

# Uniform Sarcomere Shortening Behavior in Isolated Cardiac Muscle Cells

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**ABSTRACT** We have observed the dynamics of sarcomere shortening and the diffracting action of single, functionally intact, unattached cardiac muscle cells enzymatically isolated from the ventricular tissue of adult rats. Sarcomere length was measured either (*a*) continuously by a light diffraction method or (*b*) by direct inspection of the cell's striated image as recorded on videotape or by cinemicroscopy (120–400 frames/s). At physiological levels of added  $\text{CaCl}_2$  (0.5–2.0 mM), many cells were quiescent (i.e., they did not beat spontaneously) and contracted in response to electrical stimulation ( $\leq 1.0$ -ms pulse width). Sarcomere length in the quiescent, unstimulated cells ( $1.93 \pm 0.10$  [SD]  $\mu\text{m}$ ), at peak shortening ( $1.57 \pm 0.13$   $\mu\text{m}$ ,  $n = 49$ ), and the maximum velocity of sarcomere shortening and relengthening were comparable to previous observations in intact heart muscle preparations. The dispersion of light diffracted by the cell remained narrow, and individual striations remained distinct and laterally well registered throughout the shortening-relengthening cycle. In contrast, appreciable nonuniformity and internal buckling were seen at sarcomere lengths  $< 1.8$   $\mu\text{m}$  when the resting cell, embedded in gelatin, was longitudinally compressed. These results indicate (*a*) that shortening and relengthening is characterized by uniform activation between myofibrils within the cardiac cell and (*b*) that physiologically significant relengthening forces in living heart muscle originate at the level of the cell rather than in extracellular connections. First-order diffracted light intensity, extremely variable during sarcomere shortening, was always greatest during midrelaxation preceding the onset of a very slow and uniform phase of sarcomere relengthening.

## INTRODUCTION

Application of light diffraction methods has provided some direct information about the dynamics of shortening at the level of the sarcomere in living heart muscle (Pollack and Krueger, 1976). However, the pattern of light diffracted from an isolated heart muscle preparation is not very sharp, and the diffraction method—likely more sensitive to striations that are well ordered—cannot reveal the behavior of individual sarcomeres. For these reasons our knowledge of the microscopic contractile behavior of the sarcomere in living heart muscle is imprecise. Moreover, it is uncertain (*a*) whether the maximum extent of

shortening previously seen in intact muscle is affected by the abundant extracellular tissue and interconnections in cardiac tissue or (b) whether shortening is limited by a phase of nonuniform activation within the cell, as is evident in skeletal muscle fibers (Taylor and Rüdell, 1970). As the smallest unit of the heart in which the physiological pathways of activation and control are present, the isolated myocyte constitutes an ideal model by which to assess the microscopic basis of cardiac contraction.

We describe here the microscopic nature of activation and sarcomere shortening in isolated cardiac muscle cells. Some results of this study have appeared in abstract form (Krueger and Wittenberg, 1978).

#### METHODS

##### *Preparation of Isolated Cardiac Muscle Cells*

Ventricular cells were isolated from hearts of adult, male Wistar rats (250–300 g) using a modification of perfusion methods first developed by Berry et al. (1970). The rats were decapitated, the heart was quickly excised, and retrograde perfusion of the coronary vessels was established through the aortic stump. The perfusion fluid, a calcium-free Krebs-Ringer solution fortified with 14 mM glucose and modified by replacement of phosphate buffer with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffer (HEPES-Ringer), was gassed with 100% O<sub>2</sub>. After all of the blood was washed out, 0.1–0.2% collagenase (Worthington Biochemical Corp., Freehold, N. J.) was introduced into the perfusion fluid, and the perfusate was recirculated to conserve enzyme. After 30 min, the ventricles were removed from the perfusion apparatus, minced with scissors in fresh HEPES-Ringer containing 0.05–0.1% collagenase supplemented with a minimal concentration (0.001%) of trypsin (Difco Laboratories, Detroit, Mich.), and incubated with gentle shaking for 30 min. Cells were harvested from the supernate by centrifugation at very low speeds for 2 min. The incubation was repeated three times, and the cells were combined, washed repeatedly, and resuspended in HEPES-Ringer. At this stage  $\sim 4\text{--}6 \times 10^6$  cells were obtained, of which up to 70% were visibly damaged. In the most successful preparations,  $\sim 50\%$  of the cells retained their normal morphology and contracted in response to electrical stimulation when suspended in HEPES-Ringer supplemented with 1–2 mM CaCl<sub>2</sub>.

At this stage of the purification procedure individual cells were selected for the experiments described here. Cells were stored on ice until used, but during the experimental observations, they were at room temperature (23–26°C).

Preparations strongly enriched in morphologically intact cells and free of subcellular debris may be prepared by centrifugation through metrizamide solutions. The biochemical properties of a population of cells prepared in this manner have been described (Wittenberg, 1979). Their respiration is tightly coupled to oxidative phosphorylation; intracellular myoglobin is retained. These properties indicate that mitochondria are functional and that sarcolemmal barriers are intact. We are presently optimizing conditions in this preparation to obtain a maximum yield of physiologically intact cells. A full report is in preparation.

##### *Sarcomere Length and Light Intensity Measurement*

Sarcomere length was measured by light diffraction. The diffraction patterns of the isolated cells and their respective morphological appearances were obtained with a binocular microscope fitted with a third tube as a pathway to the optical instrumen-

tation. The physical arrangement of the laser, microscope, and optical sensors appears elsewhere (Krueger and Strobeck, 1978). The features essential to the present study are described here.

The normal substage condenser lens of the microscope was replaced by a  $\times 4$ , NA 0.12, objective lens which focused the light beam from a continuous wave helium neon laser (632.8 nm wave length; 5 mW; Hughes Aircraft, Electrodynamics Div., Carlsbad, Calif.) onto the isolated cells. This lens reduced the illuminated field diameter at the specimen plane, thereby eliminating scatter from neighboring cells and increasing the effective light energy passing through a single cell. Very little diffracting material is present in an isolated cell (perhaps 1/200th the amount illuminated in an intact muscle preparation), and so the accompanying increases in diffracted light intensity markedly improved the signal-to-noise ratio of the optical sensors. In a typical experiment, the illuminated field was  $\sim 100 \mu\text{m}$  in diameter at the specimen plane. Most of the illumination was contained in a  $10^\circ$  cone so that the angle of light incident upon the cell varied by  $\pm 5^\circ$ .

Light diffracted by the cell was collected with a  $\times 50$ , NA 1.0, salt water immersion objective (Ernst Leitz GmbH, Wetzlar, W. Germany). The diffraction pattern at the rear focal plane of the objective and substage lens combination was projected onto two co-aligned optical sensors situated above the microscope. A cylindrical lens reduced the equatorial spread of the diffraction pattern on each device. The aperture of the objective lens was sufficient to collect most, if not all, of the equatorial light in each first order. In the former situation, the amount missed was small compared with the observed intensity changes.

The light intensity profile of the first-order diffraction pattern was visualized by displaying the video output of the first device, a linear array of 256 photodiodes (Reticon Corp., Sunnyvale, Calif., model LC100) on a storage oscilloscope. The photodiode array was scanned at 1 kHz, resulting in a temporal resolution of 1 ms for the video information. The display of the array's video output corresponded to the light intensity distribution across the face of the second sensor, a Schottky barrier diode. This second device, a lateral effect photodiode (United Detector Technology Inc., Santa Monica, Calif., model SC25), continuously monitored the position and intensity of the first-order diffraction pattern. First-order total intensity was measured from the sum of the cathode photocurrents at either side of the barrier diode. The contribution from zero-order scattering was blocked out, as judged from the display of the intensity profiles during contraction. Changes in first-order intensity were confirmed by determination of the area under the video profiles.

Sarcomere length was computed electronically using circuit techniques described by Iwazumi and Pollack (1979). Sarcomere length was continuously computed from the first-order light's centroidal position on the barrier diode. In later experiments, sarcomere length and diffracted light dispersion were computed every millisecond directly from the video signal of the first-order intensity profile. The position of the median (i.e., the 50th percentile of light contained in the first order) was converted to sarcomere length, and the dispersion (i.e., the breadth of the first order that included 50% of the light) was expressed relative to equivalent differences in sarcomere length. In three experiments, sarcomere length was computed from both first orders simultaneously using a second photodiode array (Reticon 256 CCPD) and computational circuit.

Sarcomere length in our system is inversely proportional to the separation,  $D$ , between the zero- and first-order diffraction patterns (see Fig. 1 *B*). This separation is proportional to the wave length and the effective focal length of the optical system (Goodman, 1968). The presence of the substage lens introduces another variable

affecting  $D$ , namely, the distance between the specimen and the collecting lens (Goodman, 1968). This distance remained fixed as long as the cells remained in focus during the experimental observations.

The optical system was initially calibrated with commercially available diffraction gratings (15,000, 7,500, and 2,500 lines/inch; Edmund Scientific Co., Barrington, N. J.). Thereafter, and often during the experiment, the sarcomere length system computation was checked indirectly by inserting a photographic replica of a Ronchi Ruling (250 and 300 lines/inch; Edmund Scientific) contact printed on Kodalith type 3 film (Eastman Kodak Co., Rochester, N. Y.) into the optical path at the slider located beyond the rear focal plane of the objective lens. The positions of the slider grating's diffracted spectra were assigned values corresponding to first-order diffraction patterns of gratings located at the specimen plane of the optical system.

In other cases, the microscopic appearance of the cells was inspected directly using an inverted microscope (Leitz Diavert). Several different forms of microscopy were utilized, as described in Results. The image of the cells was recorded on videotape (Cohu Inc., Electronics Div., San Diego, Calif., model 4400 SIT Camera; International Video Corp., Sunnyvale, Calif., model 760 1-inch videotape recorder; stroboscopic illumination, Chadwick-Helmuth Co., Inc., Monrovia, Calif., model 136) or cinematographically (Redlake Corp., Photo Instrument Div., Campbell, Calif., Locam 51 Camera, 120–400 frames/s) on 16-mm high-speed movie film (Eastman Kodak 4XR). The appearance of the cell and sarcomere length during contraction were then analyzed by stop-motion replay of the previously recorded data. Videotaped information was analyzed directly with a videoanalyzer (Colorado Video, Inc., Boulder, Colo., model 321). The 16-mm film was viewed with a Redlake analyzer (model 9006).

#### *Accuracy and Precision of Sarcomere Length Measurements*

In cells that gave the brightest diffraction patterns (generally wider specimens), a peak-to-peak noise of  $<0.01 \mu\text{m}$  was achieved: this value approximates a 200:1 signal-to-noise ratio. The first-order intensity change during contraction was estimated, in the worst case, to decrease the signal-to-noise ratio from twofold to threefold. The signal-to-noise ratio and the frequency response of the sarcomere length computation with the lateral effect photodiode was limited by the performance of the analogue divider used to normalize the first-order positive voltage by intensity changes. The manufacturer specifies that the device used (Analog Devices Inc., Norwood, Mass., model 436B) has a 0.3–0.5% accuracy at 30 kHz.

Because the cell was unattached, the possibility exists that its horizontal orientation might have changed during contraction. During the diffraction measurements, the image of the cell was observed with a  $\times 50$ , NA 1.0, objective and a  $\times 5$  eyepiece. This lens combination yields an estimated depth of focus of  $6 \mu\text{m}$  (Determan and Lepusch). Thus, an undetectable change in horizontal attitude,  $\alpha$ , could not exceed  $4^\circ$  for a cell  $100 \mu\text{m}$  long. This would decrease the sarcomere length measured in the image by a factor  $(1 - \cos \alpha)$  or 0.2% (for a cell  $50 \mu\text{m}$  long,  $1 - \cos \alpha = 0.8\%$ ). A  $5^\circ$  variation in the angle of light incident upon a simple grating of  $2\text{-}\mu\text{m}$  spacing can be shown to affect the accuracy of diffraction measurements by  $<2\%$  (Born and Wolf, 1970).

The precision of sarcomere length change measurement is affected ultimately by the dispersion of first-order light. The narrowest dispersion (i.e., width at half amplitude) in first-order light diffracted from cells in these studies was equivalent to 4% of sarcomere length; the broadest, 12%. These values undoubtedly include scattering from lens elements and broadening resulting from finite beam diameter

and the number of striations in a cell. Illumination of a uniform grating gave dispersion values ranging from 3 to 8%, depending on the number of striations included in the field (100 to 12). However, in our system, sarcomere length changes of  $0.007\ \mu\text{m}$  could be resolved during oscillatory length changes in an intact heart muscle preparation, where the dispersion of the first-order light is typically twice as large as the maximum ever seen in light diffracted from an isolated cell. For these reasons, it does not appear that cell-to-cell variations in first-order dispersion appreciably affect the precision of the sarcomere length measurement.

In practice, the principal limitation in the precision of the sarcomere length computation appeared to be contractile heterogeneities that fragment the first order and decrease its intensity. As long as the diffraction patterns remained sharp, we estimate that changes in sarcomere length may be followed to  $\pm 0.01\ \mu\text{m}$ .

#### EXPERIMENTAL PROTOCOL

##### *Observation of Cells and Initial Sarcomere Length Determination*

Approximately 2 ml of the experimental solution was placed on a microscope slide (composition [mM]: NaCl, 120; NaHCO<sub>3</sub>, 4.3; KCl, 5.8; MgSO<sub>4</sub>, 1.5; KH<sub>2</sub>PO<sub>4</sub>, 1.4; glucose, 14.1; CaCl<sub>2</sub>·2 H<sub>2</sub>O, 0.5–2.0; HEPES buffer, pH 7.3). A ring drawn with a wax pencil created a barrier of sufficient surface tension to retain the experimental solution. One drop of the calcium-free cell suspension ( $\sim 0.05\ \text{ml}$ , containing  $10\text{--}30 \times 10^3$  cells) was then gently mixed with the pool so that the cells settled evenly to the bottom of the slide. Intact cells did not stick to the glass because they would float away with the slightest mechanical disturbance. The image of the cells was projected through one eyepiece of the binocular microscope onto an observation screen. A centering telescope located in the other eyepiece projected the diffraction pattern of the cell onto a graph precalibrated to convert directly the separation of the first-order spectra into sarcomere length. This value was then recorded and used to align the optical instrumentation.

##### *Stimulation and Orientation of the Cells*

When a cell of appropriate dimension ( $\sim 20\ \mu\text{m}$  wide and  $100\ \mu\text{m}$  long) and shape (absence of side branches) was located, it was stimulated by two platinum wires (0.5 mm in diameter) spaced 1 cm apart on either side of the microscope field. Electrical stimuli 0.1–1 ms in duration and 8–15 V in amplitude were provided by a Grass S-44 stimulator. The small size of the cell and the proximity of the metallic microscope objective make the amount of current flowing through the cells difficult to determine, but the voltages employed were about twice the level we have used previously (Krueger and Strobeck, 1978) for comparable stimulation of the intact muscle; total stimulus current was  $\sim 1\ \text{mA}$ .

Diffraction patterns from specimens that contracted uniformly in response to electrical stimulation were then oriented with respect to the optical instrumentation with a dove prism situated in the microscope's optical path. With the videoscans of the diode array, the cell's first-order diffraction pattern was then positioned to correspond to the sarcomere length measured directly from the overhead graph. Sarcomere length, as indicated by a digital voltmeter, then corresponded to within  $\pm 0.03\ \mu\text{m}$  to the value measured directly.

Once aligned, the cells were stimulated at rates from 0.4–2.0 Hz. The sequence of sarcomere length and intensity changes and the videoscans of the diffraction patterns were displayed on a storage oscilloscope and recorded photographically. All data was analyzed at a later date.

## RESULTS

*Appearance, Behavior, and Selection of Calcium-tolerant Cells*

Enzymatic digestion of rat ventricular tissue produced a large number of rectangular cells exhibiting clear cross striations. When suspended in Ringer's solution with 0.5–2.0 mM  $\text{CaCl}_2$ , 50–90% of the cells beat rapidly, vigorously, and spontaneously. This behavior, well described by others, including Vahouny et al. (1970), Bloom (1970), Fabiato and Fabiato (1972), and Reiser et al. (1979) is characterized by focal shortening of 5–6 sarcomeres, the locus of which propagates along the length of the cell. However, a substantial number of isolated cells did not beat spontaneously in the presence of external calcium. These cells remained quiescent and retained the sharp striated pattern seen in cells bathed in calcium-free media. It is the nonbeating, quiescent cells that were selected for the present study, and it is these that we consider to be "calcium tolerant." The proportion of calcium-tolerant myocytes, though extremely variable, was up to 50% of the cell population with some isolation procedures.

The phase-contrast appearance of a quiescent calcium-tolerant cell whose morphology is well suited for this study is illustrated in Fig. 1 *A*. The shape of the isolated cells was quite variable and limited the number of specimens selected for the diffraction measurements. For this reason we did not systematically quantify cell size in the cell population; however, photomicrographic analysis of 32 representative specimens revealed that the average cell length was  $115 \mu\text{m}$  ( $\pm 21.6$  [SD]  $\mu\text{m}$ , range 77–165  $\mu\text{m}$ ) and the cell width was  $22 \mu\text{m}$  ( $\pm 8.1$  [SD]  $\mu\text{m}$ , range 11–30  $\mu\text{m}$ ). The cells tumble during settling from suspension or when disturbed by small fluid currents. In such cases, cells appeared elliptical in cross section, with an estimated thickness of 10  $\mu\text{m}$ . The average sarcomere length in 97 rodlike quiescent cells, measured 15–45 min after the cells had been placed on the microscope slide, was  $1.93 \pm 0.10$  (SD)  $\mu\text{m}$  (range, 1.83–2.09  $\mu\text{m}$ , selected on a basis of resting sarcomere length  $>1.8 \mu\text{m}$ ). In two preparations, cells with sarcomere lengths of 2.2–2.3  $\mu\text{m}$  were seen, but these cells were not included in the measurements because the cells were inexcitable. There was no detectable relationship between sarcomere length in the unstimulated cells and the amount of  $\text{CaCl}_2$  (0.5–2.0 mM) added to the HEPES-Ringer solution.

The appearance of a light-diffraction pattern obtained from a cell placed in the experimental system is shown in Fig. 1 *B*. The pattern's speckled appearance remained stationary, an indication that microscale movement (or dithering) of sarcomeres did not occur during the 10-s photographic exposure. The diffraction patterns were sharp, parallel, and equally spaced, as expected for a Fraunhofer interference pattern located at the rear focal plane of the objective lens. The individual orders were straight with little evidence of astigmatism. Intensity appeared to be symmetrically distributed on both sides of the diffraction pattern, although this was not always the case. Low-angle scattering from extracellular debris makes the undiffracted zero order appear broader than the respective first orders. Often, fine substructure could be seen in the first order, but this was present in the zero order as well and so cannot

be unequivocally attributed to discrete sarcomere length populations. Increasing the refractive index of the bathing media (composition: Hill, 1977) reduced zero-order scatter and substructure, and so the phenomena cannot result solely from the cell's internal structure.

*Appearance of Calcium-tolerant Cells During Contractile Activity*

Isolated cells appear to contract uniformly in response to electrical stimulus pulses as short as 0.1 ms. Direct inspection revealed that in most cases the cells shortened and elongated from both ends, a process that occurred without

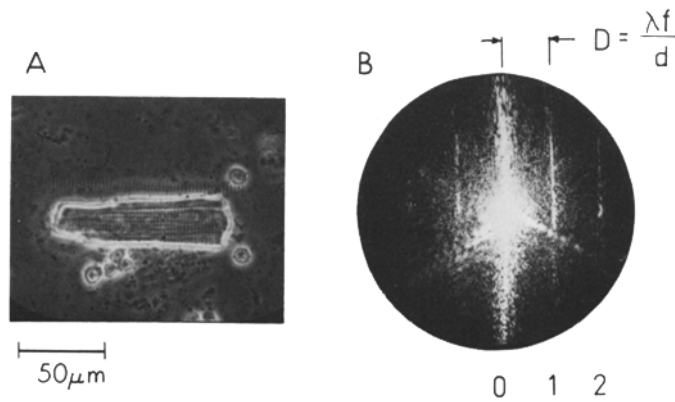


FIGURE 1. Optical appearance of isolated heart cells. (A) Phase-contrast microscopic appearance of rat ventricular myocyte whose size and shape are suitable for diffraction studies. Average sarcomere length, 2.0 μm; 1.0 mM CaCl<sub>2</sub>-Ringer's solution. (B) Light-diffraction pattern from another cell. The first-order diffracted light is sharper than the zero-order (undiffracted) light; the low-angle, diffuse scattering originates in the lens system and surrounding debris, and the 10 to 4 o'clock cross is edge diffraction from an optical aperture in the system. The first order often appears segmented, as does the zero order. The separation between each order,  $D$ , is inversely proportional to sarcomere length,  $d$ , and directly proportional to wavelength,  $\lambda$ , and the focal length,  $f$ , of the system. SL = 1.95 μm.

twisting or reorientation. Low power observation of large fields of cells demonstrated a wide variation in cellular excitability. Increase in either stimulus strength or duration caused more cells to contract in a microscope field. Once stimulus threshold was reached, a previously quiescent cell then shortened in synchrony with its more excitable neighbors. Occasionally, cells that beat spontaneously at very low frequencies (1-2 beats/min) could be stimulated to contract uniformly, especially when stimulus duration was markedly prolonged (i.e., >10 ms). However, the spontaneously beating cells did not respond in a reproducible fashion to electrical stimulation.

Sarcomeres appeared to shorten synchronously and uniformly within electrically excitable cells. Fig. 2 shows the striated pattern of a cell at rest and at the peak of shortening as recorded by cinemicroscopy. It was possible to follow

one striation throughout the complete contraction-relaxation cycle. Despite shortening to sarcomere lengths less than the length of the thick filament, there were no visible contraction bands or change in the appearance of the striations with this form of microscopy (modulation contrast [Hoffman and

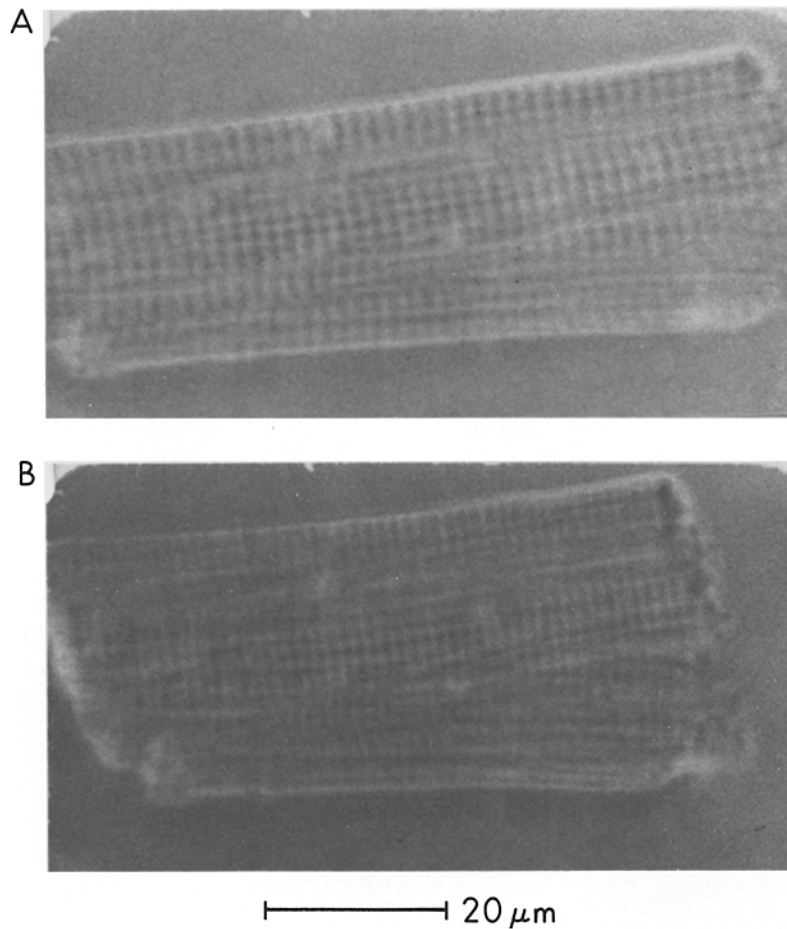


FIGURE 2. Microscopic appearance of cells during contraction. The modulation-contrast image of the cell at rest (*A*) and at peak shortening (*B*) is shown from selected frames of 16-mm film. Individual striations remain distinct and can be followed throughout the full contraction-relaxation sequence. The striated pattern remains sharp and laterally well registered, and the striation spacing always appears uniform. Average sarcomere length in *A*, 1.98  $\mu\text{m}$ ; in *B*, 1.47  $\mu\text{m}$ . 1.0 mM  $\text{CaCl}_2$ -Ringer's solution.

Gross, 1975]), in which the gradients of refractive index are directly visualized. The striations remained well registered laterally, and there was little, if any, sign of heterogeneity of striation spacing within the cell. We could not detect buckling of sarcomeres at any time in the shortening-relengthening cycle.



*Appearance of the Cardiac Cell in Passive Shortening*

The observation that cells shorten uniformly does not, by itself, resolve whether all of the sarcomeres shorten actively. This uncertainty results from the possibility that the lateral contiguity of cardiac myofibrils (Fawcett and McNutt, 1969) might inhibit buckling of the inactive elements passively shortened by active neighbors, a situation that would mask the effects of nonuniform activation were it to occur. Therefore, the effects of shortening the resting cell upon its striated pattern was investigated.

Fig. 3 shows the microscopic appearance of an isolated cell that was suspended in a gelatin-Ringer's solution (5 g gelatin [Knox Gelatin, Inc.,

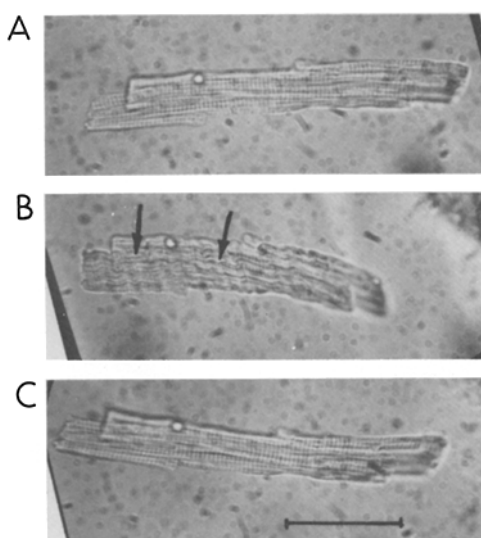


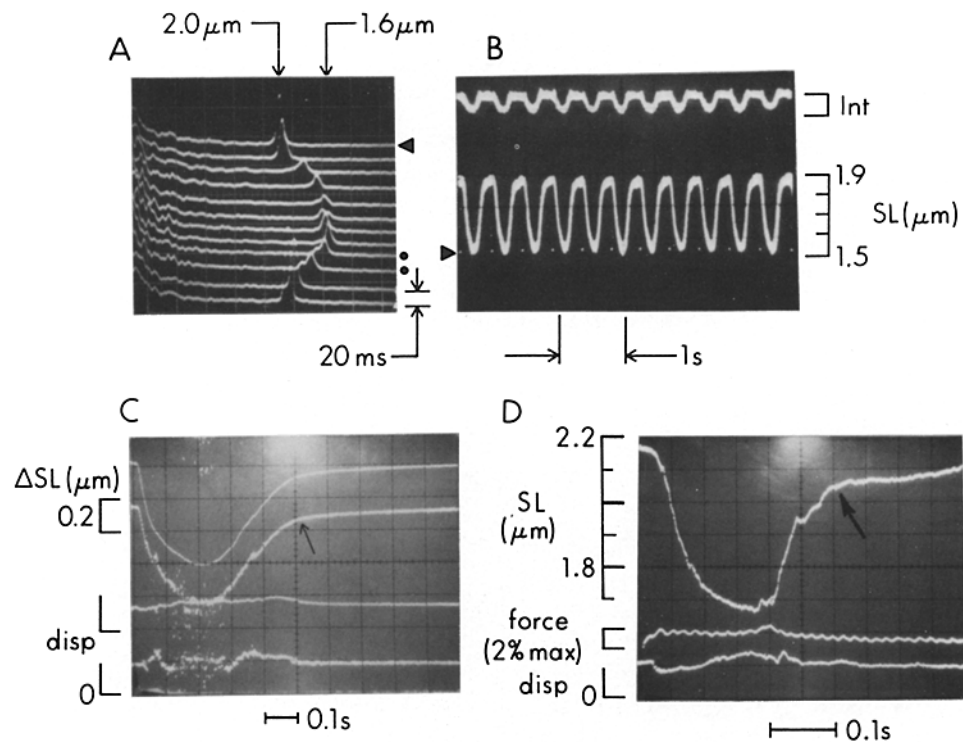
FIGURE 3. Appearance of cell during passive shortening. The microscopic appearance of an isolated cardiac muscle cell suspended in a solidified gelatin-Ringer's solution is shown (A) at rest length, (B) after compression along the cell's long axis, and (C) after stretch back to rest length. Pronounced buckling occurs with passive shortening (arrows in B) but is clearly absent in actively shortening cells. Sarcomere length (in  $\mu\text{m}$ ): A, 1.92; B, 1.44, 1.58, and 1.71 in different regions of the cell; C, 1.95.

Englewood Cliffs, N. J.] per 100 ml calcium-free Ringer's solution [Gonzalez-Serratos, 1971]). A cell in the gelled solution is shown at rest length (Fig. 3 A) and after it was compressed longitudinally by means of a micropipette, the tip of which is just visible in the micrograph (Fig. 3 B). It was difficult to maintain the alignment of isolated cells during this procedure, but the internal aspects of the compressed cells appeared noticeably buckled by passive shortening. There was appreciable nonuniformity of sarcomere length in the compressed cells: sarcomere lengths of 1.44 and 1.71  $\mu\text{m}$ , averaged over fields of ten striations, can be seen in the cell shown in Fig. 3 B. These changes were completely reversible upon relengthening the cells, as shown in Fig. 3 C. Cells

embedded in gelatin shortened in response to an electrical stimulus after superfusion with Ringer's solution containing 1.0–2.0 mM  $\text{CaCl}_2$ . The striations within the buckled cells straightened and then rebuckled during relaxation. These observations indicate that the components of cardiac cells appreciably resist shortening and cause the sarcomeres to buckle when inactive.

#### *Diffraction Analysis of Sarcomere Motion*

The limit of resolution (estimated to be  $0.45 \mu\text{m}$ ) with the lens combination ( $\times 40$ , NA 0.75, Zeiss water immersion objective [Carl Zeiss, Inc., New York]; 0.90 condenser cap) we used to inspect the image of the cell and the grain of the cinefilm made it difficult to conclude that the onset of sarcomere shortening was truly synchronous and contraction uniform within the cell. Therefore, the pattern of light diffracted by the cells was observed to further evaluate the response to electrical stimulation. Inspection of the diffraction pattern indicated that the first orders remained straight and parallel during contraction. The intensity profiles of the first-order diffracted light was displayed at precise intervals after the stimulus pulse, as shown in Fig. 4 A. Significantly, the breadth of the first-order diffracted light remained relatively narrow throughout the sarcomere shortening. The diffraction method provided a convenient way of determining the precise nature of shortening within any given cell: the appearance of the first order often distinguished cells in



which activation was synchronous, but there were slight, discrete nonuniformities in sarcomere length during shortening, as can be seen upon close inspection of Fig. 4 *A*. The slight differences shown (equivalent to differences of  $<0.1 \mu\text{m}$ ) would be difficult to resolve in even the most ideal of microscopic images.

Analysis by the diffraction method revealed that many individual cells responded to stimulation in a reproducible fashion for many contractions. Fig. 4 *B* illustrates the temporal consistency of sarcomere shortening in response to repeated stimulation at 2 Hz, the highest frequency used in these studies. Five traces of the oscilloscope encompassing 60 consecutive contractions are superimposed. The time-course and extent of sarcomere shortening—as well as the first order intensity changes—are indistinguishable over this sample period. This cell responded more than 700 times with reproducible contractile behavior, but more typically cells contracted in this fashion 100–300 times. (The cells tended to become inexcitable at the end of this period, with little alteration in their contractile response.)

The changes in sarcomere length and first-order light dispersion, recorded from a cell contracting in gelatin, are shown by a slower time base in Fig. 4 *C*.

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FIGURE 4 (*opposite*). Behavior of the diffraction pattern during contraction in electrically excitable cells. (*A*) The appearance of the first-order light intensity distribution as sampled throughout contraction, starting with the stimulus pulse (wedge at top scan of display). Sarcomere shortening is indicated by a shift to the right in the first-order position. The diffracted light remains sharp and its breadth narrow, an indication of synchronous contractile behavior. Occasionally, small subpeaks can be resolved with the first order (refer to scans 9 and 10, dots), an indication of distinct sarcomere populations at slightly different length. First-order intensity is higher during relaxation, as seen by comparing the amplitude (i.e., 3 div.) of the last scan with that of the top scan (2 div.). (*B*) Reproducibility of the contractile response. The sequence of sarcomere length (SL) and intensity (Int) changes are shown for an isolated cell. Five consecutive traces are superimposed after a period of 300–400 contractions. The first-order intensity calibration equals a 40% change. The wedge indicates the stimulus pulse. 2.0 mM  $\text{CaCl}_2$ -Ringer's solution. (*C*) Sarcomere dynamics during contraction. The onset of shortening is abrupt, and the initial velocity of shortening is near the peak value reached in contraction. The arrow denotes the onset of a very slow and uniform phase of relengthening. Sarcomere length (SL) and first-order dispersion (disp), calculated simultaneously from each first order, agree well. (The small perturbations seen in the lower length and dispersion records are an artifact of the narrow width of the sensor elements in the second photodiode array.) Cell embedded in gelatin and suffused with 2.0 mM  $\text{CaCl}_2$ -Ringer's solution. Initial sarcomere length,  $2.1 \mu\text{m}$ ; peak velocity of sarcomere shortening,  $10 \mu\text{m/s}$ . (*D*) Sarcomere shortening in intact heart muscle. The time-course of sarcomere shortening in the cells resembles that seen in muscle, shown here for comparison. The arrow denotes the onset of slow relengthening, during which the diffraction pattern remains sharp. 1.9 mM  $\text{CaCl}_2$ -Ringer's solution; temperature,  $28^\circ\text{C}$ ; peak velocity of sarcomere shortening,  $10 \mu\text{m/s}$  (methods described elsewhere [Krueger and Strobeck, 1978]).

In three experiments, sarcomere length and dispersion were computed from both first orders simultaneously with separate circuits and photodiode arrays as another test of contractile uniformity. Although differences in the sensor geometries (i.e., width of the photodiode array) made it difficult to match the signal-to-noise ratio, the overall behavior of the respective first orders appeared similar. The time-course of sarcomere shortening in the isolated cells resembled that recorded from nonenzymatically treated, intact cardiac muscle preparations shown here for the purpose of comparison in Fig. 4 *D*. (Intact papillary muscle preparations were isolated from the right ventricles of rats in the course of experiments described elsewhere [Krueger and Strobeck, 1978; Krueger and Farber, 1980].)

The relative sharpness of the first-order diffraction pattern and the similarity of the respective first orders' behavior in electrically excitable cells contrasted markedly with that seen during nonuniform contractile activity in spontaneously beating cells. Fig. 5 *A* illustrates the representative behavior of first-order light during a spontaneous beat characterized by focal, nonsynchronized sarcomere shortening within the cell. A spontaneous beat invariably gave rise to broadening and fragmentation of the first-order diffracted light and, often, an alteration in low-angle scattering associated with the zero order. The sarcomere shortening signal computed from the diffraction pattern was not reproducible in consecutive beats, and its shape and time-course differed from that shortening and lengthening sequence observed in the electrically excitable cells. Simultaneous observation of the first-order position on each side of the diffraction pattern demonstrated that the respective sarcomere length computations did not correlate well during a beat (Fig. 5 *B*). Occasionally, a period of apparent lengthening—not present in the other order's computed displacement—was seen (Fig. 5 *B*, arrow). However, when the laser spot was reduced to illuminate only a small region within the spontaneously beating cell, the shape and position of zero order could be seen to change. Despite the small size of the single cell, appreciable gradients of refractive index must exist during nonuniform contractile activity. Zero-order motion was not seen during uniform shortening in electrically excitable cells.

#### *Dynamics of Myofilament Sliding in Isolated Cells*

As long as the first-order diffraction pattern remained sharp, the fine details of sarcomere length changes could be easily measured, as shown in Figs. 4 and 6. The onset of sarcomere shortening was abrupt after a brief latent period. The initial velocity of sarcomere shortening is near the maximal value reached in contraction and often remained constant for an appreciable part of shortening. The sequence of shortening is smooth and uninterrupted, although some exceptions were recorded, as described in the Discussion. In all cells, relaxation was characterized by rapid and very slow phases of sarcomere lengthening. Comparable sarcomere dynamics were recorded upon direct inspection of the videotaped or cinemicroscopically recorded appearance of the cells. The transition between these phases was occasionally quite distinct (Fig. 6 *A*), and in no case could both phases of sarcomere relengthening be represented by a single exponential function. The first-order diffraction pattern appeared very

sharp, and the first-order dispersion remained low during the slow phase of relengthening (see the last two video scans in Fig. 4 *A* and dispersion in Fig. 4 *C*). If the interval between the stimuli was decreased so that ensuing contractions encroached on the period of slow relengthening, sarcomere length at peak shortening was reduced by an amount nearly equal to the difference between the true length at rest and that occurring at the moment of stimulation.

The peak velocity and the extent of sarcomere shortening were always maximal upon the first contraction after a resting period. The extent of sarcomere shortening and the velocity of shortening at any sarcomere length

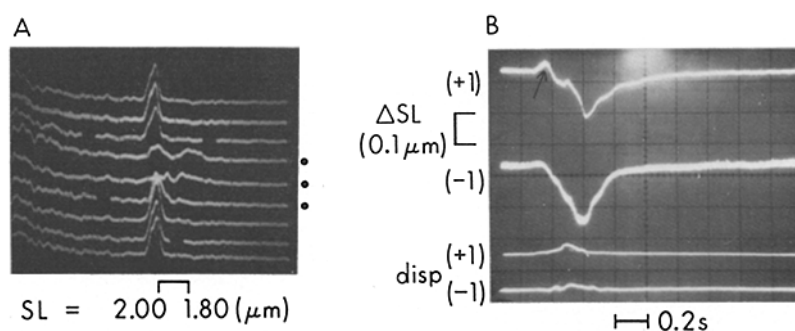


FIGURE 5. Behavior of the light diffracted from spontaneously beating cells. (A) Appearance of the first-order diffracted light intensity profile, sampled every 0.15 s. Appreciable fragmentation of the first order always accompanied by a spontaneous beat, one which here occurs between the fourth and sixth scans (dots). SL denotes the sarcomere length corresponding to the position of first order. (B) Position of each first order as computed during nonuniform and nonsynchronized contractile activity. The shape of the computed displacement ( $\Delta SL$ ) of each ( $\pm 1$ ) first order bears little resemblance to the sarcomere shortening and relengthening sequence observed in electrically excitable cells (cf. Fig. 4 *C*). The computed displacements of the respective first orders, measured simultaneously, were never well correlated. Arrow denotes a period of apparent lengthening of sarcomeres not present in the simultaneously observed behavior of the opposite first order.

then diminished to a steady level within three to four consecutive contractions. Comparable results were seen in bathing solutions containing 0.5–2.0 mM calcium chloride, as shown in Fig. 6 *C* and *D*. With steady contractile activity, the extent of sarcomere shortening was equivalent to values reported for intact heart muscle (Pollack and Krueger, 1976). For example, the average sarcomere length at peak shortening for 49 cells studied at various calcium levels was  $1.57 \pm 0.13$  (SD)  $\mu\text{m}$ . The level of calcium in the bathing medium seemed to have little effect on sarcomere length at the peak of contractile activity. The respective values of sarcomere length, standard deviations, and numbers of cells observed for solutions containing 0.5, 1.0, and 2.0 mM  $\text{CaCl}_2$  were  $1.57 \pm 0.15$  ( $n = 13$ ),  $1.56 \pm 0.10$  ( $n = 28$ ), and  $1.59 \pm 0.19$  ( $n = 9$ ).

To evaluate the level of contractile activation in the isolated cells, we

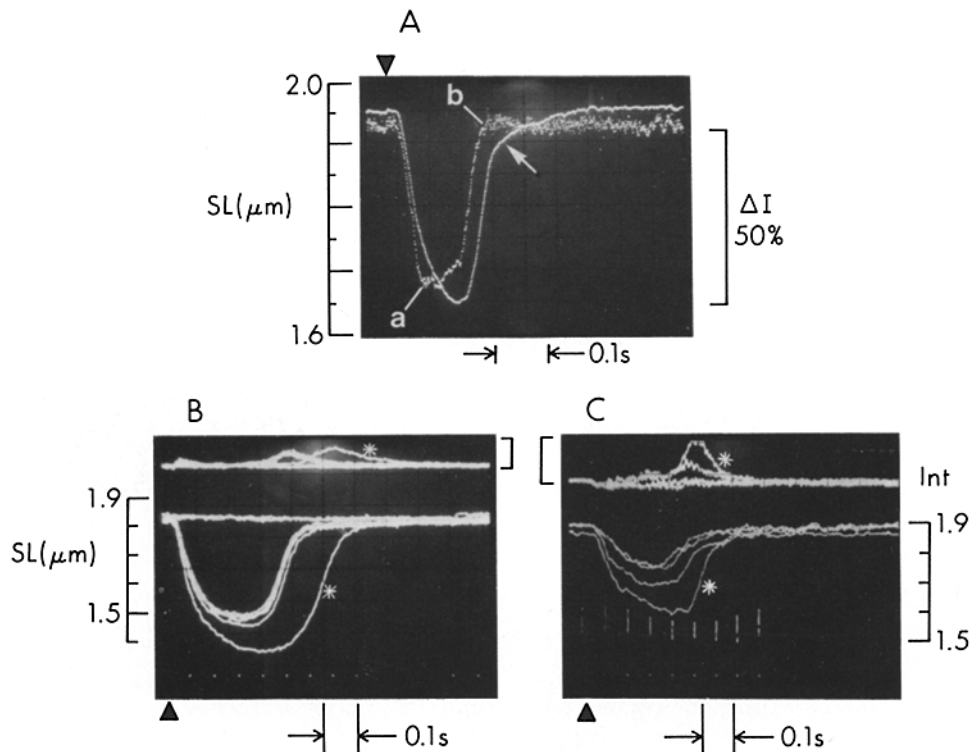


FIGURE 6. Shortening-dependent variations in first-order diffracted light intensity during relaxation. (A) First-order intensity changes ( $\Delta I$ ) typically seen during shortening and relengthening. The average of 10 contractions is shown for an unattached cell bathed in 1.0 mM  $\text{CaCl}_2$ -Ringer's solution. At any sarcomere length (SL), first-order intensity is greater during relaxation. (At  $\text{SL} = 1.75 \mu\text{m}$ , intensity has decreased by 50% [a], whereas it has returned to the initial value during relengthening [b].) Arrow denotes the slow phase of relengthening. (B and C) The sequence of SL and first-order intensity (Int) changes are shown for cells bathed in (B) 2.0 mM and (C) 0.5 mM  $\text{CaCl}_2$ -Ringer's solution. The adjustment of the contractile response to the onset of regular stimulation after a resting period is shown. The first response is denoted in each panel by an asterisk, and in B no optical changes are seen in response to the first subthreshold stimulus. Peak first-order intensity precedes the onset of the slow phase of relaxation, and its amplitude appears to be directly related to the amount of previous sarcomere shortening. The velocity of early shortening appears to mirror the level of contractile activity as determined by peak shortening. Intensity calibration, 50%; stimulus rate in B, 0.8 Hz; in C, 0.5 Hz. The wedge indicates the stimulus pulse.

measured the velocity of shortening at selected sarcomere lengths. Velocity was estimated by eye from the slope of the sarcomere shortening sequences. At a sarcomere length of  $1.8 \mu\text{m}$  the average speed of sarcomere shortening for cells bathed in Ringer's solution containing 1.0 mM  $\text{CaCl}_2$  was  $9.07 \pm 3.74 \mu\text{m/s}$ . (For the sake of comparison, the velocity of sarcomere shortening at this

length during an unloaded contraction of near maximally activated intact muscle is  $4.3 \mu\text{m/s}$ , with a standard deviation of  $\sim 1.5 \mu\text{m/s}$  (values estimated from Fig. 9, Pollack and Krueger [1976]). Despite the considerable scatter in the length-dependent behavior of absolute shortening velocity among cells sampled on different days (partly a reflection of variations in room temperature), it was clear that the maximum speed of sarcomere shortening is not impaired relative to nonenzymatically treated intact muscle; if anything, it was greater. In many cells, sarcomere shortening speed remained relatively constant over a sarcomere length range of  $2.0\text{--}1.7 \mu\text{m}$  (see Figs. 4 *C* and 6 *A* and *B*). Shortening speed appeared to be significantly reduced for the cells bathed in Ringer's solution containing  $0.5 \text{ mM CaCl}_2$ , for the average velocity of shortening was  $3.67 \pm 1.47 \mu\text{m/s}$  at a sarcomere length of  $1.8 \mu\text{m}$ .

#### *Diffracted Light Intensity Changes During Relaxation*

Pronounced changes in the intensity of the first-order diffracted light occurred during contraction which, during the shortening phase, varied appreciably from cell to cell. The first-order intensity typically decreased 30–50% with sarcomere shortening, as illustrated in Fig. 6 *A*. (A large part of this intensity decrease would be expected solely from the length-dependent properties of the cells as optical diffraction gratings (Kawai and Kuntz, 1973) and the fact that as they shorten, the cells intercept less light.) However, in many cells, first-order intensity began to increase before the occurrence of peak shortening (as seen after point *a* in Fig. 6 *A*) and, in some, there was little change in intensity despite sarcomere shortening to the postulated length of the A band, as seen in Fig. 6 *B*.

Given the cell-to-cell variability with shortening, one feature of first-order intensity behavior was striking in its consistency: the intensity of the first order always increased more rapidly than sarcomere length during relaxation. The increment in intensity could not be associated with relengthening, per se, since intensity often increased appreciably at peak shortening, i.e., during the time when sarcomeres were isometric (see Fig. 6 *A*). The maximum intensity of the first order occurred during the midportion of relaxation and preceded the onset of the slow phase of sarcomere relengthening. Though this can be seen in Fig. 6 *A*, it is most dramatic in those cases in which there was little initial intensity decrease with shortening. In these later cases it could also be seen that the peak intensity during relengthening was directly related to the previous extent of sarcomere shortening as seen during the contractile adjustments accompanying the onset of regular stimulation (Fig. 6 *B* and *C*). The existence of shortening-related changes in first-order intensity during relaxation contrasts with their absence during the period of shortening itself.

#### DISCUSSION

Our aim was to investigate the microscopic nature of activation and to obtain a precise and direct measure of sarcomere shortening dynamics in living heart muscle. This goal required a small and well-defined, physiologically controlled volume of sarcomeres whose contractile shortening was unencumbered by extracellular connections.

We describe here the microscopic appearance and the contractile behavior of unattached isolated heart cells when electrically stimulated. Previous reports have briefly described the contractile behavior of intact cells isolated from amphibian atrial tissue (Tarr and Trank, 1976) and the enzymatic isolation of rat hearts cells that briefly tolerate low (Vahouny et al., 1970; Bloom, 1970; Berry et al., 1970) and physiological levels of external calcium (Fabiato and Fabiato, 1972; Powell and Twist, 1976). We are unaware of any reports detailing the sarcomere dynamics in uniformly shortening rat heart muscle cells bathed in the calcium solutions commonly employed in intact muscle studies or of a description of the cells' physical behavior as optical diffraction gratings.

#### *Physiological Condition of Isolated Cells*

Without direct measurement of transmembrane potential it is difficult to determine the exact status of a cell's excitable membranes. Touching the cell membrane might compromise contractile uniformity (therein defeating an experimental priority), and so we chose to rely on indirect information about the physiological status of the cells.

The absence of spontaneous contractile activity (i.e., beating) in cells bathed in solutions containing 0.5–2.0 mM  $\text{CaCl}_2$  indicates that myoplasmic calcium levels are near or below the  $10^{-7}$  M concentration known to induce phasic contractions in skinned cardiac muscle preparations (Fabiato and Fabiato, 1977). For two reasons, at least, it seems unlikely that uniform contraction resulted from a direct, stimulus-induced depolarization of the intracellular calcium stores. First, sarcomere shortening is not graded with stimulus amplitude (Costantin and Podolsky, 1967), and, second, uniform shortening was triggered by 0.1–1.0-ms pulses, i.e., pulses far shorter than the 100-ms pulses required to directly influence the internal membranes in mechanically skinned cardiac muscle (Trube, 1978). Moreover, the cells did not contract when stimulated in the presence of 30 mM KCl solutions, and, furthermore, the latency period (the time from the stimulus pulse to the onset of sarcomere shortening) is nearly equal to the 15–25-ms period measured in intact muscle.

These findings indicate that the cells' excitable membranes are functional but do not reveal their exact status. Preliminary scanning electron microscopy reveals the presence of individual cells whose surface membranes appear velvety and smooth,<sup>1</sup> i.e., similar to intact muscle (McCallister et al., 1974), but unlike the corrugated surface appearance of cells spontaneously active in 1.0 mM  $\text{CaCl}_2$  solutions (Powell et al., 1978). A structural or functional resemblance to nonenzymatically treated muscle does not preclude reversible alteration of the cells' electrical characteristics (Lee et al., 1979; Powell et al., 1979). Consistent with this view, we have noticed in preliminary experiments (not included here) that 5  $\mu\text{g}$  of lidocaine per milliliter of cell suspension doubles the percentage of quiescent cells that can be stimulated after wash with lidocaine-free solutions.

The cells appear to be metabolically intact. Respiration of suspensions of

<sup>1</sup> Nagel, R., and B. Wittenberg. Unpublished observations.



cells is markedly enhanced by the addition of carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation in suspensions of these cells, and intracellular myoglobin is retained (Wittenberg, 1979). Many past metabolic studies on isolated cells have reported the integrity of the cell population as being proportional to the percentage of beating cells in unphysiologically low calcium solutions. Our experience contrasts with this view. We consider the quiescent cells to be physiologically intact, and so some conclusions of earlier studies merit reconsideration.

#### *Dynamics of Sarcomere Shortening in Isolated Cells*

We have shown that the contraction of an isolated cardiac cell can be studied with experimental solutions and techniques comparable to those used for intact muscle preparations. This permits us to more precisely resolve the microscopic nature of sarcomere shortening in living heart muscle.

With direct microscopic inspection, others have seen that the striated pattern of intact heart muscle becomes less distinct during contraction (Julian and Sollins, 1975; Wohlfart et al., 1977), an observation that parallels the decreased sharpness of light diffracted from whole cardiac muscle (Krueger and Pollack, 1975) and the presence of nonuniform activation that limits shortening in skeletal muscle fibers (Taylor and Rüdell, 1970). However, we have seen that the cell's diffraction pattern remains distinct and well ordered, with no evidence of buckling, despite shortening to sarcomere lengths as short as 1.4  $\mu\text{m}$ . Moreover, we have shown that appreciable internal buckling would be expected to occur if inactive elements existed at these sarcomere lengths. These results clearly indicate that sarcomere shortening in cardiac muscle is not limited by a phase of nonuniform activation between myofibrils.

The average sarcomere length at peak shortening in isolated cells ( $1.57 \pm 0.13$  [SD]  $\mu\text{m}$ ) agrees well with the lower limit of the sarcomeres' isometric length tension curves obtained from intact muscle at various levels of activation (Pollack and Krueger, 1976; ter Keurs et al., 1978; footnote 2). The similarities of sarcomere length at peak shortening in muscle, isolated cells, and unrestrained cardiac myofibrils partially activated at intermediate calcium levels (Fabiato and Fabiato, 1976) suggest that extracellular connections exert a negligible mechanical resistance to cardiac muscle shortening.

Although the sarcomere's functional range is similar, there are slight but possibly important differences in the *dynamics* of shortening in isolated cells when compared with observations in intact muscle. The average, maximum velocity of shortening in single cells bathed in solutions containing 1.0 mM  $\text{CaCl}_2$  was greater than that observed in intact muscles bathed in solutions containing twice as much  $\text{CaCl}_2$  (Pollack and Krueger, 1976). Based on our contention that the *mechanical* influence of the surrounding tissues on shortening is negligible, these differences may likely reflect the presence of extracellular binding sites that would lower the calcium-free ion activity at the cell surface in intact heart muscle, thereby limiting activation and reducing

<sup>2</sup> Gordon, A. M., and G. H. Pollack. 1980. Effects of calcium on the sarcomere length-tension relation in cardiac muscle. *Circ. Res.* In press.

shortening speed in cardiac *tissue*. In cells that shortened with the highest velocities, the speed of cell shortening was often relatively constant over an appreciable range of sarcomere lengths. The maximum velocity of myofibril sliding has also recently been reported to be independent of length over a similar range in tetanically stimulated skeletal muscle fibers (Edman, 1979). Though the precise reason is unclear, our results do show that the *potential* maximum velocity of shortening may be less dependent on sarcomere length than that previously seen in intact cardiac muscle (Pollack and Krueger, 1976).

Intracellular based restoring forces may contribute significantly to the rapid diastolic recoil of the heart at small end systolic volumes, for the time-course of change of the ventricular dimensions during early diastole (Rushmer, 1970) is nearly the same as the overall relengthening dynamics we observe at the level of the cell. Relengthening has been attributed to a potential energy stored within the myofibril (Gonzalez-Serratos, 1966; Parsons and Porter, 1967) and the sarcoplasmic reticulum (Fabiato and Fabiato, 1976). However, the existence of a constant velocity of shortening in highly activated cardiac cells indicates that internal loading did not occur with much of shortening, and so the restoring force cannot represent a simple, elastically stored potential energy.

Relaxation is terminated by a very slow phase of isotonic relengthening or isometric tension decline in intact muscle (Krueger and Farber, 1980) where the possibility of heterogeneity and undefined elasticities makes interpretation of its precise physical basis difficult. Our observations demonstrate that very slow relaxation phenomena exist that reflex interactions uniform at the level of the myofibril.

Inasmuch as the single cell is smaller than the area illuminated by the laser, the sample population remains constant with shortening, thereby enabling an analysis of the finer details of myofibril sliding. It has recently been proposed that sarcomere shortening occurs in a stepwise fashion, as seen by diffraction measurements in whole cardiac muscle and single skeletal muscle fibers (Pollack et al., 1977). Examples of nonsteady shortening were seen in only four cells (not shown here); in all other cases, the cells appeared to shorten and relengthen smoothly. However, the cells in this study were not generating tension, and the temporal resolution of our measurements is insufficient to rule out the possibility that pauses of very short duration (i.e., <2 ms) occur.

#### *Uncertainties about the Microscopic Nature of Activation*

One puzzling feature of our results is that with sarcomere *shortening* the decrease of first-order diffracted light was extremely variable from cell to cell. One explanation may be that, despite the fact that the cells are very thin and were illuminated by a cone of light, the volume of myofibrils contributing to the diffraction pattern varies with shortening (Rüdel and Zite-Ferenczy, 1979). It is also puzzling that (a) the first-order light never went to zero as would be predicted by application of a simple diffraction model based upon thick-fibril scattering and changes in the dimensions of the I band alone

(Fujime, 1975) and that (b) the qualitative appearance of the striations did not change. Our microscopic techniques did not have sufficient resolution to determine whether I bands existed at short sarcomere lengths (i.e., 1.6–1.4  $\mu\text{m}$ ), as has been observed in skeletal muscle fibers when activation is non-uniform (Huxley and Gordon, 1962). Thus, we cannot rule out the possibility that many myofilaments *within* the contractile lattice may be inactive in cardiac muscle, thereby resisting sarcomere shortening (Brown et al., 1970) and accounting for the unexpectedly high first-order intensity at these lengths.

The consistent observation of increased intensity during the relengthening phase (and during the short time before relengthening when sarcomeres were isometric) is not explained by current models of the diffraction process in muscle. The distribution of light within the diffraction pattern can be altered by the difference in the optical path lengths in a simple phase grating (Goodman, 1968). Consequently, the observation by Huxley and Taylor (1958) that the refractive index of the I band is decreased during relaxation after local activation in a skinned muscle fiber may have particular relevance to our observations. For example, if relaxation represents a state in which the number of myofilaments activated is altered, then not all would be under tension. This state could alter the disposition and reduce the lateral packing of the thin filaments in the myofibril—lowering the I band's refractive index—and, thereby, causing a relative increase in diffracted light intensity at any sarcomere length.

Interpretations based on other mechanisms, including variation in thick-filament registration, changes in birefringence, and differential rates of rehydration of the thick and thin filament arrays can also be advanced to explain the sequence of intensity changes during relaxation. Whatever the explanation for the increase in first-order intensity relative to sarcomere length in relaxation, the direct relation of its amplitude to the extent of prior shortening in some cells invites speculation that it is caused by physical changes related to inactivation within the sarcomere. If so, further microscopic and optical measurements on isolated cells may be fruitful in providing a more precise interpretation of graded activation in living heart muscle.

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