Morphometric Evaluation of the Contractile Apparatus in Primary Cultures of Rabbit Cardiac Myocytes

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Rabbit cardiac myocytes remain quiescent for more than 1 month when cultured at low density. During this period, myofibrillar volume density declines sixfold as myofibrils are disassembled or degraded and are replaced by actin and α-actinin–positive, myosin-negative structures that resemble myofibrils but lack thick filaments. Such structures are termed minute myofibrils. The length of the sarcomeres in these altered myofibrils is significantly less than length values obtained from freshly isolated heart cells or from contracting myocytes. A number of high density cultures develop spontaneous, synchronous contraction during the second week of culture. Myofibrillar volume density is stabilized when beating begins, and no further decline is observed in the succeeding weeks of culture. Such contracting myocytes display myofibrils typical of normal heart with no visible evidence of minute myofibrils. The volume density of the transverse tubular system also declines significantly in both beating and nonbeating myocytes, and its reduction appears more closely correlated with cell spreading than with beating per se. No quantitative changes in volume density of mitochondria or sarcoplasmic reticulum could be documented, but the structural organization of the sarcoplasmic reticulum seems to be greatly influenced by the physiological state of the heart cell. The present observations document the importance of mechanical factors in regulating the integrity of the contractile apparatus in cardiac myocytes and emphasize the utility of the cultured heart cell to directly investigate structure–function relations in individual myocytes. (Circulation Research 1991;69:86–94)

Hormonal, mechanical, and neural factors are believed to regulate and modulate the structure and function of the heart through, presumably, unique subcellular and molecular pathways.1–3 Fluctuations in any one or a combination of these environmental stimuli, and perhaps others as well, are believed to alter the structural integrity of the myofibrillar apparatus and the contractile properties of the cardiac myocyte.4 Yet, the direct contribution of these phenomena in regulating the structural and functional characteristics of individual heart cells continues to elude us. The histological complexity of the heart combined with our inability to isolate and unequivocally evaluate each of these parameters has hampered investigations, as has the absence of a suitably stable, long-term in vitro cardiac preparation. The cultured adult cardiac myocyte provides an opportunity to directly explore the role of these “putative regulators” of cardiac structure and function under conditions in which the external culture milieu can be controlled rather precisely.5,6

Calcium-tolerant, adult cardiac myocytes have been isolated and cultured successfully from several species, including the rat, rabbit, and cat.7 Each of these myocyte preparations appears to display unique properties when placed in cell culture. Adult rat heart cells acquire a spherical shape when cultured in the presence of serum-supplemented medium and lose many of their myotypic features during the first week of in vitro life.5 However, such cells ultimately respread, begin beating, and acquire a morphology resembling, in many ways, that seen in freshly isolated adult heart cells.8 Conversely, rat myocytes maintained in serum-free culture medium retain their rod-shaped morphology but fail to contract spontaneously in the absence of serum.9 The
contractile apparatus of these quiescent myocytes exhibits evidence of disrupted myofibrillar elements toward the end of the first week of culture, suggesting the development of disuse atrophy in these passively loaded but nonworking heart cells.\textsuperscript{5,10} Feline cardiac myocytes cultured under similar serum-free conditions also retain their rod-shaped configuration and remain quiescent; however, little change in the structure of the contractile apparatus is reported to develop in this myocyte model.\textsuperscript{11} Lastly, “rapidly attached” rabbit ventricular myocytes\textsuperscript{12} cultured in serum-containing medium behave somewhat differently when compared with either their rat or cat counterparts. Rabbit heart cells remain quiescent, yet display a marked disruption in their contractile elements when cultured for a prolonged period at low density.\textsuperscript{13} However, when maintained at high density, some myocyte cultures develop spontaneous, synchronous beating, and those heart cells that begin contracting reveal a cell shape and fine structure\textsuperscript{13} reminiscent of that reported in spontaneously beating rat heart cells.\textsuperscript{14,15} The observations derived from these three models implicate spontaneous beating and passive or active mechanical loading as essential elements in the maintenance of myofibrillar structure and may ultimately control the cardiac myocyte phenotype in vitro.\textsuperscript{4}

Although a considerable morphological effort has been expended to define how the adult heart cell adapts to cell culture,\textsuperscript{5,8,9,11,13,14} only one report in the literature documents the subcellular reorganization that attends this in vitro adaptation in quantitative terms.\textsuperscript{13} The results obtained from this study suggest that significant changes in the contractile apparatus, the mitochondrial compartment, and the transverse tubular (T-tubule) system accompany the spreading of cultured mature and neonatal rat cardiac myocytes. Although it is unclear whether such structural and quantitative changes reflect the dedifferentiation\textsuperscript{16,17} or the adaptation\textsuperscript{15} of adult rat heart cells to a two-dimensional culture environment, such stereological approaches appear ideal to investigate the influence of mechanical activity on the maintenance of the contractile apparatus. Previous morphological observations from this laboratory demonstrated that nonbeating rabbit myocytes lose or remodel the vast majority of their contractile elements over a 1-month interval, whereas those rabbit heart cells that acquire spontaneous contractile activity retain a well-differentiated myofibrillar apparatus;\textsuperscript{13} therefore, the principal objective of the present investigation was to quantify the subcellular reorganization that attends the prolonged culture of freshly isolated rabbit heart cells with the use of point-count stereology. The specific aim of the study was to define in quantitative terms how the acquisition of spontaneous beating modulates the relative volume fraction of those subcellular organelles implicated in mediating the contractile cycle in these cultured heart cells.

### Materials and Methods

#### Myocyte Culture

Calcium-tolerant cardiac myocytes from young male New Zealand White rabbits weighing 1.8–2.5 kg were isolated according to previously published protocols.\textsuperscript{12,13} Freshly isolated myocytes were plated into 35-mm petri dishes (Corning Glass Inc., Corning, N.Y.) or onto 18-mm glass coverslips that had been coated previously with laminin (20 \( \mu \)g/ml). Heart cells were plated at either low (10° cells/cm\(^2\)) or high (10° cells/cm\(^2\)) density and cultured in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal calf serum (Whittaker Bioproducts, Walkersville, Md.), 5% NU serum (Collaborative Research, Inc., Bedford, Mass.), 10\(^{-8}\) M insulin, 10\(^{-9}\) M selenious acid, and transferrin and antibiotics.\textsuperscript{13} Proliferation of nonmyocytic cells was inhibited by adding 10 \( \mu \)M cytosine-1-\( \beta \)-D-arabinoside to the culture medium.\textsuperscript{12,18} Cultures were incubated in an atmosphere of 5% CO\(_2\)-95% air at 37\(^\circ\)C, and the medium was exchanged every other day; serum was included to ensure maintenance of cell number beyond the first week of culture.\textsuperscript{12,18} We monitored the quality of our cultures routinely by measuring ATP and creatine phosphate content, the release of lactate dehydrogenase or creatine kinase activity into the culture medium, and the ability of the myocytes to exclude trypan blue.\textsuperscript{12,13}

#### Light, Fluorescence, and Electron Microscopy

Coverslips and petri dishes were selected after 1 hour and 1, 4, 7, 14, and 28 days of culture and were processed for the localization of actin and myosin using double-label fluorescence microscopy or were preserved in situ for transmission electron microscopy as described earlier.\textsuperscript{13} The organization of the myofibrillar apparatus was evaluated by staining briefly fixed coverslips with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, Ore.) to visualize f-actin or with a monoclonal anti-myosin antibody (CCM-52) kindly provided by Dr. William Clark.\textsuperscript{13}

For transmission electron microscopy, petri dishes were rinsed briefly in serum-free MEM and preserved in situ with 4% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) buffered with 0.1 M sodium cacodylate (pH 7.4) for 2–4 hours at room temperature. The dishes were rinsed in three changes of 0.1 M cacodylate buffer plus 7.5% sucrose, postfixed in 2% osmium tetroxide (Electron Microscopy Sciences) for 1 hour, rinsed briefly in distilled water, and stained en bloc in aqueous 2.5% uranyl acetate (pH 6.0) to enhance membrane contrast.\textsuperscript{13} The preparations were dehydrated in an ascending concentration of ethanol and then infiltrated with a 50:50 mixture of absolute ethanol and Medcast (Ted Pella Inc., Redding, Calif.) overnight. The dishes then were drained of epoxy, and a thin layer of fresh plastic was applied; five to eight Beem capsules were inserted into the plastic over previously marked groups of beating or nonbeating myo-
cytes, and the plastic was polymerized for 18 hours at 55°C. The dishes were removed from the polymerized plastic, and the capsules were filled completely with the epoxy resin and polymerized for an additional 48 hours at 55°C.

**Preparation of Samples for Point-Count Stereology**

A thin (approximately 2-mm thick) disk of epoxy, which contained the embedded heart cells, was removed from each petri dish, and each disk was cut into four pie-shaped wedges. Each wedge was trimmed and reembedded into Beem capsules at 90° to the original cell surface and filled with Medcast. After repolymerization, transverse thin sections of selected myocytes could be acquired routinely from this preparation. This sectioning protocol was chosen for cultured heart cells because it is the only plane of section in which all portions of a myocyte have an equal opportunity to be sectioned and visualized; therefore, only transverse profiles were used for quantitative purposes in this investigation. Light gold ribbons of these transversely sectioned myocytes were picked up on uncoated 300 mesh copper grids and stained with 2.5% aqueous uranyl acetate followed by 0.1% lead citrate. Grids were scanned at low magnification (×5,000) with a 100CX electron microscope (JEOL U.S.A., Inc., Peabody, Mass.) until a grid square containing the adherent cardiac myocytes was located; the edge of the upper grid bar and that portion of the myocyte included in the ×5,000 field then was photographed. The next grid square was recorded identically and so on until that section was systematically photographed in its entirety. All the myocyte profiles then were photographically enlarged to ×10,000 or ×20,000 and subjected to the morphometric analysis described below. All blocks of embedded myocytes were double blinded so that the age of the culture or the physiological status of the experimental preparation remained unknown to the electron microscopist. Each disk yielded four wedges so that four different grids were photographed from each disk. At each experimental interval, five disks were chosen for study, each of which came from a separate myocyte isolation; therefore, at least 20 different grids were photographed and analyzed from five different myocyte preparations for each experimental point. Although only small portions of any one myocyte were ever analyzed, a minimum of 6,500 μm² of total myocyte area was tabulated from thin sections to obtain a statistically valid sample size. A transparent rectilinear test grid with points 1.0 cm apart was used to measure the volume fraction of mitochondria and myofibrils, and a second lattice with points separated by 0.5 cm was used to quantify T-tubule and sarcoplasmic reticulum volume density. The test grid was placed over each micrograph and was oriented parallel to the serum line on the culture substratum. The percent fraction of myocyte volume (VₒMYO) occupied by any subcellular organelle, X(Vₒ,X), was calculated from the following relation:

\[ \frac{V_o X}{V_o MYO} = PX/PMYO \times 100 \]

where PX represents the number of points falling over organelle X, and PMYO reflects the total number of points that fall over the heart cell. Point counts were tabulated using an Apple IIe computer equipped with BIOQUANT (R&M Biometrics, Inc., Nashville, Tenn.) stereology software program. All volume density measurements were expressed as percent mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to determine significant changes in organelle volume density measurements with time in culture; a two-way ANOVA was used to determine significant differences between beating and nonbeating cultures as a function of time in vitro. One-way and two-way ANOVAs were followed by post hoc analysis with a Student-Newman-Keuls test. Differences in volume density were considered significant if they fell above the 95% confidence interval.

**Results**

When rabbit cardiac myocytes are cultured at low density (i.e., approximately 10⁴ cells/cm²), the rod-shaped cells acquire a flattened configuration after 2 weeks in culture but fail to display any evidence of spontaneous contractile activity. Under these culture conditions, the pattern of actin and myosin redistribution that develops after 3–4 weeks of culture illustrates that few recognizable myofibrils are visible (Figures 1a–1c). Those myofibrils that can be observed in these preparations lack any reinforcing registry (Figure 1a) but do display a periodic distribution of actin (Figure 1b) and α-actinin. Most of these fibrils, however, lack myosin; instead, myosin assumes a predominately diffuse, perinuclear location (Figure 1c). Thin sections reveal that these fibrils disclose repeating Z-lines that are interconnected by numerous thin filaments, but few, if any, thick filaments are apparent in most instances (Figure 1d). The Z-line dimensions measure approximately 1 μm, significantly less than sarcomeres in freshly isolated myocytes, which measure approximately 1.8 μm. If myocytes from the same preparation are plated at a higher density (i.e., approximately 10⁶ cells/cm²), a small proportion (approximately 10%) of the cultures begin beating spontaneously during the second week of culture (i.e., between days 10 and 12 of culture). These contracting heart cells retain a well-developed contractile apparatus for a month and be-
FIGURE 1. Phase (panel a), fluorescence (panels b and c), and transmission electron (panel d) micrographs of quiescent rabbit myocytes cultured for 3 weeks. Panel a depicts fine fibrils that interdigitate with one another in adjacent well-spread heart cells. Panel b reveals that these fibrils exhibit a periodic distribution of actin (arrows and arrow within circle) at 1 μm intervals. Panel c illustrates that some fibrils (arrows) possess myosin, and others (arrow within circle) do not. Much of the myosin is located in a diffuse pattern surrounding the nuclei (N) of each of the neighboring heart cells. The circle in panels a, b, and c demarcates the same region of the myocyte. Panel d shows an area similar to that depicted in the circle in panels a, b, and c. The myofibril possesses discrete z-bands interconnected by thin filaments (arrows) but lacks thick filaments. The sarcoplasmic reticulum (SR) in such cells is poorly organized. Magnification, panels a, b, and c: ×1,125; panel d: ×27,750.

FIGURE 2. Fluorescence (panel a) and transmission electron (panel b) micrographs of 1-month-old heart cells that have been contracting for 16 days. Panel a illustrates the distribution of actin in the myofibrillar apparatus. Sarcomeres (arrowheads) reveal a high degree of registry in beating myocytes. Panel b is a cross section of an area similar to that depicted in panel a. Portions of three myofibrils are apparent, with the central myofibril displaying well-developed z-bands from which thin filaments are seen to interact with thick filaments (arrows). Magnification, panel a: ×1,425; panel b: ×13,800.

Beyond (Figure 2a) when compared with the myofibrils visible in their paired, nonbeating counterparts (see Figure 1b versus Figure 2a). The fine structure of these contracting heart cells exhibits, even in distal processes, well-ordered myofibrils that possess recognizable thick filaments (Figure 2b). The length of the sarcomeres in these myofibrils measures approximately 1.8 μm, a value not significantly different from that derived from freshly isolated myocytes.

The relative volume density (Vv) of the mitochondrial compartment (VvMIT) remains unchanged in myocytes cultured for as long as 28 days (Figure 3). Moreover, when these values are compared with VvMIT obtained from either whole heart or freshly isolated myocytes, no differences could be documented in the Vv of this organelle in any of the preparations. Likewise, VvMIT values derived from contracting heart cells that had been beating either for 2 days (i.e., 14-day culture) or 16 days (i.e., 28-day culture) also are not significantly different from one another, regardless of culture age or their physiological status (Figure 3). Previous morphological observations revealed that few changes in mito-
Conversely, myofibrillar volume density ($V_{v, MYF}$) declines precipitously in quiescent myocytes cultured for 1 month (Figure 4), with $V_{v, MYF}$ being reduced approximately sixfold ($p<0.01$) when heart cells cultured for 1 day ($V_{v, MYF}=56.7\pm2.6\%$) are compared with nonbeating cells cultured for 28 days ($V_{v, MYF}=10.2\pm3.5\%$). Significant reductions in $V_{v, MYF}$ also could be demonstrated at the end of the first week of culture, where $V_{v, MYF}$ is reduced approximately 50% when $V_{v, MYF}$ derived from whole heart (59.1\%\pm2.7%) or 1-hour-old cultures (57.5\%\pm1.9%) is compared with 7-day-old (31.5\%\pm3.8%) nonbeating heart cells ($p<0.01$). This decline in $V_{v, MYF}$ is linear through 2 weeks of culture, but then the apparent rate of myofibrillar loss is reduced between days 14 and 28 of culture. The development of spontaneous beating in some myocyte cultures during week 2 of culture arrested the anticipated decline in $V_{v, MYF}$ normally encountered in nonbeating myocytes; in fact, no further change in $V_{v, MYF}$ could be documented once the heart cells began contracting (Figure 4). Those myocytes that displayed spontaneous beating exhibited myofibrils that terminated directly into adhesion plaques (Figure 2a) or reassembled intercellular junctions (Reference 13 and D.G. Simpson, R.S. Decker, unpublished data, 1991). Such myofibrils disclosed both thick and thin filaments (Figure 2b), unlike their nonbeating counterparts (Figure 1d). The aberrant actin aggregates and minute myofibrils that characterize 14-day-old nonbeating myocytes (Figures 5a and 5b) are not observed in any 14-day-old contracting heart cells (Figure 2a). Although the $V_{v, MYF}$ in beating myocytes is significantly less than that derived from either whole heart or freshly isolated cells ($p<0.01$), 1-month-old spontaneously beating heart cells had a significantly greater $V_{v, MYF}$ than myocytes from paired quiescent cultures ($p<0.01$), and the acquisition of contractile activity prevented any further myofibrillar loss (Figure 4) or structural alterations (Figure 2).

In the present investigation, the volume density of the sarcoplasmic reticulum ($V_{v, SR}$) (i.e., junctional and nonjunctional sarcoplasmic reticulum) appeared to decline gradually over the month-long culture interval, although no significant difference in $V_{v, SR}$ could be documented in nonbeating heart cells at any point in vitro (Figure 6). Likewise, acquisition of spontaneous beating provoked no significant change in $V_{v, SR}$. This is not to say that there are no structural changes in the sarcoplasmic reticulum, however, because its topological organization seemed closely coupled to contraction and myofibrillar integrity. The classic “honeycomb” morphology of the sarcoplasmic reticulum found in 1-day cultured (Figure 7a) rabbit myocytes is lost by day 14 in nonbeating cultures. The sarcoplasmic reticulum in those myocytes discloses a poorly ordered reticular pattern when compared with the more classic plexiform morphology that ensheaths myofibrils in paired beating heart cells (Figure 7c). No attempt has been made to assess the volume density of junctional sarcoplasmic reticulum and compare it to nonjunc-
tional membrane, because the volume density of the transverse tubular system (T-tubules) is reduced significantly in both beating and nonbeating myocytes. Nevertheless, junctional sarcoplasmic reticulum continues to retain its close association with the T-tubule in both beating and nonbeating heart cells.13,15

The change in the relative volume density of the T-tubule ($V_{\text{vT-T}}$) system follows a complicated pattern in quiescent heart cells. When compared with values derived from whole rabbit heart, $V_{\text{vT-T}}$ increases significantly after the rabbit heart cells are isolated but then declines to very low values in heart cells cultured for 2 weeks or longer (Figure 8). Past fine structural observations suggest that this change in $V_{\text{vT-T}}$ is related to a dilation of the T-tubule that accompanies the isolation of the myocytes rather than an increase in the number of T-tubule profiles observed in thin sections.13 The $V_{\text{vT-T}}$ of myocytes contracting for 2 days (i.e., myocytes cultured for 2 weeks) is significantly greater than corresponding values obtained from nonbeating cells (0.8±0.3% versus 0.3±0.2%; $p<0.05$), but at 1 month, no significant differences in $V_{\text{vT-T}}$ could be documented in either of these preparations (Figure 8). The range of T-tubule volume densities obtained in the present study were similar to those values derived from well-spread adult rat cardiac myocytes cultured on laminin-coated surfaces.15

**Discussion**

The results derived from the present morphometric investigation clearly confirm our previous morphological observations that illustrated that nonbeating rabbit heart cells disassemble or degrade their myofibrils when maintained in vitro for prolonged intervals.13 Ultimately, in the absence of mechanical activity (i.e., beating), the mature myofibril apparently is replaced by a minute fibrous structure, termed a minute myofibril,13 that retains a periodic distribution of actin and $\alpha$-actinin but becomes depleted of its myosin content and thick filaments (Figure 1). At present, the origin of these minute myofibrils is unknown, but they appear in nonbeating myocytes as myofibrillar disruption progresses (Figure 5). Such structures may represent remnants of preexisting myofibrils or may assemble de novo; however, it is clear that these fibrils do not resemble leptomeres, because their $Z$-line–to–$Z$-line dimensions are significantly greater than those exhibited by the leptomere.21 If the rabbit myocytes begin beating spontaneously, this progressive myofibrillar "atrophy" is arrested, and at 1 month of culture, contracting heart cells disclose a well-developed contractile apparatus (Figure 2). Stereological measure-
ments further reveal that the $V_v$MYF of 28-day old beating myocytes is significantly greater than values derived from nonbeating myocytes, and extrapolating such values back to the point when the rabbit cells began beating suggests that contractile activity stabilizes $V_v$MYF and precludes further myofibrillar atrophy (Figure 4). Such observations further suggest that passively loaded (i.e., attached, nonbeating heart cells) rabbit myocytes are incapable of retaining an intact contractile apparatus in a quiescent state, in contrast to results derived from nonbeating adult feline myocytes, which maintain a relatively high degree of myofibrillar organization when cultured in a serum-free environment.\textsuperscript{11} The present results convey the impression that work generated by contracting myocytes is essential to the maintenance of myofibril structure in cultured rabbit cardiac myocytes.

Changes in the relative volume density and structural organization of other subcellular organelles known to participate in the contractile cycle also are altered when adult myocytes are placed in primary culture. When comparing their data with that from previous reports on $V_v$T-T derived from either whole rat heart\textsuperscript{21,22} or freshly isolated rat heart cells,\textsuperscript{20} Delcarpio et al\textsuperscript{15} first demonstrated that a significant reduction in $V_v$T-T developed in cultured rat heart cells. Our observations confirm these results and further suggest that the decline in $V_v$T-T appears to be related to cell spreading rather than the acquisition of contractile activity per se, for the reduction of $V_v$T-T is apparent in both beating and nonbeating rabbit heart cells (Figure 8). Perhaps, the need for a well-developed T-system to initiate Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is minimized, because the distance that Ca\textsuperscript{2+} ions must diffuse during excitation-contraction coupling is reduced to as little as 1–2 $\mu$m in these flattened heart cells.\textsuperscript{13,15}

Conversely, the relative volume density of the mitochondrial compartment and the sarcoplasmic reticulum is not altered significantly in cultured rabbit ventricular myocytes (Figures 3 and 6), although the organization of the latter membranous compartment is markedly influenced by the physiological state of the heart cell (Figure 7). The stability of the rabbit myocyte mitochondrial population ($V_v$MIT) is further supported by constant creatine phosphate

FIGURE 7. Transmission electron micrographs of day 1 (panel a), day 14 nonbeating (panel b), and day 14 beating (panel c) myocytes. In day 1 (panel a) and day 14 beating (panel c) myocytes, the sarcoplasmic reticulum (arrowheads) displays its "honeycomb" conformation in association with myofibrils (M). In nonbeating myocytes (panel b), many myofibrils lack thick filaments, and the sarcoplasmic reticulum (arrows) no longer retains its classic association with the myofibril but is found scattered throughout the cytoplasm. m, Mitochondria; T, T-tubule. Magnification, panel a: ×12,375; panel b: ×15,000; panel c: ×11,625.

FIGURE 8. Percent volume density of the transverse tubular system ($V_v$T-T) in intact heart, freshly isolated myocytes, and nonbeating and beating heart cells. Values reflect % mean $V_v$T-T±SD. $V_v$T-T declines precipitously in nonbeating and beating myocytes as the cells flatten during prolonged culture. $V_v$T-T at 14 and 28 days of culture is significantly different from in situ heart, freshly isolated cells, and 1-day cultures (p<0.01), regardless of physiological state of the cells. Day 14 contracting myocytes have a significantly greater T-tubule $V_v$ than day 14 quiescent cells (p<0.05), but the $V_v$T-T values of 1-month-old cells are statistically indistinguishable from one another.
and ATP levels measured from these in vitro preparations\textsuperscript{12,13} and contrasts with a reduction of V\textsubscript{v}MYF documented for cultured adult rat cardiac myocytes.\textsuperscript{15} Adult rat heart cells undergo a significant degree of subcellular reorganization\textsuperscript{8,14,16,17} when placed in long-term primary culture; therefore, the reduction in V\textsubscript{v}MIT observed in the latter study\textsuperscript{15} may reflect a loss of mitochondria during the early phase of myocyte rounding\textsuperscript{14}—a feature not frequently observed as rabbit heart cells adapt to a cell culture environment.\textsuperscript{12,13} The V\textsubscript{s}SR also is not altered during the course of cell culture (Figure 6); however, in nonbeating heart cells, its classical plexiform morphology is transformed into a relatively disorganized reticular pattern (Figure 7). Although the importance of this reorganization is not readily apparent nor has it been described previously in cardiac myocytes derived from normal or diseased hearts, Croop et al.\textsuperscript{23} reported that a similar structural reorganization of the sarcoplasmic reticulum developed when cultured skeletal muscle myotubes were exposed to the phorbol ester, tumor promoter, 12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA). Under these circumstances, the reorganization of the sarcoplasmic reticulum paralleled a disruption of the contractile apparatus and a reduction in intracellular calcium content. Moses and Claycomb\textsuperscript{24} recently illustrated that cultured adult rat cardiac myocytes responded to TPA treatment by also disassembling their contractile elements and apparently reordering the sarcoplasmic reticulum. One feature of the TPA-induced subcellular reorganization of cardiac myocyte structure that has a parallel in the nonbeating rabbit heart cells is the apparent maintenance of a stable association between the T-tubule and junctional sarcoplasmic reticulum in both myocyte models,\textsuperscript{13,24} suggesting that this relation survives significant subcellular remodeling. Even though the mechanisms mediating the loss of myofibrils and the morphological transformation of the sarcoplasmic reticulum have yet to be resolved, Claycomb\textsuperscript{25} reported that TPA also suppressed the expression of the muscle-specific genes for myosin heavy chain (MHC) and the M-form of creatine kinase and activated several proto-oncogenes. Because the actions of TPA are believed to be mediated by a Ca\textsuperscript{2+}-dependent protein kinase C\textsuperscript{26} and metabolites of the diacylglycerol pathway, fluctuations in [Ca\textsuperscript{2+}]\textsubscript{i} may modulate contractile protein gene expression and therefore the organization of the contractile apparatus and, perhaps, the sarcoplasmic reticulum as well.

Although we have not evaluated myofibrillar gene expression in our quiescent myocyte preparations, the synthesis of contractile proteins (i.e., actin and MHC) is severely depressed (approximately 95%) in nonbeating rabbit heart cell cultures (Reference 27 and R.S. Decker et al, unpublished data, 1991) as can be seen when compared with similar measurements made on in vivo rabbit preparations.\textsuperscript{28,29} Because total protein degradation is markedly elevated, especially during the second week of culture (R.S. Decker et al, unpublished data, 1990), the reduction in V\textsubscript{v}MYF reported in the present study may reflect an imbalance between the synthesis and degradation of contractile proteins in these nonbeating (i.e., nonworking) myocytes. Although rates of actin and MHC degradation have not been measured either directly or indirectly, an exponential fit of the data derived from Figure 4 reveals that the apparent t\textsubscript{1/2} of the myofibril in nonbeating heart cells is approximately 10 days.\textsuperscript{30} Because the disappearance of myofibrils in these quiescent myocytes appears to follow first-order kinetics, a fractional rate of “myofibrillar” degradation (K\textsubscript{d}) can be estimated from the “myofibrillar half-life.” Such a calculation yields a K\textsubscript{d} of approximately 6.5% per day, indicating that the apparent rate of myofibrillar degradation is significantly greater than the synthesis rate of its constituent proteins (i.e., actin and MHC) (Reference 27 and R.S. Decker et al, unpublished data, 1990). Thus, in the absence of contractile function, myofibrillar breakdown in vitro, which does not appear to be significantly different from those values obtained for the degradation of actin or MHC in situ,\textsuperscript{28,29} may mediate the observed decline in V\textsubscript{v}MYF. Potassium-arrested neonatal heart cells maintained in culture also exhibit a significant reduction in the fractional rate of RNA and protein synthesis,\textsuperscript{31} as well as a reduction in the relative rate of myosin synthesis and myosin content, when compared with their spontaneously contracting counterparts.\textsuperscript{32} Furthermore, protein degradation was not altered significantly in these nonbeating neonatal heart cells, suggesting that cardiac myofibrillar protein turnover is regulated primarily through modulating synthesis rather than degradation.\textsuperscript{1,28,29,31} The observations derived from this investigation and those of McDermott and coworkers\textsuperscript{31,32} further strengthen the implication that work is the principal factor that regulates myofibrillar organization and the turnover of contractile proteins in cultured heart cells.

Several other investigations support the hypothesis that heart cells must generate work (i.e., must be extrinsically loaded) to maintain their structural and functional properties.\textsuperscript{4} Transection of the chordae tendineae of feline right ventricular papillary muscle induces a rapid and progressive atrophy of these cardiac myocytes.\textsuperscript{33} This atrophic response is characterized by a marked reduction in myofibrillar volume density, a concomitant decline in both actin and myosin content, and a depression in the contractile function of the unloaded papillary muscle.\textsuperscript{34} A unique feature of this unloaded papillary muscle is that it continues to contract in synchrony with the right ventricle,\textsuperscript{33} implying that changes in extrinsic load control the composition and organization of the contractile apparatus. When the papillary muscle is reloaded, it quickly regains its normal structure and function.\textsuperscript{34} The morphological dissolution of papillary myofibrils\textsuperscript{35} associated with the atrophy in this model closely resembles the events described previously,\textsuperscript{13} where the focal loss of Z-line material precedes the disruption of myofibrillar order. More
recent observations and comparisons with paired preparations of unattached, beating cells further illustrate that cultured, beating neonatal myocytes grow larger if attached to a substratum. Moreover, the attached, contractile myocytes retain their myofibrillar organization, whereas unattached cells display a poorly ordered contractile apparatus. Such results implicate the extrinsic work load that the heart cell must overcome when it contracts as the principal factor that regulates the composition and structure of the contractile apparatus in the isolated heart cell. The present observations provide additional support to this hypothesis and further demonstrate that alterations in the structure of the contractile apparatus are paralleled by specific changes in the organization of the sarcoplasmic reticulum.

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