Developmental Changes in the Ultrastructure and Sarcomere Shortening of the Isolated Rabbit Ventricular Myocyte

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Adult myocardium develops greater tension per cross-sectional area than immature myocardium. Exploring the causes of this difference is fundamental to understanding how contraction is modulated at the cellular level.

A maturational enhancement of the amount of force per cross-sectional area could be due either to changes in tissue properties or to changes in intrinsic cellular characteristics. Tissue properties could change as a result of an increase in the relative amount of contractile material or of a change in tissue organization in the characteristics of the extracellular matrix. Intrinsic cell properties that could change the contraction waveform include alterations in the control of activator calcium and in the properties of the regulatory proteins and of myosin ATPase.

Friedman found greater developed force per cross-sectional area in myocardium from adult sheep than in fetal lamb but no difference in the muscle shortening velocity. He, therefore, suggested that the increased force in the adult heart was due to a larger amount of contractile material. However, Uthraler et al. found a maturational increase in unloaded shortening velocity in the dog: this cannot be accounted for solely by an increase in contractile material but requires changes in intrinsic cellular properties during maturation.

In this study, the effects of maturation on sarcomere shortening characteristics in isolated cardiac cells were measured and assessed. In multicellular preparations, the assessment of developmental changes in sarcomere shortening can be obscured by tissue complexities and by maturational changes in the extracellular matrix. By measuring sarcomere shortening in isolated cells, the difficulties caused by the extracellular matrix and by varying geometries of cell-to-cell connections encountered in the multicellular preparation were avoided. This approach allows for the determination of whether the differences in the responses of immature and adult myocardium are a result of maturational changes in cellular properties or are due to changes in tissue organization.

Previous studies of isolated calcium-tolerant myocytes have been limited to cells from the adult myocardium. In the present study, calcium-tolerant myocytes from ventricles were examined in three-week-old and adult rabbits. The rabbit was selected for study because rabbit myocardium has many properties in common with the human heart, e.g., prominent postextrasystolic potentiation, a positive force-frequency relation, and a positive response to an increase in extracellular calcium concentration. The rabbit myocardium responds differently from most mammals to changes in the rate and pattern of stimulation and to...
changes in extracellular calcium concentration. The properties of restitution and postextrasystolic potentiation are an integral part of the complex system of excitation-contraction coupling and beat-to-beat modulation of contraction in the mammalian myocardium. That the rabbit myocytes continue to exhibit these properties after isolation demonstrates their ability to control calcium normally. Furthermore, these properties provide us with a powerful tool to examine developmental changes in the calcium-control systems of the cell.

In this study, a new method is described that allowed for the quantitative determination of sarcomere motion in the living myocyte and that allowed for the examination of the ultrastructure of the same cell. In the light of cell-to-cell variability in single-cell characteristics, this approach is necessary to avoid assumptions involved when the function of one cell is related to the structure of another.

The combined analysis of function and structure of single cells indicates that the maturational changes in contraction waveform are a consequence of intrinsic cellular differences. Specifically, in the immature myocyte, the cellular architecture may result in a greater internal load, and the membrane systems that control cytosolic calcium appear less differentiated. Both of these factors may contribute to the decreased contractility of the immature myocardium.

Materials and Methods

Preparation

New Zealand white rabbits, 3 weeks of age (weighing from 150–300 g, mean 230 g, n = 9) and 3 months of age (1.8–3.0 kg, mean 2.5 kg, n = 11) were administered heparin (1,000 U/kg i.p.) before they were killed by cervical fracture. The chest was opened, the aorta cannulated, and the heart removed. The coronary system was perfused with Solution I (described under "Solutions") for 3–5 minutes or until the heart stopped contracting. The heart was then perfused with Solution II for 40 minutes or until it appeared pale and softened, followed with 100 ml of Solution III.

The heart was removed from the cannula. The right and left ventricular free walls and septum were cut into small pieces and placed in small vials containing Solution III. The vials were shaken gently. Cells were obtained for study within 3 hours after the initiation of the mechanical disaggregation. During the study of sarcomere dynamics, the cells were placed in Solution IV.

Solutions

The solutions were similar to those described by Brady et al.29

A. DISAGGREGATION SOLUTIONS. Solution I, aerated with 95% O₂–5% CO₂, contained (mM): NaCl 127, KCl 4.0, MgCl₂ 1.0, NaHCO₃ 13, Na₂HPO₄ 0.44, glucose 5.6; insulin 10 mU/l. Solution II was Solution I with 0.25 mM CaCl₂, 4.0 mM ATP (Sigma Chemical Co., St. Louis, Mo.), 0.1% collagenase (Woerhington, Malvern, Penn.), 0.2% hyaluronidase (Sigma), and 0.0025% elastase (Sigma); pH 7.2. Solution III was Solution I with 1 mM CaCl₂ (total) and with 20 mM phosphate replacing the bicarbonate; pH 7.2.

B. SOLUTION USED IN EXPERIMENTS. Solution IV contained (mM): NaCl 127, KCl 4.0, MgCl₂ 1.0, NaHCO₃ 13, Na₂HPO₄ 0.44, glucose 5.6, MOPS (3-[N-morpholino]propane-sulfonic acid) 10, CaCl₂ 1.0 or 2.0; insulin 10 mU/l; pH 7.4.

Experimental Apparatus

The cells were examined in a chamber with a microscope cover glass used for its bottom. The bath was mounted on the stage of a Zeiss inverted microscope fitted with a trinocular eyepiece and a 40× (numerical aperture 0.75) water-immersion objective. The microscope was fitted with the Nomarski differential interference contrast system that, when used with the 40× objective, provided a shallow depth of field (approximately 1 μm). The cells were illuminated with a tungsten-halogen lamp. During data recording, a xenon-flash light source was used.

Criteria for Selecting Cells for Study

Every cell selected for the study of sarcomere dynamics fulfilled the following criteria: 1) the cell was quiescent during exposure to millimolar concentrations of calcium, 2) the cell was electrically excitable with an "all-or-none" response in sarcomere shortening that was synchronous and homogeneous throughout the cell, 3) the cell was free of external loading during contractions, 4) there were no visible oscillations of sarcomeres or contractions that propagated along the cell, and 5) the sarcomere contraction waveform at a constant pacing rate was reproducible from contraction to contraction.

Criteria for Acceptance of Physiologic Measurements

Physiologic measurements were accepted only when the cells had fulfilled the above criteria. In addition, measurements of peak velocity and amount of sarcomere shortening were obtained under externally unloaded conditions, i.e., the cells were floating freely. When a cell adhered to the floor of the tissue chamber, a range of sarcomere-shortening waveforms was observed within that cell: sarcomeres on the side of the cell in contact with the tissue chamber shortened the least amount and with the lowest velocity, but those at the top of the cell shortened the most and the fastest. Data from cells that were found damaged on subsequent ultrastructural examination were excluded from the final data analysis.

Experimental Protocol

Cells were paced at a constant rate (15 min⁻¹; 1–10-msec pulse duration) at room temperature (22–24°C). An additional pacing pattern was used to examine the restitution of sarcomere shortening and postextrasystolic potentiation: following a steady-state contraction at the basic pacing interval, an extrasystole was introduced at a known, variable test interval, tₑ, after
every eighth contraction at the basic rate. Restitution was determined by measuring the amount of sarcomere shortening of the extrasystole as a function of $t_E$. Postextrasystolic potentiation was determined by comparing the waveform of the contraction following the extrasystole with that at the regular rate.

**Physiologic Data Recording**

The image of the single cell was recorded with a television camera (fitted with a Plumbicon tube; Dage-MTI, Michigan City, Ind.) and a video cassette recorder (Sony, SLO-323MD, New York). A segment of the raster line was brightened to mark the time of stimulation. The video camera was fitted with a 50-mm objective, focused at infinity, and mounted above the third eyepiece (10×) of the microscope. The camera was oriented so that the axis of the cell was parallel to the television raster lines. The xenon flash and the stimulus generator were synchronized to the vertical synchronizing pulses of the video camera.

**Overall Cell Dimensions**

The length of each cell was measured from the light microscope image displayed on the video monitor. Two cross-sectional dimensions were determined under the light microscope by measuring the width of the cell in both the horizontal and vertical planes. This was performed by focusing the microscope (using the 40× water-immersion objective) on the top and bottom surfaces of the cell and noting the difference in readings of the scale on the focusing knob. The scale was calibrated by focusing on the top and bottom surfaces of a piece of microscope cover glass of known thickness. Because of the complex shapes of most cells, accurate measurements of overall cell dimensions under the light microscope were not practicable in this study. However, the measurements are adequate to illustrate the ranges of size and shape observed.

**Sarcomere Measurement**

The video images were replayed frame by frame. The two interlaced video fields were separated, one field at a time being displayed on the monitor. Two vertical cursors were positioned to span a group of sarcomeres (usually 10–20) that was measured throughout the time course of a contraction. A number proportional to the distance between the cursors was displayed on a digital readout. With the 40× objective, the least significant digit corresponded to 0.02 μm for 15 sarcomeres.

The image of a stage micrometer of 2 μm/division was recorded as a calibration signal during each experiment. This micrometer was compared to a stage micrometer (Graticules Ltd., Tonbridge, England) calibrated by the National Physical Laboratory, Teddington, England, and the corrected calibration factor was used to compute sarcomere lengths. Nonlinearity of the system did not exceed 1.7% in the center of the field of view where measurements were made. All the readings were corrected for nonlinearity. The long-term stability of the system was excellent.

The sarcomere lengths were measured throughout each contraction and were stored for analysis using an IBM PC computer. The amount of sarcomere shortening was calculated as the difference between the rest sarcomere length and the length at peak shortening. The same method was used to determine the amount of sarcomere shortening in early extrasystoles where the contraction waveform was fused with that of the previous regular contraction (peak shortening in the extrasystole occurred at a time when the previous contraction would have returned to the rest sarcomere length).

After smoothing the data by taking a three-point moving average of sarcomere length as a function of time, the maximum velocity of sarcomere shortening was obtained using a computer program that fit a "moving" regression line; this line had a length equal to the line segment connecting the two points on the waveform that corresponded to 10 and 60% shortening. Maximum reextension velocity was determined in a similar manner. Lines representing the maximum shortening and reextension velocities were displayed on the sarcomere contraction waveform and checked for their reasonableness. The algorithm, in most cases, was found to result in velocities that agreed with those obtained by visual curve fitting. Rest sarcomere length, the amount of sarcomere shortening, and the maximum velocities of sarcomere shortening and reextension were examined. The duration of the contraction was determined by measuring the interval between the time of stimulation and the time at which the sarcomeres had extended by an amount equal to half that of shortening. In a number of cells, this was done in multiple areas throughout the cell.

**Ultrastructural Analysis**

After the physiologic recordings were made, the cell was drawn into a micropipette containing Solution IV with no added calcium and then gently ejected into one well of a Corning polystyrene Cell Well Plate (Fisher Scientific, Silver Spring, Md.) containing 0.1 ml of fixative (3% glutaraldehyde, 0.2–0.4% tannic acid in buffered Ringers, pH 6.8–7.0, room temperature). The cell was left undisturbed for approximately 10 minutes before the well was filled to capacity (0.2 ml) with the fixative.

The cells were kept in the primary fixative for 30 minutes to 1 hour. After being rinsed with buffered Ringer’s solution, the postfixative [1% OsO$_4$ (EMS) in 100 mM KH$_2$PO$_4$ + K$_2$HPO$_4$ buffer with 10 mM MgCl$_2$, pH 6.1] was added. The postfixative was initially ice cold and was allowed to warm to room temperature. After 30 minutes, the cells were rinsed with distilled water and block stained (2% uranyl acetate in H$_2$O) for 15 minutes at room temperature, or, if overnight, ice cold and then rinsed with distilled water and dehydrated in a graded ethanol series at room temperature. After dehydration, the cell was suspended in Araldite 506™ plus DMP 30 at 65° C for 15–25 minutes and was then transferred to a sheet of polypropylene. The cell was oriented for embedding using an eyelash mounted on a handle. A tiny volume of
Araldite resin containing the cell was partially polymerized (2 hours at 65°C) to a tacky stage at which time a freshly filled capsule (size 00; Better Equipment for Electron Microscopy, Inc., Bronx, N.Y.) was inverted over the secured cell. The blocks were cured for 48 hours at 65°C.

Cross-sections and longitudinal sections ranging from gray to gold were cut with a Diatome diamond knife (Electron Microscope Sciences, Port Washington, Penn.) on a Reichert OMU-3 ultramicrotome (American Optical-Reichert, Edison, N.J.) and picked up on 200-mesh carbon-coated grids. The number and order of the sections were recorded to make it possible to reconstruct the entire cell. The sections were stained for 15 minutes with aqueous 2% KMnO₄, rinsed with water and Pal’s bleach (3 drops of 0.5% Na₂SO₃ and 0.5% (COOH)₂H₂O added to 10 ml distilled water) and stained with Sato lead stain for one minute. Images were photographed (Kodak S0163 E.M. film) using a Siemens 101 electron microscope (80 kV, 50-μm objective aperture). Micrographs were recorded at 2,000, 10,000, and 18,000 X. The microscope was calibrated several times during the course of the work with a cross-grating replica (Ernest Fullam, Schenectady, N.Y.); the lenses were regularly normalized, and the objective current was recorded for all images so that the percent deviation from the nominal magnification caused by variation of current could be corrected.

The three-dimensional characteristics of each cell were displayed by tracing the outline of each of 6 to 10 levels of the cell directly from 2,000 X negatives onto acetate sheets. Length and width measurements of the whole cell were taken from these tracings. Sarcomere lengths were measured directly from the 2,000 X and 10,000 X negatives using 10–12 sarcomeres per measurement and also from 2.5 X prints.

Results

Cell Appearance and Size

The shapes of the adult cells were more complex than those of most cells from the three-week-old heart. The adult cells usually had several step changes along their lateral borders (Figure 1C), while a few were branched. Only 1 adult cell could be described as a simple flattened cylinder (Figure 1B). In the adult cells, the major and minor axes of the cross-section were unequal.

Three-week-old myocytes generally had a smooth outline with blunted and sometimes tapered ends (Figure 1A). The cross-section was approximately circular. Two single immature cells were found that had the same large size and complex shape as the adult cells. Some other cells from three-week-old hearts that appeared by light microscopy to be similar in size to the adult cells were found by electron microscopy to be a complex of small, simple-shaped cells, approximately 40–50 μm in length. The characteristics of sarcomere shortening were not useful in helping us identify whether such a preparation was a single cell or a complex of several cells; the amount and velocity of sarcomere shortening were the same throughout the preparation.

The mean dimensions of the adult and three-week-old myocytes are given in Table 1. In adult cells, length and major and minor diameters were significantly larger than in cells from the three-week-old heart (p<0.001; see Table 1 and Figures 1 and 2). Among the cells from each age group, a wide variation in cell size was found, and the data from the two age groups overlapped.

Rest Sarcomere Length

The mean rest sarcomere length in the adult cell, 1.91 ±0.071 μm, was significantly longer than that in the immature cell, 1.82 ±0.09 μm (p<0.001). Within each age group, the distribution of lengths was sufficiently broad so that the data from the two age groups overlapped: 1.76–2.09 μm in the adult, and 1.61–1.91 μm in the immature cells (see Table 1). Sarcomere lengths were measured in multiple areas of some cells. In the smaller, less complex adult cells and in the immature cells, rest sarcomere length throughout a given cell was quite uniform (the differences were less than 0.02 μm). Sarcomere lengths measured by light microscopy did not differ significantly from those obtained by electron microscopy from the same cells.

Sarcomere Dynamics at a Constant Pacing Rate

Amount of Sarcomere Shortening. The range of sarcomere shortening is summarized in Table 1. The mean amount of sarcomere shortening was significantly (p<0.001) greater in the adult (0.31 ±0.05 μm) than in the three-week-old cells (0.17 ±0.06 μm).

Sarcomere length at the peak of the shortening waveform ranged from 1.75 to 1.42 μm in the adult and 1.81 to 1.44 μm in the immature cells. Although the ranges of sarcomere length at peak shortening of the two age groups overlapped, there was a statistically significant difference between the means (p<0.05).

In both age groups, cells capable of contracting to a sarcomere length of 1.44 μm, a length well below the thick filament length of 1.55–1.60 μm were found. Thus, collision of the thick filaments with the Z disks does not place an absolute mechanical limit on sarcomere shortening. Despite contracting repeatedly to such short sarcomere lengths, these cells showed no ultrastructural evidence of myofilament disarray.

Sarcomere Shortening Velocity. The peak velocities of sarcomere shortening are summarized in Table 1. The mean shortening velocity for adult cells was significantly (p<0.001) greater than immature cells.

Cell-to-cell variability in sarcomere shortening velocity occurred within each group (Table 1). Such variability may be related to cell-to-cell variability in rest sarcomere length: the shorter the rest sarcomere length, the greater the potential, earlier in the contraction, for interference between thick and thin filaments in the M line and between thick filaments and the Z disks. However, examination of the data did not reveal any relation between rest sarcomere length and either peak velocity of shortening or the sarcomere
FIGURE 1. Longitudinal sections through the near central region of 3 myocytes from rabbit heart. The contraction waveform of each cell is beneath its electron micrograph. Sarcomere length (SL) is plotted as a function of time (1 mM Ca). A, an average-sized three-week-old myocyte. B, a small-sized adult myocyte. Even this relatively small adult cell had a greater amount of and a faster sarcomere shortening than that of the average-sized immature cell. C, an adult cell of average size. All cells in Figures 1 and 2 are shown at identical magnification.
Table 1. Myocytes From the Adult and Immature Rabbit: Observations by Light Microscopy*

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<th></th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
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<td>Length (μm)†</td>
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<td>31.0</td>
<td>27</td>
<td>73–152</td>
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<td>Minor diameter (μm)</td>
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<td>25</td>
<td>7–14</td>
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<td>1.9</td>
<td>16</td>
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<td>Major diameter (μm)</td>
<td>16–35</td>
<td>25.8</td>
<td>5.6</td>
<td>27</td>
<td>9–23</td>
<td>14.0</td>
<td>4.1</td>
<td>19</td>
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<td>Sarcomere shortening</td>
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<td>Rest sarcomere length (μm)</td>
<td>1.76–2.09</td>
<td>1.91</td>
<td>0.07</td>
<td>27</td>
<td>1.61–1.91</td>
<td>1.8</td>
<td>0.09</td>
<td>19</td>
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<td>Amount of shortening (μm)</td>
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<td>0.05</td>
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<td>0.08–0.30</td>
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<td>Velocity of shortening  (μm/sec)</td>
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<td>0.2–1.4</td>
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<td>Velocity of reextension (μm/sec)</td>
<td>0.9–3.8</td>
<td>2.14</td>
<td>0.75</td>
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<td>0.1–2.3</td>
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<td>Duration of shortening (msec)</td>
<td>357–1,325</td>
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<td>194.0</td>
<td>25</td>
<td>744–1,436</td>
<td>1,103</td>
<td>225</td>
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*All the means listed show a statistically significant difference between the 2 age groups (Student's two-tailed t test, p<0.001).
†Lengths of immature cells given in the table may be overestimates since some of the measurements were multiple cells and could not be distinguished always as such under the light microscope.

length at which shortening velocity began to decrease. Some of the cells that had the shortest rest sarcomere length also had the fastest shortening. This further suggests that limitation of sarcomere shortening velocity by mechanical constraints, such as the collision of thick myofilaments with Z disks, does not account for the range of shortening observed.

Duration of the contraction. The overall duration of the contraction waveform was significantly longer in the immature cells than in the adult cells (see Table 1). This suggests that cytosolic calcium is removed more slowly by the sarcoplasmic reticulum (SR) or the Na–Ca exchange in the immature cell than in the adult cell. The cell-to-cell variability in duration of contraction at each age resulted in overlap of the two ranges.

**Velocity of sarcomere reextension.** In general, the greater the amount and velocity of sarcomere shortening, the greater the velocity of reextension. The velocity of reextension almost always exceeded the shortening velocity. In the adult cell, the onset of sarcomere reextension was usually abrupt, and the maximum velocity of reextension was reached rapidly. In immature cells, the onset was gradual; the maximum velocity was attained later in the contraction, and the velocity was significantly smaller. The ranges of the two ages overlapped (see Table 1).

**Sarcomere Dynamics in Extrasystoles**

The restitution of contractility between contractions was examined by introducing an extrasystole into the basic pacing rate (see Figure 3). In the adult cell, the most premature extrasystole (one that was elicited at the shortest tE) had the smallest sarcomere shortening (Figure 4B). Sarcomere shortening increased with increasing tE until the waveform of shortening in the extrasystole became identical to that at the basic rate.

In the immature cell, the restitution of contractility was markedly different from the adult cell. In the immature cell, sarcomere shortening in the most premature extrasystole was already as large as it was in contractions at the basic rate (Figure 4A). In contrast, the most premature extrasystole in the adult myocyte had a very small amount and velocity of sarcomere shortening. The range of tE used in the two age groups was similar.

**Sarcomere Dynamics in the Postextrasystolic Contraction**

Sarcomere shortening in the contraction following an extrasystole was examined for potentiation. In both the adult and the immature cell, potentiation was present and was greater the earlier the extrasystole, i.e., the shorter the tE. Potentiation occurred in the amount and in the maximum velocity of sarcomere shortening as shown in Figure 3. The amount of potentiation in the
immature cells was similar to that measured in the adult cells.

Ultrastructural Analysis

The ultrastructural characteristics of the isolated myocytes at both ages were, in general, similar to those reported for multicellular preparations. The reader is referred to those publications, and comment will be made only on those observations that are important to the present analysis or conclusions.

The wide range in single-cell size and shape observed using light microscopy was confirmed by examining serial sections in the electron microscope. Adult cells of extraordinarily large size with noncylindrical shapes were indeed single cells. Single immature cells were usually smaller than adult cells and were cylindrical (Figures 1A, 2D, 2E, and 2F).

Large preparations from the three-week-old heart were often complexes of two or three small, cylindrical cells.

General cell architecture. The adult and immature cells had different organizations of myofibrils relative to the nuclei and mitochondria (Figure 5). Diagrams of the different arrangements are shown in Figure 6.

In the adult, 16 of the 17 cells examined had 2 nuclei, and all had a small perinuclear aggregation of mitochondria. The majority of the mitochondria were arranged in rows adjacent to the sarcomeres (Figure 7). There were 8–12 myofibrils across the main body of the cell and 3 or 4 across the stepped ends.

In the small, immature myocyte, there were always 2 nuclei. These nuclei were surrounded by a large aggregation of mitochondria (Figure 5). The myofibrils were arranged in a shell, usually 2 myofibrils deep, around the large central mass of nuclei and mitochondria (Figure 8). Relatively few mitochondria were lined up between the sarcomeres.

Although three-week-old hearts yielded mostly cells of the "immature" type, 2 adult-type "mature" myocytes were seen among the cells from three-week-old ventricles. In the mature-type cells, the contractile material was distributed more evenly throughout the myocyte, and the mitochondria formed rows among the myofibrils. These cells were almost indistinguishable from adult cells except that they were somewhat smaller. In a preliminary survey of myocytes from younger and older animals, only small immature-type cells from the two-week-old heart and small mature-type cells from the four-week-old ventricles were observed.

Cytoskeleton. The cytoskeleton was evident in cells from both ages (Figure 9D). A network of intermediate filaments, desmin, surrounded and apparently linked adjacent Z bands and held the Z bands in register across the cell in adult cells (Figure 7). These anchor fibers frequently appeared to merge with the transverse tubular (T tubule) membrane. The attachments of adjacent Z lines to a T tubule can be seen in Figure 9C, where 2 myofibrils have moved out of register. At the cell periphery, the Z and I bands often had dense, amorphous material extending from them to the sarcolemma (Figure 9A, B, and C). This density may
FIGURE 5. Longitudinal sections through an adult myocyte (A) and a three-week-old myocyte (B) show the differences between the two ages in cell shape and in the disposition of mitochondria and nuclei relative to the myofibrils. The second nucleus of the adult cell lies just off the top of the page.
ally, and their motion is affected by that of other
connections join the outermost sarcomeres to the
periphery. White areas (B) represent nuclei and mitochondria, and shaded areas represent
myofibrils. Central mass of mitochondria and nuclei occupies a larger fraction of the immature cell than of the adult cell.

Represent vinculin, arranged in "costameres" that link the peripheral Z or I bands to the cell membrane. Since myofibrils are linked to one another across the cell and connections join the outermost sarcomeres to the sarcolemma, sarcomeres are clearly constrained laterally, and their motion is affected by that of other sarcomeres and by the presence of mitochondria and the membrane structure.

In the adult, the linking of myofibrils through Z disks across the cell appeared to delineate transverse compartments consisting of levels of single sarcomeres surrounded by longitudinal sarcoplasmic reticulum (LSR) and flanked by mitochondria and microtubules (Figure 7). This arrangement may effectively constrain the sarcomeres longitudinally and laterally. The nuclei and mitochondria at their poles were the only apparent interruption in this system of linked myofibrils. In addition, the step changes in cell boundaries often resulted in groups of myofibrils that did not extend the full length of the cell.

In the immature-type myocyte, the nuclei and mitochondria occupied the central core of the cell almost from end to end (Figures 5 and 8). In these immature cells, the Z bands were connected laterally to one another around the periphery, but the intermediate filaments did not cross the central mass to connect myofibrils on opposite sides or form transverse levels across the cell. The immature-type cell was usually a smooth cylinder and did not have steps so that nearly all myofibrils appeared to extend the full length of the cell.

**Glycocalyx.** The glycocalyx appeared well stained by the fixative containing tannic acid (Figures 7 and 9). In all cells, the glycocalyx appeared intact over the entire cell surface, except for the intercalated disk region and could be seen to accompany the T tubule profiles into the depths of the cells. Fine filaments were embedded in the glycocalyx and were often aligned parallel to the cell's long axis. No obvious differences were observed in the glycocalyx among these physiologically intact cells (nor were any cells observed, even those that contracted spontaneously, that completely lacked this density outside the sarcolemma). Intact glycocalyx provides further evidence of the integrity of these cells.

**Transverse tubular system and sarcoplasmic reticulum.** T tubules and junctional sarcoplasmic reticulum (JSR) profiles (diads and triads) were present in all cells at both ages (Figures 7 and 8). No difference in structure between particular examples of adult and immature triads could be detected, but in the adult, the T tubules were present at more Z lines. At both ages, the LSR and the corbular sarcoplasmic reticulum (CSR) were evident (Figures 10 and 11).

In the adult, pale-staining longitudinal elements of the SR were abundant (Figure 10). The dense, footed profiles of the CSR, connected by narrow necks to the LSR, formed a ring around many Z lines and were even seen at some Z levels adjacent to triads.

CSR profiles in the immature cell were similar in appearance to those of the adult and were occasionally seen budding off the LSR (Figure 11). However, in contrast to the adult myocyte, the dense material inside the CSR in the immature myocyte continued into the LSR (Figure 11). In the immature cell, the connecting sections between the CSR and LSR appeared wide, as opposed to the narrow connection observed in the adult myocyte. In addition, a dense coat sometimes continued along the surface of the connecting segments between the CSR and LSR in the immature cell, making the demarcation between the corbular and longitudinal elements indistinct.

Coated vesicles in immature cells were quite different in number and distribution from those in adult cells. In the adult, a few bristle-coated pits and vesicles were seen under the sarcolemma and at some Z lines. The clathrin coat of these vesicles was different in appearance from the foot processes covering the CSR (Figure 12). The interiors of the coated vesicles were lightly stained, whereas CSR profiles were filled with dense material, presumably calsequestrin. In immature cells, accumulations of coated pits and vesicles, ribosomes, and filamentous material were seen. Coated vesicles were also numerous in the vicinity of the nucleus and around the sarcomeres and T tubules.

**Discussion**

The present findings in the isolated ventricular cell suggest that the amount and velocity of sarcomere shortening increase with maturation. This is consistent with the developmental increase in force per cross-sectional area in the intact myocardium and suggests that the isolated myocyte retains its in situ properties. These results also suggest that differentiation of the SR...
FIGURE 7. *Longitudinal section of an adult cell. T-tubule profiles are penetrating at the Z-line levels of the cell surface, on the right, and appear as triads flanking each Z line. Rows of mitochondria and myofibrils that alternate across the image typify the arrangement in the adult cell. Myofibril closest to the cell surface passes out of the section plane for a short distance, allowing for the observation of mitochondria that envelop each myofibril. A vesiculated gap junction is at the right (arrowhead).*
and changes in cellular architecture may be the basis of these developmental changes in cell shortening.

However, the possibility of subtle damage during the isolation procedure could not be ruled out completely as the cause of the decreased sarcomere shortening in the immature cells. To minimize the possibility of artifacts due to damage, each cell had to meet a series of stringent criteria before its data were included in the developmental comparison. First, the cell had to demonstrate quiescence in physiologic concentrations of calcium; this state demonstrates that the cell can control its cytosolic calcium concentration.39 Second, a brief electrical stimulus had to elicit an all-or-none contraction (another criterion for cell intactness39). Third, sarcomere shortening had to be uniform and simultaneous throughout the cell. Cells that responded in this manner have normal resting and action potentials.21,27 In addition to the reproducible response at a constant rate, the cells also had to respond reproducibly and uniformly to a perturbation in the pacing pattern; both the extrasystole and the postextrasystole had to satisfy all the criteria.

The differences in shortening waveform observed between adult and immature myocytes may be attributed to a number of factors, including differences in 1) internal load (restoring force), 2) amounts of activator calcium, 3) amount of contractile material per cross-sectional area, and 4) myofilament proteins (calcium sensitivity, myosin ATPase). Results suggest that two of these factors may be major contributors to the developmental differences in sarcomere shortening. First, the conspicuous differences in cell organization between immature and adult cells may result in significant differences in internal load and in the ratio of amount of contractile material to internal load. Second, the developmental differences in the organization and structure of the LSR and CSR may result in significant differences in the amount and modulation of activator calcium. The sensitivity of the myofilaments to calcium is unlikely to play a major role in these differences. In the rat, the difference in sensitivity of the myofilaments to calcium between the two-day prepartum and the adult is small, and the prenatal rat is more sensitive to calcium than the adult.52 Although some differences in calcium sensitivity possibly exist between three-week-old and adult rabbit myocytes, results from this study can be interpreted without invoking such differences.

Although myosin ATPase isoforms were not measured, the following arguments suggest that myosin isoforms are not the major determinants of shortening velocity in these myocytes. Myosin ATPase is related to unloaded shortening velocity.53 The two myosin isoforms, V1 and V3, have different rates of ATP splitting; V1 is 3 times faster than V3. Adult rabbit papillary muscle, in which the predominant myosin isoform had been changed from V1 to V3 by thyroxin treatment, had a higher unloaded shortening velocity than muscle from untreated rabbits in which the predominant isozyme is V1.54 In general, the relative amount of V1 increases with development,8,9 which would lead one to expect the unloaded shortening velocity to be less in the adult. On the other hand, myofibrillar ATPase either increases24 or decreases25 in the rabbit with maturation. Indeed, myofibrillar ATPase was significantly greater in the left ventricle, which had relatively less V1 than the right.26 and ATPase activity has been shown to vary from one region of the heart to another.57

The presence of an internal load would make it unlikely that the measured velocity of sarcomere shortening would be determined solely by the relative amounts of myosin isoforms present in the cells. Present observations suggest that a greater internal load exists in the immature cells. For these reasons, interpretation of the present results focuses on differences in cell organization (and internal load) and on the differentiation of SR to explain the differing shortening characteristics of immature and adult rabbit myocytes.

Two distinctions become clear when the ultrastructure and function of adult and immature myocytes are...
FIGURE 9. Composite illustrating the relation of the Z band to the sarcolemma and to the intermediate filaments. A., longitudinal section of an adult cell shows 2 rows of myofibrils and mitochondria. Sarcolemma is on the right, and a nucleus is at the left. The sarcolemma appears fused with the Z-I area of the sarcomere at the arrow. B., a cross section of a comparable area of another adult cell. The density between the sarcolemma and the Z-I band area as seen here was frequently observed. C., attachment of T tubule to the Z line is suggested by this image in which the T tubule profile maintains a close proximity to 2 Z lines that are out of register. D., slightly oblique longitudinal section of a three-week-old myocyte illustrates the ring of intermediate filaments surrounding the Z line.
FIGURE 10. Longitudinal section of an adult cell. The repetitive arrangement of CSR and LSR is typical of the mature myocyte. One myofibril passes slightly out of the section plane and reveals a ring of CSR (black arrows) at each Z band and the network of LSR (white arrows and braces) around the A band.
compared: First, small (immature) myocytes with central aggregations of nuclei and mitochondria never exhibited fast, large contractions, while, conversely, briskly contracting adult myocytes never had immature-type cellular organization. The large central mass, against which the immature myofibrils must contract, may present an internal load that is relatively greater than in the adult. Second, immature cells with the slower, smaller contractions always showed pools of sarcoplasm filled with numerous coated pits and vesicles associated with irregularly and loosely arranged CSR and LSR profiles. Also, immature cells had fewer JSR and T tubule profiles than adult cells. Since these structures participate in the process of excitation-contraction coupling and in the control of intracellular calcium, the observed differences suggest that changes in these membrane systems may contribute to the increase in shortening velocity during development.

As to the first distinction, changes in cellular architecture probably will have consequences for both passive and active characteristics of myocytes. An example of the effect of cell organization on passive characteristics can be seen in the following: a significant difference in rest sarcomere length was found between immature and adult cells. This may be related to the maintenance of different rest levels of cytosolic calcium in different cells. However, variations in rest sarcomere length were also seen among adult intact cells (Krueger et al, Robinson et al, Roos et al, Leung, and this study) and also in cells with completely dissolved membranes that were exposed to a relaxing solution with a uniform calcium concentration. (De Clerck et al and observation by the authors).
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Furthermore, the present study indicates that changes in extracellular calcium concentration do not result in changes in rest sarcomere length. Therefore, the existence of different levels of cytosolic calcium concentration do not appear a likely cause for different rest sarcomere lengths between adult and immature cells or among cells of the same age.

The cytoskeleton provides another basis for variations in rest sarcomere length, which may apply to both the differences between adult and immature cells and those among cells from the same age group. The organization of the cytoskeleton is complex, and it includes microtubules and intermediate filaments that connect Z disks to one another and that link the myofibrils to the T tubules, mitochondria, and to the nuclear envelope. In addition, vinculin may link the Z-I areas to the sarcolemma. Because of the constraints imposed on the mitochondria, myofibrils, and nuclei by the cytoskeleton, this structural organization probably determines to some extent the rest sarcomere length. Brady has also suggested that the cytoskeleton plays a role in the variation of rest sarcomere length.

The organization of the cytoskeleton and its relation to the organelles change with development. In the immature cell, the central mass of nuclei and mitochondria appears to prevent the intermediate filaments from linking the myofibrils on one side of the cell to those on the other; the myofibrils appear to be linked as a thin shell around the periphery of the cell. In contrast, in adult cells, mitochondria are interspersed among myofibrils with intermediate filaments connecting myofibrils from one side of the cell to the other. The step-changes at the cell borders and the different relations of the myofibrils to nuclei and mitochondria in the adult myocyte could create mechanical constraints for the sarcomeres different from those in immature cells. Figures 5 and 6 illustrate the marked differences in cell organization between the age groups. Such developmental changes in cell organization are probably determinants for the differences in rest sarcomere length measured in the two age groups.

The cell architecture also can affect active characteristics of contraction waveforms, specifically by conferring internal loads. That a restoring force exists...
within the cell is now accepted.\textsuperscript{52,63} The fact that cells with dissolved membranes can shorten when exposed to calcium and extend when calcium concentration is reduced (Fabriato and Fabriato,\textsuperscript{64,65} and observations by the authors) implies that the restoring force is not primarily due to the membrane structures. The cytoskeleton is not removed by chemical skinning\textsuperscript{66} and would continue to exert its influence on sarcomere length and shortening.

The differences in the organization of the cytoskeleton, described earlier, can affect the relative internal loads of the immature and adult cells. In the immature cell, the relatively large amount of centrally located eton, described earlier, can affect the relative internal loads of the immature and adult cells. In the immature cell, the relatively large amount of centrally located material is augmented by the presence of 2 nuclei, even in the smallest cells. Visual observation of cells under the microscope shows clearly the deformation (axial compression) of the nuclei as the cells contract. Assuming the classic inverse force-velocity relation, a small difference of internal load can result in a relatively large difference in shortening velocity since the slope of the velocity vs. load curve is steep at low loads. Fabriato and Fabriato\textsuperscript{64} measured restoring force in skinned adult cells and found it to be a small fraction of the full contractile force developed by the cell. To our knowledge, restoring forces have not been measured in immature myocytes. If the differences in cellular organization of the immature and adult cells, described above, produce internal loading that is significant and greater in the immature than in the adult, this by itself could result in the smaller amounts and velocities of shortening observed in the immature cell.

Immature cells have longer contraction duration and reextend more slowly than adult cells and exhibit faster restitution (see below) of sarcomere shortening. These differences cannot be accounted for by differences in internal load. The slower reextension and longer duration could result from slower calcium uptake from the cytosol. The faster restitution also suggests that control of calcium may differ between adult and immature myocytes. The presence of potentiation in immature cells indicates that the SR is functioning in controlling calcium (see below).

The JSR and CSR (thought to be analogous to the extended JSR of the bird heart, Dolber and Sommer\textsuperscript{65}) are considered release sites of calcium, important in the modulation of contraction.\textsuperscript{52,55,65} Although they are both found in the area of the Z disk, they have different dispositions relative to the sarcolemma. The JSR, which is more frequently observed in the adult cell, is closely apposed to the sarcolemna or the T tubule membrane, but the CSR, which is prominent in the immature cell, is relatively distant from the sarcolemma.

If the amount of calcium released from the JSR and CSR is modulated by a diffusible substance that comes from the cell membrane, the relative positions of these structures within the cell would be functionally significant. Specific evidence for such a mechanism is provided by Fabriato,\textsuperscript{66} who found that the triggered release of calcium from intracellular stores depended on the rate of increase of the trigger calcium concentration. The rate of increase of cytosolic calcium, following trans-sarcomemmal calcium influx, is slower the greater the distance from the sarcolemma. Consequently, the release of calcium from sites separated from the sarcolemma, i.e., CSR, would be less than that of membrane-associated SR. It may be that the greater amount of JSR relative to the CSR in the adult cell could result in higher levels of activator calcium, which would contribute to a greater amount and velocity of sarcomere shortening in adult cells.

Changes in the pattern of stimulation of mammalian cardiac muscle result in changes in the distribution of calcium in cell compartments\textsuperscript{67} and in characteristic beat-to-beat changes in contractility.\textsuperscript{14,33,68} Two manifestations of such changes are restitution and postextrasystolic potentiation. When adult myocardium is paced at a constant basic rate and an extra contraction is interpolated between two beats, the earlier the extra beat (extrasystole) is elicited in the basic interval, the smaller the contraction. When the extra contraction is elicited later and later in the interval, the contraction increases in magnitude until it equals the preceding contraction at the basic rate. This increase, the restitution of contractility, reflects a change in the availability of activator calcium: cytosolic calcium, as determined from the aequorin luminescence, increases from a very low value in the early extrasystole (immediately following a contraction) to the steady-state value of the contraction at the basic rate.\textsuperscript{18} The beat following the extrasystole is potentiated and the corresponding aequorin signal is larger than normal\textsuperscript{18} as a result of the release of a larger amount of activator calcium from the SR.\textsuperscript{68} Both restitution and postextrasystolic potentiation are independent of load,\textsuperscript{14,15} a property that distinguishes the effects of differences in internal load from differences in calcium availability.

Alteredations in the stimulus pattern were used as a probe of the effects of changes in calcium concentration in isolated myocytes and were used to assess the developmental differences in the availability of activator calcium and its control in the cell. The comparison of the restitution of contractility in immature and adult myocytes demonstrates a significant difference between the two ages, indicating that a difference in calcium availability exists. In the adult myocyte, restitution is gradual, but in the immature, restitution is so rapid that the earliest extrasystole elicited was equal to a steady-state contraction. Maylie\textsuperscript{69} also reported a developmental difference in the restitution: it was much faster in the neonatal cat than in the adult. Since restitution is independent of load, this difference between immature and adult myocytes cannot be explained by a difference in the restoring forces. The presence of postextrasystolic potentiation in the immature myocyte implies that the flat restitution curves obtained in these cells are not a result of the cells being maximally activated (i.e., operating at the peak of their force-pCa curve). The maturational change in restitution is, therefore, not explained by a change in the calcium sensitivity of the myofilaments, but it is related to differences in the membrane systems that deliver and recycle calcium.
In the mammalian ventricular myocardium, intracellular stores play a major role in the uptake and release of activator calcium. The calcium is released in response to a trigger (e.g., calcium, which enters the cell during the action potential). In frog myocardium, in which activator calcium is believed to come from extracellular pools mediated by voltage dependent processes in the sarcolemma, postextrasystolic potentiation and restitution of contractility are not present. In the rabbit myocardium, both restitution and potentiation were strongly affected when calcium from the SR was blocked by ryanodine: restitution became faster and postextrasystolic potentiation was abolished.

If SR in the immature rabbit cells is too undeveloped to participate in such a complex scheme of calcium regulation and if these myocytes were dependent on trans-sarcolemmal influx of activator calcium, the presence of postextrasystolic potentiation in immature cells would not be expected. The fast restitution exhibited by the immature cell, if considered by itself, could be explained by a relatively large dependence on trans-sarcolemmal calcium influx for activation. However, the present finding that the immature myocytes exhibit marked postextrasystolic potentiation comparable in magnitude to that of the adult indicates that compartmentalization of calcium within SR is occurring and that this calcium pool is effective in modulating contractility in the three-week-old cell.

Restitution is not limited to species with T tubules, and it occurs in the cat before the development of T tubules. Thus, the observed differences in restitution at the two ages are probably not a result of differences in the amount or development of the T system.

Differences in SR or the sarcolemma between myocytes at the two ages may result in differences in the time course of restitution. The delivery of calcium to the myofilaments and the route taken in its uptake and return to the release sites may be crucial determinants of the time course of restitution of contractility. Thus, functional differentiation of the LSR from the JSR and CSR may underlie the maturational changes in the restitution of contractility. Present results indicate that the compartments of the CSR and the LSR in the adult cell are distinct and separate structures; the connections between them appear narrow. In contrast, the two structures appear less well demarcated in the immature cells; the connections between them appear relatively wide. Electron-dense material, presumably calquestrin, which fills the CSR profiles, sometimes appears to extend into the LSR of these immature cells.

The presence of the LSR, JSR, and CSR in rabbit hearts at either age would allow the basic process-controlling calcium movement to be qualitatively similar. However, the observed differences in the SR might result in quantitative differences in this process. The rapid restitution of the immature cells is a likely consequence if two conditions are met. First, the amount of activator calcium available for the extrasystole is determined by the time necessary for calcium to be moved from the uptake to the release sites. Second, the lessened structural and functional demarcation between the LSR and CSR of the immature cell shortens the time necessary to move calcium from the uptake site (LSR) to the release sites (CSR and JSR).

The combined functional and structural analysis of the same isolated cell demonstrates that a developmental enhancement in sarcomere shortening occurs. The increase is intrinsic to the cell and is not related to developmental changes in tissue complexity or in extracellular organization of the intact myocardium. This functional increase is associated with changes in cell organization and in the membrane systems that control cytosolic-calcium concentration. These changes in cell organization may produce a relatively greater internal load in the immature cell. Although a greater internal load would result in the smaller amount and velocity of sarcomere shortening observed, it does not explain the lower velocity of sarcomere reextension and the longer duration of the contraction of the immature cell. The maturational changes in restitution of contractility in association with differences in structure of the SR between adult and immature cells suggest that alterations in compartmentalization and in delivery of calcium to the myofilaments may contribute largely to the developmental increase in contractility.

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