic labeling experiments were then performed to determine whether the lysosomal amine chloroquine (25 nM to 25 μM) reduced the accelerated degradation of actin in arrested cells. This agent did not appear to affect cellular viability at the concentrations used (data not shown). Of note, we have previously shown that chloroquine at the highest

TABLE 2. Pulse-Chase Biosynthetic Experiments

<table>
<thead>
<tr>
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<th>Contracting cells</th>
<th>Arrested cells</th>
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<tr>
<td>$K_v$ (%/h)</td>
<td>1.3±0.1</td>
<td>3.4±0.2*</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>67</td>
<td>22</td>
</tr>
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$K_v$, actin fractional degradative rate; $T_{1/2}$, actin apparent half-life. Values are mean±SEM for $K_v$.

Contracting myocytes were biosynthetically labeled (1 or 24 hours) with $[^3]$S]methionine (4 μCi/mL) and "chased" for 0 to 96 hours in unlabeled medium containing 2 nM methionine. In half the culture dishes, verapamil (10 μM) was added to produce immediate and sustained contractile arrest during chase. $K_v$ was determined as described in "Materials and Methods." $K_v$ was significantly increased in arrested cells. $T_{1/2}$ was significantly reduced in verapamil-arrested cells compared with contracting cells. The pooled data from 15 different pulse-chase experiments (each from a separate isolation) are depicted.

$^*P<0.001$ by paired t test.
FIG 6. Panel a: Bar graph showing response of contracting neonatal rat ventricular myocytes biosynthetically labeled (24 hours) with [35S]methionine (4 μCi/mL) and “chased” for 24 hours in unlabeled medium containing 2 mM methionine in the presence (V) and absence (C) of 10 μM verapamil. Increasing concentrations (from 2 nM to 2 μM) of the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) were also added to the chase medium of verapamil-arrested myocytes to determine whether activation of the protein kinase could suppress the accelerated proteolysis associated with contractile arrest. To account for variation in labeling and fluorography conditions data are presented as the percentage of densitometric intensity found in contracting myocytes. PMA partially suppressed the accelerated actin degradation in arrested myocytes in a dose-dependent manner. *P<.05 by one-way blocked analysis of variance. Panel b: Bar graph showing response of myocytes maintained in the presence (V) or absence (C) of 10 μM verapamil for 48 hours. In additional wells, 200 nM PMA was added to the culture medium. Total cellular actin was assessed by densitometric scanning of Coomassie brilliant blue-stained gels. PMA stimulated the accumulation of actin in contracting myocytes and partially prevented the loss of actin from verapamil-treated cells. Data are the mean±SEM of 7 to 12 isolations. *P<.05 vs. C; +P<.05 vs. V.

PMA Treatment of Arrested Myocytes Partially Suppresses Actin Degradation but Does Not Lead to the Formation of Myofibrils

To evaluate the role of PKC activation in the regulation of actin accumulation in arrested myocytes, similar pulse-chase biosynthetic labeling experiments were performed in the presence of verapamil and PMA or in the presence of verapamil and 4α-phorbol. As seen in Fig 6a, PMA partially suppressed the accelerated actin degradation in arrested myocytes in a dose-dependent manner. In contrast, 4α-phorbol (inactive phorbol) had no effect on actin degradation in arrested myocytes (data not shown). The PMA-mediated suppression of accelerated actin proteolysis partially prevented the loss of total cellular actin in verapamil-arrested myocytes (Fig 6b). However, morphological examination (by confocal microscopy) of verapamil-arrested PMA-treated myocytes revealed that actin filaments failed to reform in the absence of spontaneous contractile activity (Fig 7).

Restoration of Contractile Activity Results in the Reaccumulation of Sarcomeric Actin

Removal of verapamil from the culture medium at 24 to 48 and 72 to 96 hours of maintenance culture resulted in restoration of spontaneous contractile activity usually within 1 hour, albeit at a much slower rate (5 to 6 beats per minute). Cells that were arrested for 48 hours and then allowed to resume contractile activity for up to 24 hours regained a more rapid rate of contraction and exhibited a morphology similar to control cells (Figs 8a and 8b). Actin fibers reappeared within the cytoplasm and resumed their densely packed sarcomeric alignment. The myocytes had also regained their elongated morphology. Myocytes inhibited from contracting for 72 to 96 hours were also able to recover their extensive actin fibril network but at a much slower rate. After 24 hours of restored contractile activity (Figs 8c and 8d), actin fibrils had reformed but appeared as singular fibrils with few points of attachment to adjacent filaments. The fibrils had no alignment in regard to their Z lines or I bands and had a wavy appearance. Resumption of contractile activity for 48 hours (Figs 8e and 8f) resulted in better alignment and additional sites of attachment. After 72 hours of restored contractile activity, the cells exhibited normal rates of vigorous contractions and demonstrated an elongated morphology similar that of control cells (Figs 8g and 8h). Biochemical analysis of actin content in contracting, arrested, and recovering myocytes also indicated the reaccumulation of actin in these cells (Fig 9). In summary, the time-dependent reformation of actin fibrils, their attachment to one another, and their sarcomeric alignment clearly indicated that the disassembly of sarcomeric actin was reversible and that the maintenance of these structures was dependent on contractile activity.

Discussion

The role that mechanical tension development plays in cytoskeletal rearrangements has been documented in several cell types. Cytoskeletal responses to mechanical tension are especially interesting in cardiac muscle since such forces are both “active” (generated by the spontaneous contractile activity of the cells themselves) and “passive” (generated by surrounding cellular and extra-
cellular matrix interactions). McDermott et al\textsuperscript{30} and Samarel and Engelmann\textsuperscript{23} have demonstrated that the elimination of active tension development by inhibiting the spontaneous contractile activity of neonatal cardiac myocytes results in the selective loss of myosin heavy chain (MHC). Goncharova et al\textsuperscript{32} and Hileskii et al\textsuperscript{35} have also demonstrated that the removal of passive forces acting on cardiac myocytes by eliminating certain cell to cell and cell to matrix interactions with N-cadherin and B\textsubscript{3} integrin antibodies results in myofibril disassembly and sarcomeric misalignment.

In the present study, the role active tension development plays in the regulation of the actin cytoskeleton in neonatal rat cardiac myocytes has been examined. Inhibition of spontaneous contractile activity resulted in the coordinated disruption of sarcomeric actin, changes in cell shape, and the ultimate disappearance of actin staining from the cells. Morphological evaluations of changes in cellular actin were supported by pulse and equilibrium labeling as well as pulse-chase experiments.

Although previous studies by Samarel and Engelmann\textsuperscript{23} have shown decreased mRNA levels for $\alpha$-cardiac actin in response to contractile arrest, our studies indicate that the accelerated loss of actin from the cells could not be explained by decreased actin synthesis alone. Increased rates of sarcomeric protein degradation must have been responsible for the accelerated loss of protein. Similar findings have also been reported in unloaded skeletal muscle studies in vivo.\textsuperscript{21} Surprisingly, polymerized actin was present during the first 24 hours of contractile inhibition (Fig 2) when the rate of total actin disappearance was the highest (Fig 3). These findings suggest that the monomeric pool of actin was more susceptible to degradation than the actin in polymerized filaments. Bandman and Strohman\textsuperscript{24} have speculated that decreased contractile activity may in some way prevent the incorporation of newly synthesized contractile proteins into sarcomeres, leading to the enhanced degradation of newly synthesized but unincorporated contractile protein. In vitro studies have indeed shown that polymerized forms of actin are inherently stable because of their association with various actin-binding proteins such as tropomyosin.\textsuperscript{34,35}

Since it is not yet possible to accurately measure the F-actin and G-actin pools separately, such findings are not conclusive.

Intracellular proteolytic processing of contractile proteins thus appears to be as important as their synthetic processing. Therefore, the intracellular sites for actin's degradation were studied by the use of chloroquine, an inhibitor of lysosomal degradation. Actin's partial reaccumulation in response to this agent indicated that some of its degradation is occurring in lysosomes. These data are in contrast to previous findings for MHC, which indicate a nonlysosomal degradative pathway for this protein.\textsuperscript{29} Immunofluorescence studies by Hileskii et al\textsuperscript{35} have demonstrated that ubiquitin, a protein associated with cytosolic proteolysis, is closely localized to the sarcomeric complex.\textsuperscript{36} Such observations indicate that sarcomeric proteins may be differentially regulated through separate means of proteolytic processing.

The mechanisms controlling protein synthesis and degradation in response to mechanical force are not well understood. PKC transduction mechanisms are thought to play a major role, since this kinase is known to be activated by many agents producing growth and hypertrophy of the heart.\textsuperscript{37,38} Kumoro et al\textsuperscript{17} have shown that physically stretching myocytes produces increased phosphoinositide turnover, which is known to activate PKC. Allo et al\textsuperscript{39} have also demonstrated that PMA treatment of contractile arrested cells leads to a reversal of sarcomeric protein loss from neonatal myocytes. Conversely, PKC inhibitors in the presence of spontaneous contractile activity inhibit much of normal cellular growth.\textsuperscript{17,39} However, it has not been shown whether the stimulation of the PKC pathway is necessary for the development and maintenance of myofibrils. This study shows that despite PMA's ability to stimulate protein accumulation in contractile arrested myocytes, it does not promote the formation of myofibrils. It is possible that, although PKC pathways are necessary to control the synthetic or degradative machinery of the cell, they are not responsible for the incorporation of proteins into myofibrils. These data are indicative of the fact that rather than having a single system of transducing mechanical signals into cytoskeletal organization, cells may use several systems in unison to regulate myofibril assembly.

Unlike treatment of arrested myocytes with PMA, restoration of contractile activity by removal of the arresting agents was clearly associated with the reformation of actin fibrils. These results were not unexpected, since it has been shown that mechanical stimulation of endothelial,\textsuperscript{12} epithelial,\textsuperscript{14} and fibroblast\textsuperscript{12} cells leads to increased amounts of polymerized actin within such cells. The formation of myofibrils in our cells began with the reformation of singular actin filaments, which
eventually coalesced into robust myofibrils. This pattern of myofibrillogenesis was similar to that described by Antin et al.\textsuperscript{26} in chick myocytes.

Previous studies of MHC synthesis and degradation have shown findings similar to those of actin in this report.\textsuperscript{28} Both MHC and actin degradation are increased in response to contractile arrest. In both cases, accelerated degradation was greater when contractile arrest was produced by L-type calcium channel blockade rather than by K\textsuperscript{+} depolarization. The reason for these differences are not known but may be related to their initial effects on cellular calcium levels. Membrane
FIG 9. Bar graph showing that restoration of contractile activity results in actin reaccumulation. Myocytes were maintained in the presence (V) or absence (C) of 10 μM verapamil for 96 hours. In additional wells, myocytes were treated with verapamil for 48 hours and then allowed to resume spontaneous contractile activity for 48 hours by removing the drug from the media (V/C). Total cellular actin was assessed by densitometric scanning of Coomassie brilliant blue-stained gels (see "Materials and Methods"). Restoration of contractile activity resulted in the reaccumulation of actin in verapamil-arrested myocytes. *P<.05 by one-way blocked analysis of variance.

depolarization initially floods the cell with calcium by opening voltage-gated channels, while calcium channel blockers prevent the entry of calcium through voltage-gated L-type channels.41 There are also other differences between actin and MHC degradative processes in response to contractile arrest. As previously mentioned, MHC degradative rates were not affected by the lysosomotropic amine chloroquine, whereas rates of actin degradation were partially suppressed. This finding indicates that separate pathways for regulating the degradation of these proteins may exist. A second difference between actin and MHC was that in the presence of contractile arrest, PMA elevated total cellular actin, whereas myosin was not effected by this agent; thus, PMA may play a more active role in regulating the synthesis and degradation of actin than of myosin. However, PMA stimulation of PKC-signaling pathways did not promote the restoration of myofibrils in either case.

Because this study has used a calcium channel blocker to inhibit mechanical activity of the cardiac myocyte, it is not able to differentiate between the need for mechanical stimulation as the direct means of signal transduction for actin filament polymerization and the need for phasic membrane voltage changes and ion fluxes. One possible approach to this difficult problem would be to mechanically load the contractile arrested myocytes via a physical stretch system. Studies of this nature are currently being undertaken. However, recent findings that calcium channels may be associated with integrin receptors indicate that mechanical stretching of cells may also produce calcium transients.42 Further investigations into how mechanical activity is transduced into information regulating protein synthesis, degradation, and filament assembly are clearly needed.

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References


Contractile activity modulates actin synthesis and turnover in cultured neonatal rat heart cells.

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