Nonuniform volume changes during muscle contraction

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ABSTRACT We measured dynamic changes in volume during contraction of live, intact frog skeletal muscle fibers through a high-speed, intensified, digital-imaging microscope. Optical cross-sections along the axis of resting cells were scanned and compared with sections during the plateau of isometric tetanic contractions. Contraction caused an increase in volume of the central third of a cell when axial force was maximum and constant and the central segment was stationary or lengthened slightly. But changes were unequal along a cell and not predicted by a cell's resting area or shape (circularity). Rapid local adjustments in the cytoskeleton evidently keep forces in equilibrium during contraction of living skeletal muscle. These results also show that optical signals may be distorted by nonuniform volume changes during contraction.

INTRODUCTION

Important functional roles are attributed to the cytoskeletal matrices of skeletal muscle (Street, 1983; Wang, 1984; Magid et al., 1984; Goldstein et al., 1987). But average values from many cells or static measurements from cells modified irreversibly raise questions about the conformity of these results to single living cells where events recur in a fraction of a second. The problems with using whole muscle are well documented for studies of volume changes during contraction (Baskin and Paolini, 1966). Uncertainties relate to the facts that the measurements are made on a closed system, a volume change caused by a water shift within or between muscle cells and the bathing medium would elude measurement, and the vectors for axial force and forces producing a volume change are not coincident in a multi-cellular preparation (Sato, 1954; Baskin and Paolini, 1966).

A system that permits (*a*) multiple two-dimensional imaging of microscopic portions of a cell, (*b*) frame read-out times shorter than a few milliseconds, (*c*) image synchronization with extrinsic stimulation and, (*d*) simultaneous measurement of active force, allowed us to map two-dimensional and three-dimensional features in real physiological time (Taylor and Roos, 1990). We used the optical-mechanical apparatus built originally to study how a muscle cell adjusts its volume after a change in osmotic pressure of the bathing medium (Blinks, 1965). We combined this apparatus, with some modification, to our imaging system which exposes frames very briefly, gives a linear output, has a very wide dynamic range, high signal-to-noise ratio, and is lag-free compared with photographic emulsions or silicon intensified target video cameras (Roos et al., 1989). We used this to study how a muscle cell adjusts its volume in response to brief contractions involving no detectable shortening of the central segments. We found that the volume of serial sections along the axis of single cells changed rapidly during contraction to maintain a dynamic equilibrium between axial and radial forces. Changes were closely reproduced in each contraction with a pattern unique for each cell and each cell segment. These results not only show the dynamic role of the cytoskeleton in muscle, they have general implications for optical measurements of physiological events. Optical signals are difficult to interpret when a sustained or transient geometric change occurs (Taylor et al., 1975; Baylor, 1983). Our results show that optical signals may also be inaccurate because volume changes occur nonuniformly along a cell.

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MATERIALS AND METHODS

Cells were dissected from the zone of the tibialis anterior muscle of the British frog, *Rana temporaria*, furthest from the nerve where short (4–7 mm), relatively large, fatigue-resistant fibers are located (Smith and Ovalle, 1973). In selecting a particular zone of an amphibian muscle from which to dissect fibers one can obtain cells with either a broad spectrum of biochemical and physiological properties or homogenous properties if the fibers are selected with exclusionary strictness (Lannergren, 1987). We selected homogeneous fibers for this report using criteria described in detail elsewhere (Cecchi et al., 1986). In addition, we excluded fibers that did not show a negative slope of the length/tetanic tension *and* the length/twitch tension relationships with stretch beyond slack length (Close, 1972). This alone eliminates one-third of the single fibers tested from those included in this report and forestalls consideration of some unknown factor involved in activation that is related to the cross-sectional dimensions of individual

0006-3495/91/04/926/07 $2.00

muscle fibers (Close, 1972). Furthermore, we selected fibers ($n = 5$) in which some segments remained stationary during the plateau of each tetanus, a few segments elongated, and none shortened compared with the six fibers used to define and simulate the relationship between tension creep and segment length changes (Edman and Reggiani, 1984). This eliminated fibers in which the cross-sectional area of a segment might increase because the segment shortens (Sato, 1954). These selection criteria eliminated factors that would add uncertainty to our comparison with studies showing an increase in the Z-band lattice spacing in the maximally activated state (Goldstein et al., 1987, 1988).

Our cells were dissected and stimulated at least 18 h before the final length-tension relationships were determined. Experiments were conducted at the same temperature to which the frogs were adapted (0 to 4°C). Brief electrical stimuli were delivered from platinum electrodes built in the assembly that held a force transducer (RCA 5734, Harrison, NJ), which allowed us to simultaneously measure axial force and image cross-sectional area (Blinks, 1965).

RESULTS

Each cell included in our analysis maintained constant (0 to <3% change) axial tetanic force during an imaging period. Each series of images contained isometric segments in series with segments that showed a relatively small (<3%), reproducible axial lengthening during the plateau of each tetanus nearly a day after isolation (Fig. 1).

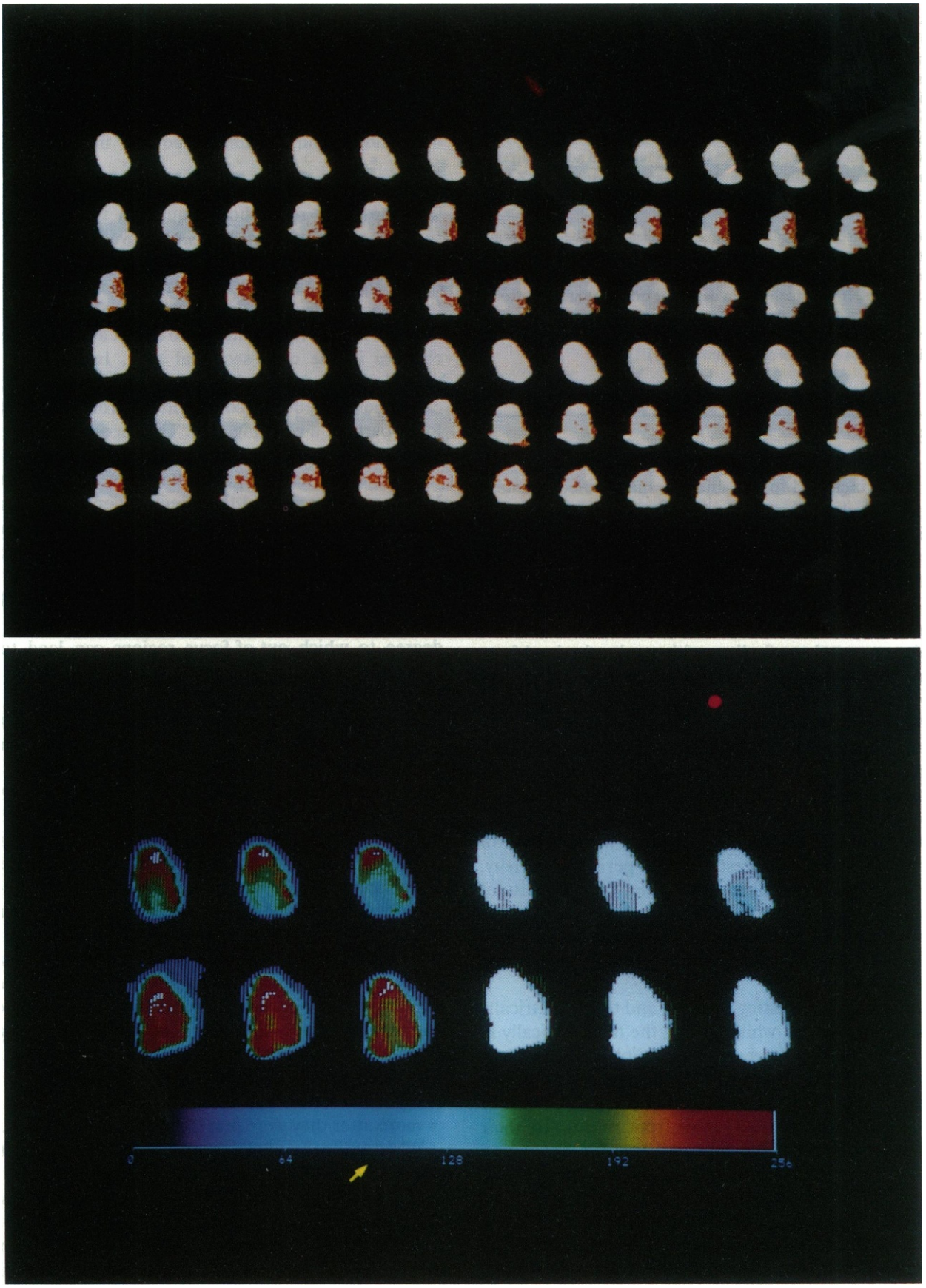
We compared our findings with results obtained from cine-photomicrographic records (Edman and Regianni, 1984) as well as with x-ray diffraction where *shortening* of ~5% is considered isometric (Hazelgrove, 1983). The fact that our images of the middle segments showed axial *lengthening* ranging from zero to 2.5% of a fiber's segment length (1 mm), implies that axial translations were negligible to small, shortening was not a factor, contraction was propagated along the whole length of fibers that had the longest possible period of activity, and these were physiologically stable, fatigue-resistant fibers. These data were used in tests for correlations between (*a*) the circularity and resting cross-sectional area and (*b*) the resting area and percent change during contraction.

We scanned the resting segment and the isometrically contracting segment while moving the fiber vertically in one direction for a given contraction (Fig. 2), then moved it in the opposite direction in the next contraction (Fig. 3). Cells were moved perpendicular to the plane of the sensor, 1 mm either away from or toward their approximate center. We repeated this for upper and lower fiber segments over several hours to assure that the volume changes along a length that ranged from ~25 to 50% of a whole cell, were reproducible. We did not image the extreme ends because they are conical, the peripheral myofibrils terminate before the central myofibrils do, and the tendons cover parts of the cell's surface (Blinks, 1965; Ishikawa et al., 1983). When the end of a fiber *is* imaged to maintain an unchanging site of measurement during shortening, the cross-sectional area reportedly increases equally along the end segment in some fibers and thickens unequally in others (Sato, 1954).

Fig. 1 *a* shows images during the plateau of a tetanus. The top three rows show sequential two-dimensional sections along a fiber as it was moved through focus while at rest. The bottom three rows show the same fiber during the plateau of an isometric tetanus. Background light was so low that it was unnecessary to enhance contrast in the original images to detect and measure the changes in the cross-sectional area. In addition, the variable frame integration time (4 ms or less in these experiments) reduced contributions from dim, out-of-focus parts of a cell (Fig. 1 *b*). The theoretical depth of focus of long working distance objectives required for such experiments is large, perhaps several millimeters. However, we measured the effective depth of focus for millisecond exposures of images from both inanimate objects as well as living cells, and found that all detectable light from an object in focus disappeared as the focal plane was displaced less than ±150 μm. Others have made similar measurements to determine the degree to which out-of-focus regions can lead to an erroneous estimate of actual cell size (London et al., 1986).

This operational shallowness in depth of focus allowed us to discover that deviation from the mean cross-sectional area at 25 μm intervals along a cell was unexpectedly large (Blinks, 1965; Elzinga et al., 1989). Figs. 1–3 show that the change during isometric contraction of this fiber varied from ~1–40%. The spatial distribution of the volume change was unique for each fiber. The pattern during a scan in one direction compared with a scan in the opposite direction was virtually the same for a given segment in back-to-back contractions over a period of several hours. The images associated with tetanic force also showed no axial movements like those that one would expect from an injured cell, one that might not allow action potentials to propagate over its entire excitable surface and, therefore, contract only locally. Fibers routinely developed isometric tetani at regular intervals like those shown in Figs. 1–3, a day or more after they were first isolated and stimulated.

When we analyzed twitches we found that segments at a single point of focus also increased in cross-sectional area after a single stimulus. But because we did not scan along the length of a fiber we could not rule out the possibility that shortening occurred at the single point of focus. Volume appeared to change during the initial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transition from rest to activity in the same way that it did during the plateau of a sustained tetanus (Fig. 1 *a*). This suggests that traveling waves or vibrations owing to the stepping movements probably cannot account for the differences in area we observed as resting or stimulated fibers were moved through focus. Such effects can clearly be ruled out as factors accounting for the area change during the tetanus plateau.

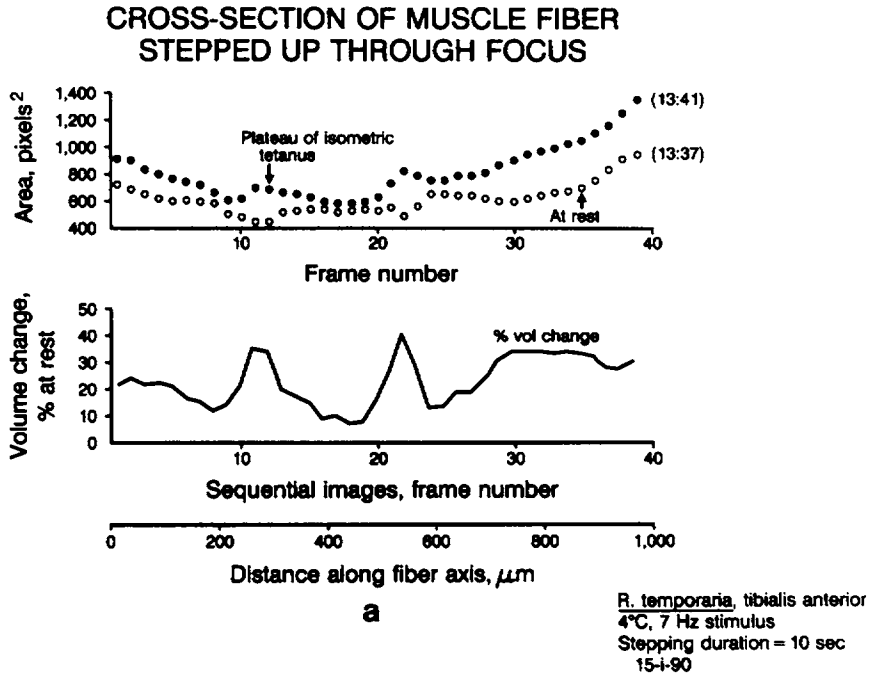
FIGURE 1 (*Top*) Cross-sectional images of a 1-mm segment of an isolated frog fiber at rest and during the plateau of an isometric tetanus. Images of the resting fiber (top three rows) and tetanized fiber (bottom three rows) were taken while the cell was moved through a slit of xenon light and force was recorded simultaneously as shown in Fig. 2. The bar on the bottom = 100 μm. The distance between the immersion objective and sensor (a 128 × 128 solid-state array [EG&G Reticon, Sunnyvale, CA] coupled to a gated, second generation, dual microchannel plate, proximity focused model XTO 141 image intensifier [Litton, Tempe, Arizona]) was adjusted after each preparatory contraction until the area filled slightly less than one-quarter of a frame. The axis of a fiber rarely coincided exactly with the *Z* axis of the objective (UMx20/NA 0.33 long-working distance objective and water-immersion meniscus [Wild Leitz, Wetzlar, Germany]). Hence we set the overall magnification to ×50.8 by reducing the optical path length to ~170 mm. This kept the fiber in the field of view and allowed us to follow it without interruption if it shifted when moved up or down through focus or moved during contraction. (*Bottom*) Images from the same cell shown in Fig. 1 *a* are displayed here to illustrate that neither computer enhancement nor differences in the pixel intensity distribution between resting and tetanized fibers can account for our findings. The top row shows images from the resting fiber. The bottom row shows images from the fiber during the plateau of a tetanus. The six images on the left show the original data (12 bits on an 8 bit display monitor) with the grayscale scale values displayed in false color. The bar across the bottom shows the grayscale values associated with a given color. The signal-to-noise ratio in the original data was sufficiently large that no subtraction of a cell-free background image or readjustment of the transfer function of a grayscale histogram was necessary to manually trace an outline that excluded the dim values represented by blue. The same six images are shown on the right after grayscale values were assigned to pixels above and below thresholds that included the range from the middle of the green through the far red and into the white. Hence the brightest intensity values equalled the dimmer values of the outline of a cell at the same position in focus. The brightest scatterers of light usually corresponded to dust or connective tissue adhering to the cell surface (Blinks, 1965). The low clipping removed the blue values too dim to be detected by eye if the same raw data were displayed in black and white. We measured area (*a*) by manually tracing the border of unprocessed images and, (*b*) by setting the value of the dimmest pixels to zero, then plotted the remaining nonzero pixels as pixels^2 (Figs. 2 *a* and 3 *a*). The results were qualitatively the same with each method although the absolute values were dependent in the traced measurements on the precision and accuracy with which a person could move the mouse across the tablet. For one of the fibers (11.i.90) in which we compared the two methods of measuring cross-sectional area the average increase during the tetanus with upward movement was 20% and 22%, and for the next tetanus with downward movement the increase was 29% and 32% when the images were measured, respectively, by manual tracing vs. automatic summation of nonzero pixels.

DISCUSSION

Active force development along a fiber's axis evidently produces outwardly directed radial forces that clearly cannot be attributed to cell shortening (Figs. 2 and 3). Several static methods of studying molecular relationships between muscle structure and function have led to the same inference, namely that a tetanus *must* induce forces with perpendicular components that are absent in muscle that is passively stretched or shortened (Goldstein et al., 1988). We suggest an interpretation that unifies these findings.

The surface membrane of a frog fiber is firmly attached to elements of the cytoskeleton that normally restrict lateral expansion of an intact fiber (Maughan and Godt, 1979; Magid and Reedy, 1980). When this membrane is disrupted or removed, a fiber swells. But when the contractile filament lattice spacing is decreased osmotically and the fiber placed in rigor, the cross-bridges exert a lateral force that may be attractive or repulsive depending on the existing filament separation (Matsubara et al., 1984). There is no change in the volume of the contractile filament lattice during contraction in the region where each thick filament is surrounded by six thin filaments (Hazelgrove, 1983). As the thin filaments extend from the overlap zone toward Z disks at the ends of each sarcomere, their lattice arrangement is transformed from a hexagonal pattern into one that is predominantly small squares (Franzini-Armstrong and Porter, 1964; Rowe, 1971; Goldspink, 1983; Goldstein et al., 1988). Cross-bridge attachment and the power stroke in portions of the myosin molecule that push actin past myosin causes oblique pulling by actin on the Z disks and expansion of the small square lattice by 20% on average (Goldstein et al., 1988; Edwards et al., 1989; Goldspink, 1983). Formation of cross-bridges and active contraction cause radial forces to dynamically change the elastic cytoskeletal network primarily in the Z disk. M line bridge proteins and connecting filaments between myosin filaments and the Z disk also stabilize the contractile lattice (Street, 1983; Wang, 1984; Magid et al., 1984; Goldstein et al., 1987; Goldspink, 1983; Goldstein et al., 1988; Edwards et al., 1989). The fact that the changes are reproducibly nonuniform rather than just a single broad increase in the central segment suggests that intrinsic differences in the cytoskeleton may determine the patterns of nonuniformity.

Our observations of isolated, living cells are not subject to the uncertainties of working with whole muscle without the ability to measure reversibility, reproducibility, or the timing between the rise and fall of tension and rapid changes in volume. Elastin and connec-



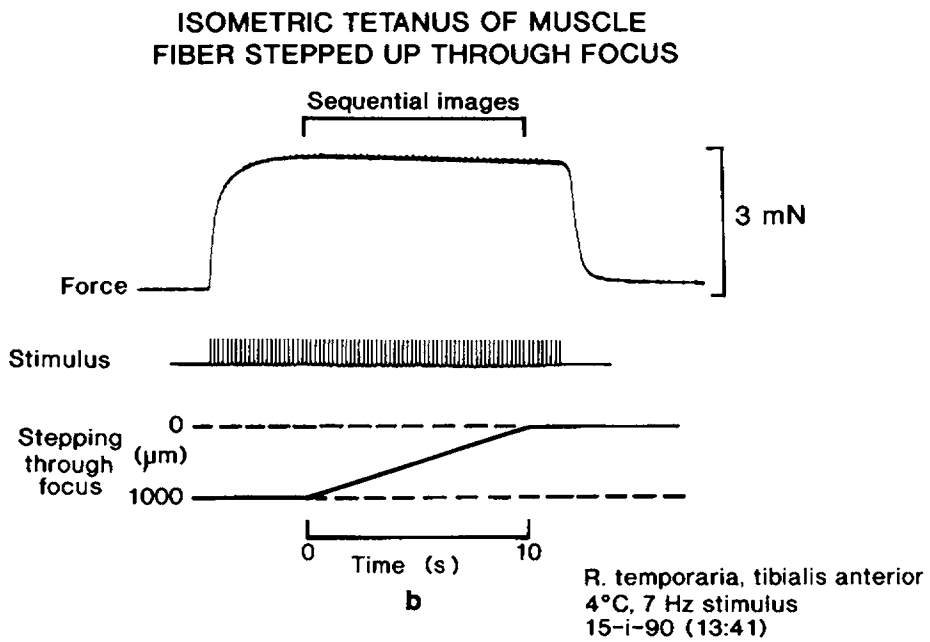


FIGURE 2 (*a*) Graph of areas from Fig. 1. (*Top*) Raw data; (*Bottom*) The *difference* in segment areas during the tetanus in Fig. 2 *b*. The fiber was stepped through focus during the plateau of the tetanus. Fiber length was set so that both twitch force and tetanic force were on the descending limb of their length-tension relationships. Stretch reduced movements that tended to blur images, and assured that all the results were obtained near the same striation spacings, ~2.6–2.8 μm (Close, 1972; Lopez et al., 1981). The rise in $[Ca^{2+}]_{int}$ exceeds the level for saturation at these spacings (Cecchi et al., 1986). (*b*) Record of isometric force corresponding to the image data in Fig. 2 *a*. The fiber and transducer assembly (Blinks, 1965) were mounted on a Huxley-type micromanipulator. The spring-loaded arm that controlled movement along the optical axis of the objective was coupled to a nonrotating spindle driven by a micro-stepper. When a fiber reached the plateau of tetanic force the motor moved the fiber and force transducer 1.00 mm in 10 s under computer control.

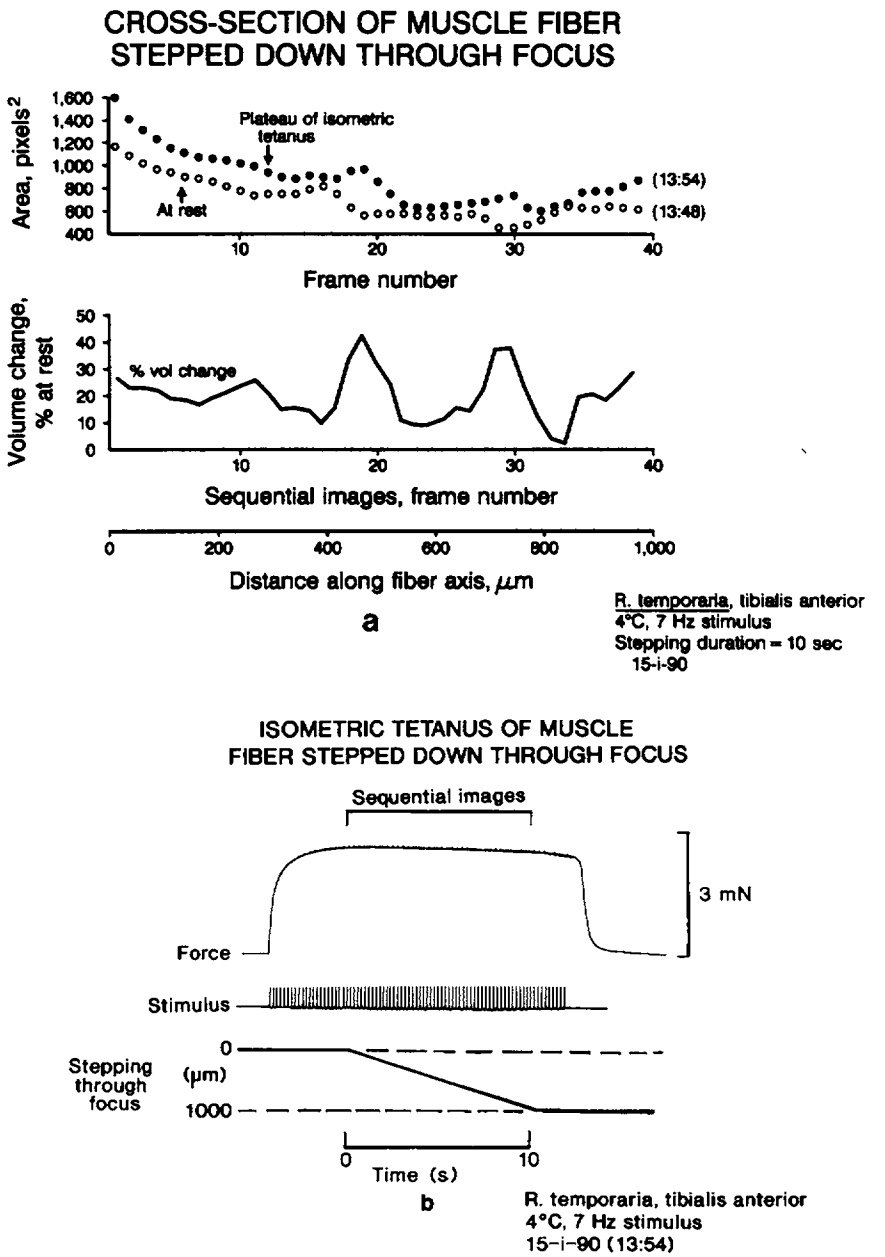


FIGURE 3 (*a*) Graph of the same segment in Figs. 1 and 2. However, the fiber is moving in the opposite direction through the slit of light. Note that changes in a given segment show the same pattern and, therefore, are independent of time during a brief tetanus. In addition, note that regions with the same area at rest can show markedly different changes during the plateau of isometric contraction. (*b*) Record of isometric force corresponding to the image data in Fig. 3 *a*.

tin provide the stability that centers the myosin filaments in a *resting* sarcomere (Horowits and Podolsky, 1987; Pierobon-Bormioli et al., 1989; Funatsu et al., 1990). The dynamic changes we found must now be included in models of a muscle's cytoskeleton that describe elasticity and mechanical stability in general. The angle between dynamic forces must change to neutralize one another during contraction and maintain equilibrium. If this did not occur the resultant forces would push, twist, and turn the cell further from its asymmetrical noncylindrical shape and cause it to collapse. One way to cancel unequal forces is to redistribute them through the

cytoskeleton when large axial forces develop from active contraction (Goldspink, 1983; Goldstein et al., 1988; Edwards et al., 1989). Radial expansion increases the moment of inertia (i.e., the stability against turning) and decreases the angular velocity as axial force increases during contraction, as expected from the law of conservation of angular momentum.

Area increased and reversed on the same time scale as force development and relaxation and the pattern of nonuniform changes were similar whether we imaged a cell segment near the beginning or end of the tetanus plateau. The changes are related to a quick-acting mechanism involved in auto-regulation of contraction and are not time dependent. This mechanism is different from those involved in swelling that follows either extreme shortening or intense, prolonged contraction, which probably are the result of water movement between the cell and the extracellular medium (Sato, 1954; Lannergren, 1990).

Differences in radial forces among cross-sectional areas in series (e.g., Figs. 2 *a* and 3 *a*) could be owing to variations in (*a*) the relative amount of contractile material, (*b*) the elastic characteristics of the extracellular matrix, or (*c*) the assembly of the intracellular cytoskeleton and connections that transmit force to the tendons. Volume compensation followed a change in axial force, differed in adjacent segments although they were in series and had the same axial force, and is not likely related to the first possibility. The first two possibilities cannot account for our findings because the volume changes and tension were not related to the cross-sectional area at rest and the circularity. Circularity is the ratio of the area of the fiber to the area of the smallest circle that most closely encompasses the fiber's area.

Cross-sectional area of *resting* fibers is often used to relate contractile protein density with tension development per unit area. But volume and tension have not previously been measured simultaneously in single contractions of isolated, intact muscle fibers. We compared area of resting fibers with a circle to determine whether or not the more circular fibers correlated with changes that suggested the cytoskeleton was balancing higher tetanic forces (Elzinga et al., 1989). Circularity of a resting segment did *not* correlate with either the size of an area or its change during the tetanic plateau. Therefore, neither resting area nor resting circularity are necessarily correlated with the active tension generated by a contracting fiber.

The volume compensation for radial forces that develop in contraction is probably owing to water redistribution. Some, if not all, of this water may come from the extreme ends of the cell which are known to behave differently from the middle two-thirds of a cell (Edman and Reggiani, 1984; Burton et al., 1989). The extreme ends shorten whether or not the central portion of the cell is isometric or stretched and the ends probably squeeze cell water into the middle segments.

The measurement of any event in contracting muscle by an optical signal may also be affected by these nonindicator related changes (Taylor et al., 1975; Baylor, 1983). A signal that depends on a change in light intensity to monitor an event may be distorted by the geometrical changes we observed in the brief time that a muscle cell contracts vigorously.

We are greatly indebted to John R. Blinks for the loan of microscope objectives and his experimental chamber, to Norman R. Lee for dissecting single muscle fibers, and to K. P. Roos and J. R. Blinks for their criticisms.

Supported by the Australian NH & MRC (to Dr. Neering) and National Institutes of Health NS 22369, National Science Foundation DMB-85034964, and Pittsburgh Supercomputing Center DCB-890009P (to Dr. Taylor).

Received for publication 8 October 1990 and in final form 8 January 1991.

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