

Periodic Organization of the Contractile Apparatus in Smooth Muscle Revealed by the Motion of Dense Bodies in Single Cells

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Abstract. To study the organization of the contractile apparatus in smooth muscle and its behavior during shortening, the movement of dense bodies in contracting saponin skinned, isolated cells was analyzed from digital images collected at fixed time intervals. These cells were optically lucent so that punctate structures, identified immunocytochemically as dense bodies, were visible in them with the phase contrast microscope. Methods were adapted and developed to track the bodies and to study their relative motion. Analysis of their tracks or trajectories indicated that the bodies did not move passively as cells shortened and that nearby bodies often had similar patterns of motion. Analysis of the relative motion of the bodies indicated that some bodies were structurally linked to one an-

other or constrained so that the distance between them remained relatively constant during contraction. Such bodies tended to fall into laterally oriented, semirigid groups found at $\sim 6\text{-}\mu\text{m}$ intervals along the cell axis. Other dense bodies moved rapidly toward one another axially during contraction. Such bodies were often members of separate semirigid groups. This suggests that the semirigid groups of dense bodies in smooth muscle cells may provide a framework for the attachment of the contractile structures to the cytoskeleton and the cell surface and indicates that smooth muscle may be more well-ordered than previously thought. The methods described here for the analysis of the motion of intracellular structures should be directly applicable to the study of motion in other cell types.

A major goal of cell biology is to understand the organization of living cells, how various structures and organelles within cells interact with one another, and how these interactions change with time. In the case of motile or contractile cells it is of particular importance to understand the structure and dynamics of the cellular elements responsible for producing motion or generating force and to understand how these elements are integrated into the overall architecture of the cell. To accomplish this it is necessary to have both spatial and temporal information about cell organization. Although there is an increasing interest in obtaining and analyzing temporal information (see, for example, Webb and Gross, 1986; Jacobson et al., 1989) to date, most research effort has been devoted to obtaining spatial information at a fixed time point and, while this may be adequate for predicting the temporal behavior of structures in some cases, in many instances it is insufficient. It is therefore important that methods be developed to study and analyze the motion of structural elements and organelles within cells. The goals of the work we present here are to develop and adapt such methods for the study of the contractile process in smooth muscle.

The well-ordered contractile apparatus in skeletal and cardiac muscle has proven amenable to study with the light and electron microscope and from such studies it has been

possible to directly observe or infer information about the contractile process itself. In smooth muscle cells, however, such order is not readily apparent and these approaches have revealed much less information about the overall organization of the contractile system (see the recent review by Bagby, 1986) and little can be inferred about the dynamics of contraction at the structural level. It is not known, for example, in what directions forces act within cells during contraction, how the force generating elements are arranged with respect to one another, how this arrangement changes during shortening, how these elements are linked to the cell surface and cytoskeleton, and how force is transmitted from cell to cell within smooth muscle tissue. Although the structural and biochemical evidence that is available has led to the suggestion that a sliding filament mechanism is responsible for the development of tension in smooth muscle, even this hypothesis must remain somewhat tentative because of the paucity of direct evidence about the organization of living contractile cells.

The development of a skinned single cell preparation in our laboratory (Kargacin and Fay, 1987) has provided us with a unique opportunity to directly study the motion of structures in smooth muscle cells during shortening. The skinned cells are highly permeable so that their shortening can be precisely controlled by the Ca^{++} and ATP added to the bathing medium. Moreover, the cells are optically lucent so that dense bodies are visible within them under the phase

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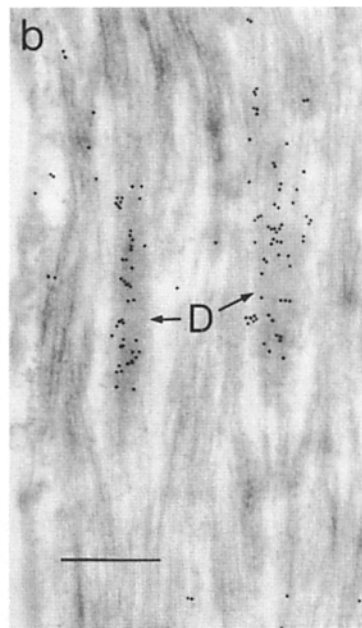
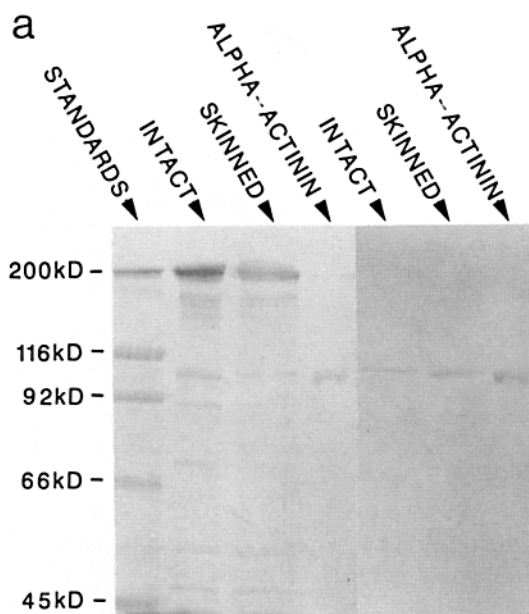


Figure 1. Specificity of the alpha actinin antibody to smooth muscle alpha actinin and to dense bodies in saponin permeabilized cells. (a) First through fourth lanes, SDS-PAGE gel stained with Coomassie Blue. Fifth through seventh lanes, immunoblot. Both intact (second lane) and skinned (third lane) smooth muscle cells contained a protein that migrated on SDS-PAGE gels with purified toad alpha actinin (fourth lane). This protein and purified alpha actinin were specifically marked with alpha actinin antibody on immunoblots (fifth, sixth, and seventh lanes). (b) Electromicrographs of dense bodies (*D*) in a skinned single cell. The dense bodies were specifically labeled with 10-nm colloidal gold particles (*dark spots*) conjugated to a secondary antibody directed against an alpha actinin antibody.

contrast microscope. These structures are believed to be the attachment sites for actin filaments in smooth muscle (Pease and Molinari, 1960) and thus analogous to the Z disks in skeletal muscle. This hypothesis is supported by the finding that both dense bodies and Z disks are rich in alpha-actinin (Schollmeyer et al., 1976) and by the observation that actin filaments have opposite polarity on opposite ends of dense bodies (Bond and Somlyo, 1982; Tsukita et al., 1983). With the skinned cell preparation we were therefore able to study, for the first time, the movement, during contraction, of structures intimately associated with the contractile apparatus in smooth muscle.

From digital images of cells, collected at fixed time intervals during shortening, we identified individual dense bodies, tracked their movement during contraction and analyzed this motion. The results of this analysis suggest that dense bodies in smooth muscle are organized into semirigid structures that are found at $\sim 6\text{-}\mu\text{m}$ intervals along the long axis of the cell and that most of the shortening in the cell occurs between these structures. This work, which suggests that smooth muscle is more highly organized than previously thought, provides, for the first time, two-dimensional temporal information about contraction that can be used in conjunction with three-dimensional spatial information obtained from optical sectioning to provide a more complete description of the contractile structures in smooth muscle and their relationship to other cellular elements. The methods we have used in our analysis provide a general approach to the study of the motion and should be directly applicable to the study of movement of structures in other cell systems. Preliminary accounts of this work have appeared previously.

Materials and Methods

Preparation of Cells

Single smooth muscle cells from the stomach of the toad *Bufo marinus* were

isolated as described by Fay et al. (1982) and permeabilized as described by Kargacin and Fay (1987). For the experiments described here, cells were permeabilized or "skinned" in Rigor solution with 25 $\mu\text{g}/\text{ml}$ of saponin. The skinned cells were resuspended in Rigor solution containing 0.1 mM free Ca^{++} , and shortening was induced with contracting solution superfused through the cell chamber (see below). Rigor solution contained 150 mM K^+ , 5 mM EGTA, 3 mM Mg^{++} , 6 mM Cl^- , 10 mM 3-[*N*-Morpholino] propanesulfonic acid (MOPS), and 135 mM propionate (pH 6.5; ionic strength 0.16). Ca^{++} -Rigor solution contained, in addition to the above, 0.1 mM free Ca^{++} with K^+ and propionate adjusted to maintain ionic strength at 0.16. Contracting solution contained 0.1 mM free Ca^{++} , 3 mM free Mg^{++} , 1-5 mM ATP, 10 mM MOPS with K^+ , and propionate adjusted to maintain ionic strength. All solutions contained PMSF (75 mg/liter) to inhibit proteolysis and DTT (5 mM) to prevent -SH oxidation.

Antibody Preparation and Specificity

Frozen aliquots (10 ml) of whole immune serum from rabbits, developed against purified alpha-actinin from toad smooth muscle (Fay et al., 1983), were thawed, and immunoglobulin-enriched fractions were precipitated by addition of ammonium sulfate to 40% of saturation at 0°C. The precipitated protein was collected by centrifugation, subfractionated by anion exchange chromatography, and conjugated to tetramethylrhodamine isothiocyanate isomer R (TRITC)¹ according to procedures outlined by Forni and de Petris (1984).

The specificity of binding between TRITC-labeled protein and toad smooth muscle alpha actinin was assayed by immunoblotting it against the major polypeptides of whole isolated smooth muscle cells from toad stomach muscularis and purified alpha actinin after SDS gel electrophoresis and transfer to sheets of nitrocellulose, following modified methods of Towbin et al. (1979) and Hawkes et al. (1982). The immunoblots showed that the antibody specifically marked a 100-kD protein from intact and skinned cells that had the same relative mobility on SDS-PAGE gels as purified toad alpha actinin (Fig. 1).

The specificity of the antibody for dense bodies was examined with immunoelectron microscopy. Sectioned, isolated, skinned smooth muscle cells were embedded in Lowicryl K4M, using the rapid method of Altman et al. (1984). Thin sections on Ni grids were incubated sequentially in (a) whole preimmune serum, (b) TRITC-labeled antibody fractions to alpha actinin, (c) PBS, and (d) antibodies to rabbit IgG, coupled to 10-nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium). Under the electron microscope the colloidal gold particles were found to specifically mark cytoplasmic (Fig. 1 b) and membrane (not shown) dense bodies.

1. Abbreviation used in this paper: TRITC, tetramethylrhodamine isothiocyanate isomer R.

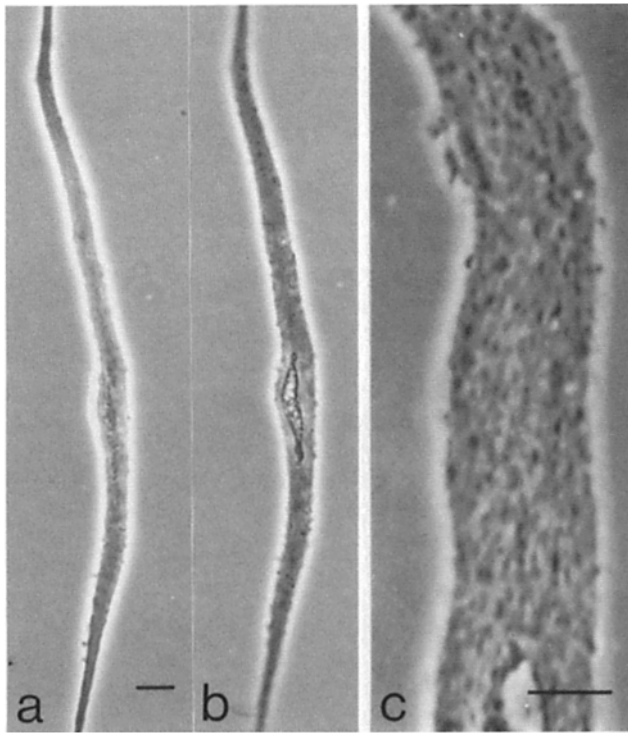


Figure 2. Changes in the optical properties of isolated smooth muscle cells after saponin permeabilization. Phase contrast micrograph of an isolated cell (*a*) before saponin treatment and (*b*) after permeabilization. Note the enhanced visibility of the nucleus and the increase in cell diameter in *b*. (*c*) High power phase contrast digital image of an isolated cell showing the punctate phase dense structures that became visible after saponin permeabilization. Bars, 5 μm .

For comparison of phase contrast and fluorescence images of single cells, TRITC-labeled protein fractions, at concentrations between 100 and 200 $\mu\text{g}/\text{ml}$ with F/P ratios from three to five (calculated from optical densities at 280 and 555 nm), were mixed with saponin skinned, isolated cells in PBS containing 1% rabbit preimmune serum. Cells were incubated with the antibody for 2–24 h. Labeled cells were recovered by centrifugation at 60 g for 10 min, and mounted in a medium for fluorescence (Johnson and Araujo, 1981).

Observation of Cells

Digital images of cells were obtained at fixed time intervals during shortening with a Zeiss inverted microscope and the imaging system described by Fay et al. (1986). Cells were pipetted into a chamber made from a piece of parafilm sandwiched between two glass coverslips (Kargacin and Fay, 1987). This chamber could be inserted between an oil immersion objective (100 \times ; NA 1.3) and condenser (NA 1.4) on the microscope and provided a means of continuously superfusing the skinned cells with solution while they were under observation at high power. Cells pipetted into the chamber settled down onto the bottom coverslip after a few minutes and usually remained well enough attached so that they did not wash away when the superfusion was started. Although the cells remained in place on the coverslip, they were, nevertheless, able to shorten when superfused with contracting solution suggesting that they were most strongly attached at only one point.

Video images were collected with a Newvicon camera (model 68; Dage-MTI Inc., Michigan City, IN) and digitized with an Imaging Technology (Imaging Technology Inc., Woburn, MA) video analyzer controlled by a PDP 11/73 microcomputer. The images were digitized to a resolution of 512 \times 480 pixels with each pixel assigned an intensity value between 0 and 255 and were transferred to a VAX 11/750 minicomputer for analysis. For the timed sequences, images, which were the sum of four video frames, were collected at 2- or 3-s intervals during shortening. These images were col-

lected either directly as cells shortened or from video tape recordings of contracting cells.

Analysis of Dense Body Motion

To mark the locations of dense bodies in digital images, the images were displayed on a solid view system (model 3400; Lexidata Corp., Billerica, MA). An interactive computer program was used to label each selected body in a cell image and to store the coordinates of the body in a data file. To permit specific bodies to be easily followed during a contractile sequence, their positions were marked on one image of the sequence and this image, with a map of the selected points overlaid on it, was displayed beside the next one in the sequence while it was being marked. The coordinates of the bodies determined in this way were then translated into the coordinate system described in Results.

To determine the accuracy with which the centers of dense bodies could be marked, 10 bodies in each of three images were marked by an observer and this process was repeated 10 times. The error in the positions of the bodies was found to be approximately one pixel in any direction (average standard deviation of the position in x was 0.64 pixels; average in y 0.81 pixels).

To analyze the relative motion of dense bodies, the total distance between each pair of bodies in an image was found for every image in a timed sequence. As a measure of the change in the distance between two bodies over any time interval, the ratio of their separation at the end of the interval to their separation at the beginning of the interval was computed.

Results

Optical Changes Induced by Skinning

When isolated smooth muscle cells were skinned they became somewhat swollen and optically lucent (Fig. 2, *a* and *b*). The latter change was made most obvious by the enhanced visibility of the nucleus in the skinned cells and by the appearance of fine fibrillar structures (see Fig. 4) and dark punctate bodies (Figs. 2 *c* and 4) within the cells when they were viewed at higher power with the phase contrast microscope. The phase dense bodies were 0.5–1.0 μm in length and \sim 0.25 μm in diameter. As illustrated by the image plane shown in Fig. 2 *c*, the bodies appeared to be distributed more or less evenly throughout the cytoplasm.

Identification of the Phase Dense Structures

The number, distribution, and appearance of the phase dense structures in the skinned cells (Fig. 2 *c*) was strikingly similar to that of the cytoplasmic dense bodies that have been observed previously in isolated smooth muscle cells labeled with fluorescent antibodies to alpha actinin (see Bagby, 1980; Fay et al., 1982). To see if, in fact, the phase dense structures were dense bodies, skinned cells stained with a fluorescent antibody to toad alpha actinin (see Materials and Methods) were examined under both the phase contrast and fluorescence microscope.

In skinned cells the distribution of dense bodies revealed by the fluorescently labeled antibody (Fig. 3 *b*) appeared to be identical to the distribution of phase dense structures seen with the phase contrast microscope (Fig. 3 *a*). To test this objectively, the interactive computer program described in Materials and Methods was used and the positions of the structures in the digital phase contrast images of cells were marked (as illustrated in Fig. 3 *c*). The maps thus generated were digitally overlaid on the corresponding fluorescence images (Fig. 3 *d*). In the fluorescence images of three cells a total of 784 bodies labeled with the antibody were visible; 728 of these bodies (93%) were correctly marked on the corresponding phase images of the cells. Many of the bodies

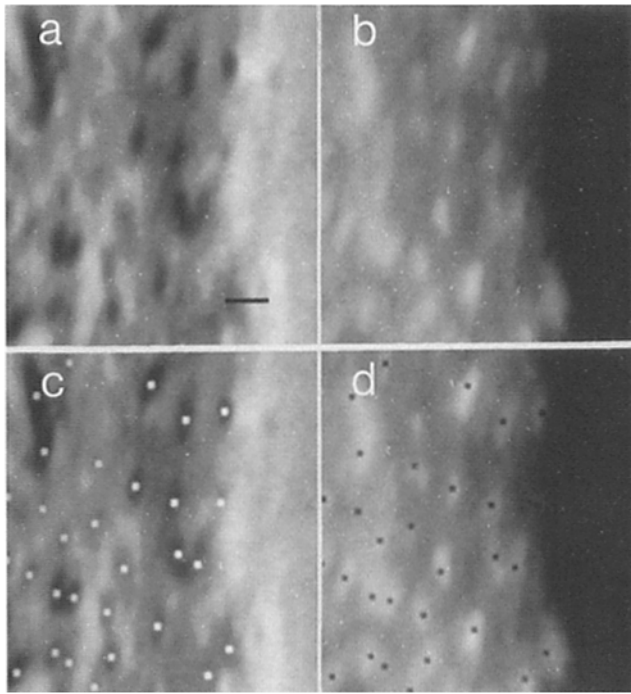


Figure 3. Coincidence of dense bodies and phase dense structures. (a) Digital phase contrast image of a saponin permeabilized single smooth muscle cell. (b) Digital fluorescence image of the cell in a showing alpha actinin rich structures marked with TRITC-conjugated alpha actinin antibody. (c) Phase image after the locations of the phase dense structures were marked with the interactive computer program discussed in Materials and Methods. (d) Digital overlay of the phase map (c) on the fluorescence image in b. Bar, 1 μm .

visible in the fluorescence images but not seen in the phase images were obscured by other phase dense objects such as the nucleus. About 10% (63) of the structures marked in the phase images did not coincide with a fluorescently labeled dense body. Most of these structures were found along the edges of the cells, however, and may have been phase dense surface features. The excellent coincidence of the structures in the two types of images indicated that most of the phase dense structures were indeed dense bodies.

In the analysis that follows, phase contrast images of contracting cells were examined to study the motion of dense bodies during shortening. Although, as indicated by the above results, cells labeled with fluorescent alpha actinin antibody could also have been used for these experiments, there appeared to be three advantages to the use of phase images. (a) Cells could be studied immediately after permeabilization and did not have to be incubated with antibody and subsequently centrifuged and washed to remove unbound antibody. (b) The possibility that shortening of the cells was altered by the antibody itself did not have to be considered. (c) Because the phase images were brighter than the fluorescence images it was not necessary to sum as many frames to obtain acceptable digital images. This minimized loss of resolution due to blurring induced by motion.

Movement of Dense Bodies during Contraction

If dense bodies in smooth muscle cells are the structural

equivalent of the Z disks in skeletal muscle and form the attachment sites for actin filaments they should be an integral part of the contractile apparatus. Their movement during cell shortening might thus be indicative of the way in which forces are distributed in the cytoskeleton during the contractile process. To see if dense bodies could be followed during cell shortening, video tape recordings of contracting cells were examined and digital phase contrast images (Fig. 4, a-c) of the cells were collected at fixed time intervals either in real time or from the video tapes. Although some dense bodies moved into and out of the plane of focus of the microscope during shortening, most bodies (see below) in the digital images could be identified over relatively long time intervals. This was especially true if the shortening rate of the cells was reduced by the use of lower ATP (1 mM) in the superfusion fluid (see Kargacin and Fay, 1987) and a series of images collected at short time intervals was examined. In Fig. 4 d, 10 images of a portion of the cell in Fig. 4, a-c are shown. These images were collected 3 s apart and in them two bodies (marked with arrows in the first and last panels) can be followed during the contraction.

To determine what percentage of dense bodies remained in the focal plane of the microscope (estimated to be 3-4- μm -thick in phase images observed with the 100 \times ; 1.3 NA objective) during 25-30 s of cell shortening, 20 arbitrarily chosen bodies in each of three cells were marked in an initial digital image and then identified in a series of subsequent images collected at 2-3-s time intervals. After ~ 30 s of shortening (27, 28, and 24 s, respectively) $87.5 \pm 7.5\%$ of the originally marked bodies could still be identified in the cells. For the analysis reported here images were collected with the microscope focused at the center of the cells. This was done to extract the most information from the cells because the relatively thick focal plane of the microscope ($\sim 1/3$ the cell diameter) would make more dense bodies visible in the central plane than in more peripheral planes.

Although it was possible to observe the movement of dense bodies in skinned smooth muscle cells during contraction, visual analysis of this movement was not highly informative because of the complexity of the intracellular organization. There were many dense bodies visible in each cell and it proved to be impossible to describe their motion since, at any one time, only a few bodies could be successfully followed by eye. It was thus necessary to find a way to label the bodies and to locate them with respect to some frame of reference. For analysis of their motion, 80-100 dense bodies were labeled, as described in Materials and Methods, in each image of a series collected 2 or 3 s apart. As illustrated by the images in Fig. 4, a-c, however, some cells translated in the field of view during contraction. If the locations of the marked bodies were plotted on a coordinate system fixed with respect to the field of view, the coordinates of individual bodies at different times would reflect not only their positions within the cell but also the overall translation of the cell. To avoid this, the positions of individual dense bodies were determined with respect to a reference system that was fixed on the cell. The y axis of this coordinate system was located along the midline of the cell and the origin established at the top of the image if the cell contracted upward (Fig. 5 a) or at the bottom if it contracted downward. Although such a reference system would not be satisfactory if portions of a cell bent with respect to one another during shortening, for the

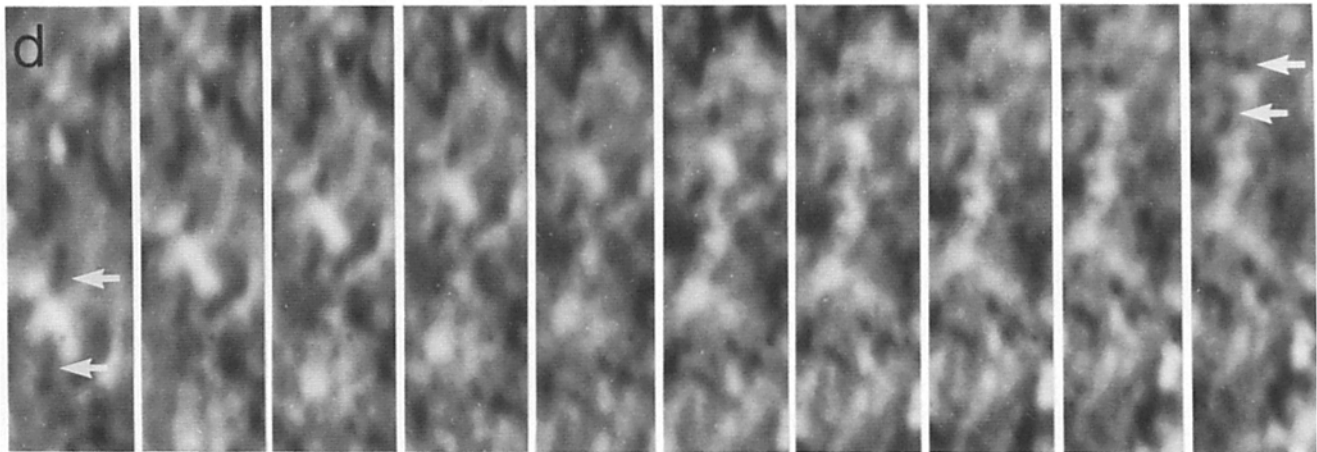
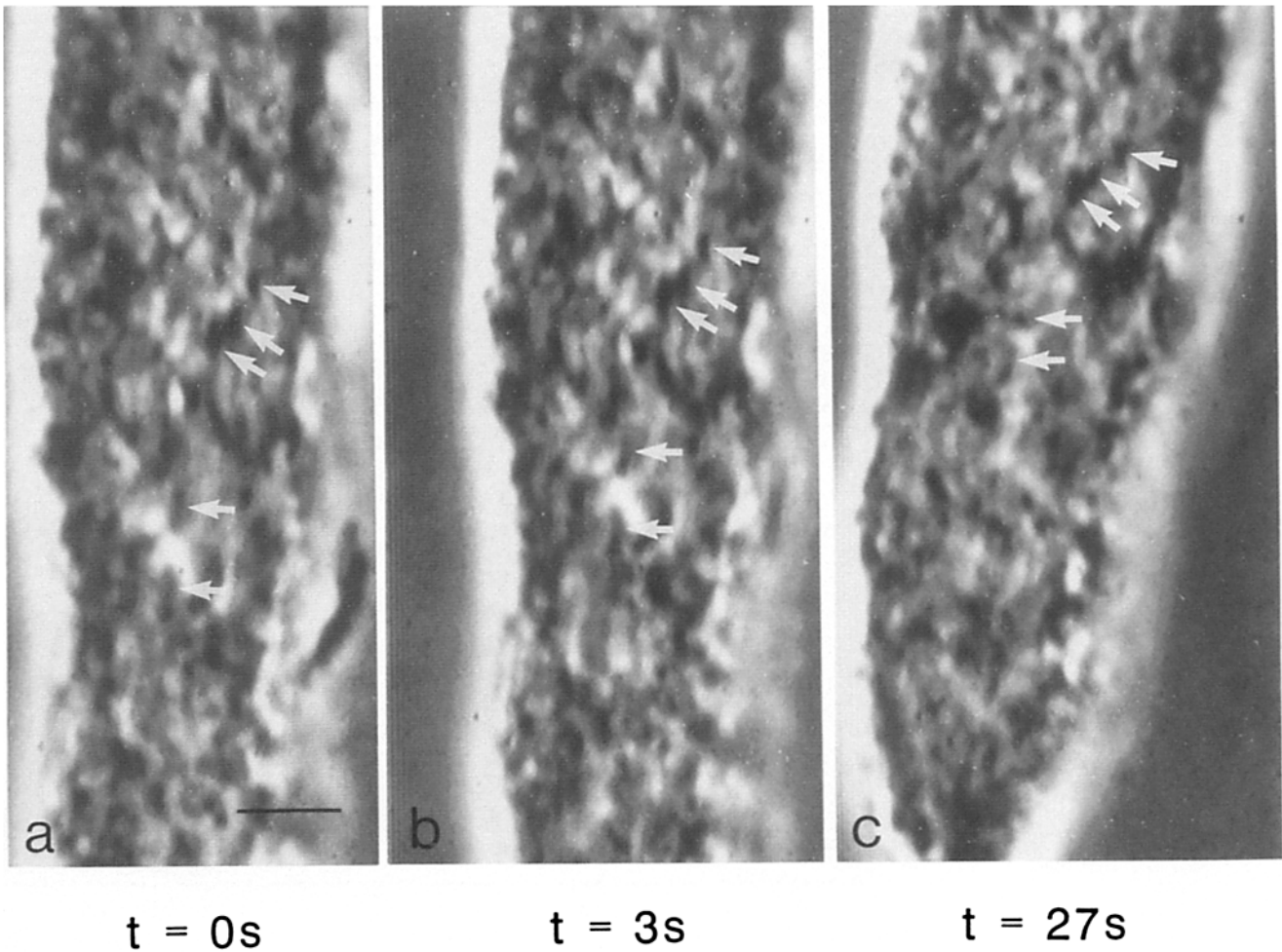


Figure 4. Digital phase contrast images of a saponin permeabilized single smooth muscle cell at various stages of contraction. The initial positions of five dense bodies in the cell are marked by the arrows in *a*. The same five bodies are marked by the arrows in image *b*, collected after 3 s of shortening and in image *c*, collected after 27 s of shortening. From comparison of a series of images collected at short time intervals as in *d*, it was possible to follow the same dense bodies over long intervals. The two bodies marked in the first and last section of the series of images in *d* are the same bodies marked by the lower two arrows in *a*, *b*, and *c*. Bar, 5 μm .

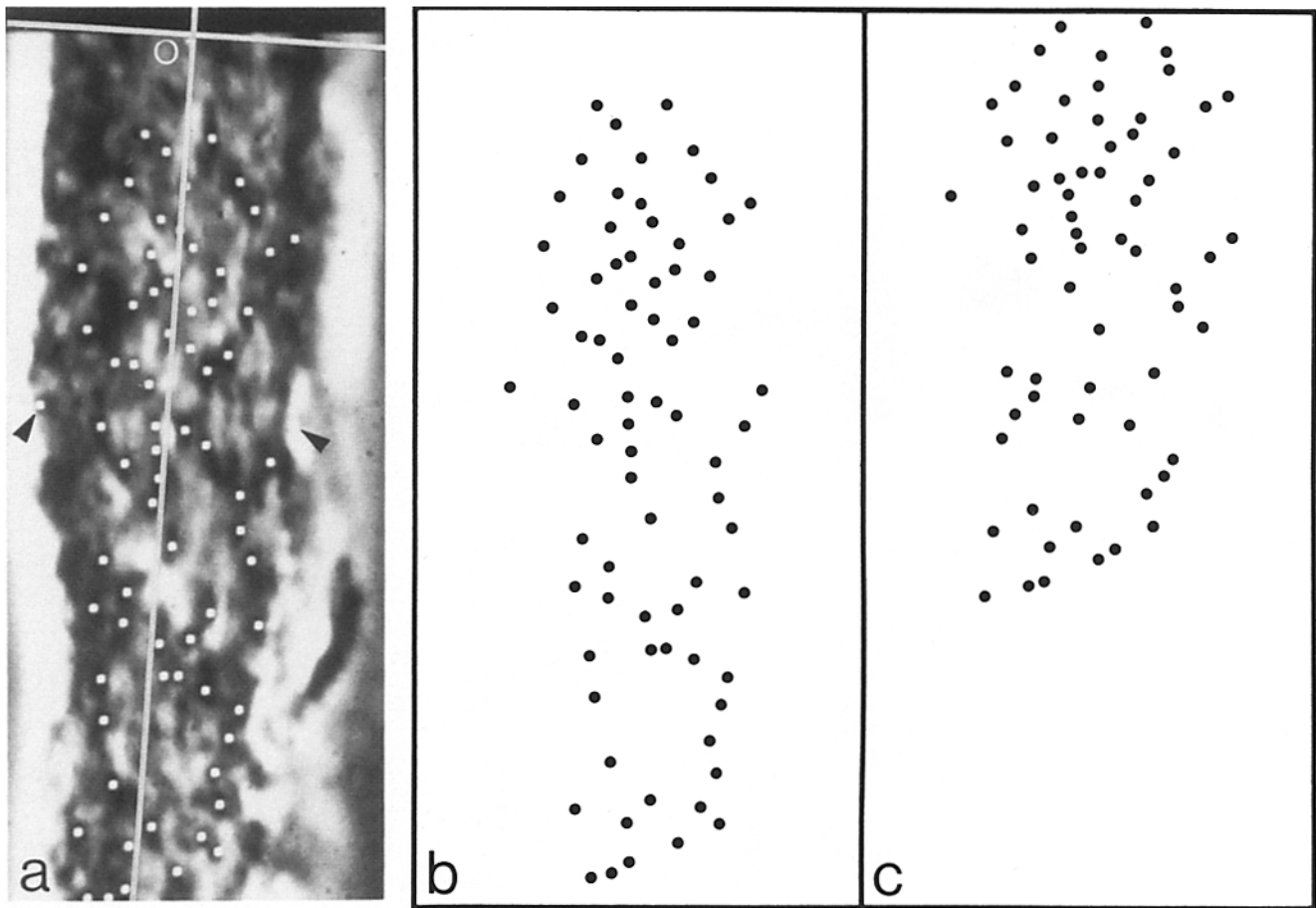


Figure 5. Generation of dense body maps from digital images of contracting smooth muscle cells. (a) Dense body positions in cells (white spots) were plotted on a coordinate system oriented along the long axis of the cell. The origin of this system (*o*) was established at the top of the frame and midway between the sides of the cell in this example. The y axis passed through the origin and midway between two surface structures (arrowheads) that could be identified throughout a contractile sequence. (b and c) The positions of the dense bodies in a are shown at the beginning (b) and end (c) of a 27-s contractile sequence. The coordinate system and the dense body maps have now been rotated so that the y axis is vertical.

relatively short cell segments studied here such bending did not appear to be significant. Fig. 5, b and c show the initial and final positions of some of the dense bodies in the cell in Fig. 5 a plotted on a coordinate system, as described, that followed the cell as it moved in the field of view.

Trajectories of Dense Bodies

When a smooth muscle cell shortens its diameter increases. If the cell was organized so that the forces acting on dense bodies were distributed uniformly throughout the cytoplasm or if dense bodies moved passively in cells during contraction, it might be possible to accurately predict the final positions of the dense bodies within a cell during contraction from their initial positions if the extent of shortening and concomitant diameter increase were measured. If, as seems more likely, this is not the case, it might nevertheless be possible to obtain information about the organization of a cell from any discrepancies between the actual motion of dense bodies and that predicted by such a model. The following experiments were conducted to examine these possibilities.

To predict the way in which dense bodies would move during a contraction if they were acted on by uniformly dis-

tributed forces or if they moved passively, the shortening and diameter increase of skinned cells were measured. Shortening was determined from the initial and final positions of points within cells that were far apart axially. The diameter increase was determined from points that marked surface features that could be identified throughout a contraction. A linear transformation based on these parameters was used to predict the final positions and trajectories of dense bodies given their initial positions in a cell. Fig. 6 a is a schematic drawing of a contracting smooth muscle cell showing the position of the same dense body at three times during the contraction. These positions, when plotted in a single reference frame, trace out a trajectory for the body. Fig. 6 b shows the trajectories of all the marked bodies in a cell with the trajectory (arrow) of one body emphasized with darker symbols. This trajectory is shown again in Fig. 6 c on the left. The open circles are 4 μm in diameter and represent an estimate of the maximum error (see Materials and Methods) in the measured positions of the body. On the right is shown the trajectory predicted by the model for the same body moving passively or acted on by evenly distributed forces. Comparison of the trajectories in Fig. 6 c indicates that the motion

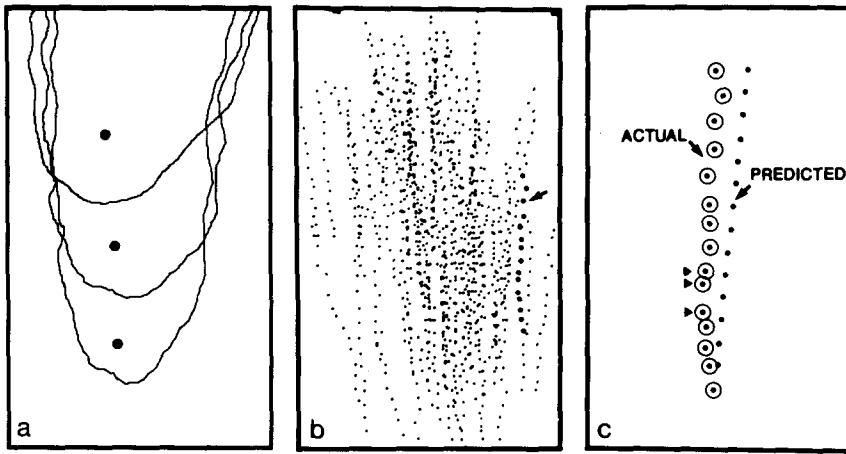


Figure 6. Trajectories of the dense bodies in a contracting cell. (a) Schematic drawing of a contracting cell showing the track or trajectory of one dense body. (b) Actual trajectories of all the marked dense bodies in one cell during a 28-s contractile sequence. The cell contracted toward the top of the figure. The trajectory of the dense body shown in c is indicated with darker symbols and the arrow. The trajectories indicate that most of the motion of the bodies was axial although a slight radial flaring of the trajectories with time can be seen, reflecting the increase in diameter of the cell as it shortened. The positions of the bodies are plotted at 2-s intervals. (c) The actual trajectory of a dense body is shown on the left. The open circles surrounding the points

are 4 μm in diameter and represent the possible error in the marked positions of the dense body. The trajectory predicted by the uniform motion model for the same dense body is shown on the right. The initial position of the dense body and the extent of shortening and diameter increase of the cell were used to construct the predicted trajectories.

of the body deviates throughout the whole contraction from that predicted for a body moving passively or acted on by evenly distributed forces. Furthermore, it seems unlikely that this discrepancy could be accounted for entirely by an error in the marked positions of the body. Analysis of the trajectories of the other marked bodies in this and other cells also indicated that dense body motion was not uniform. Overall, when predicted and actual trajectories were com-

pared there was no obvious pattern to the nonuniformities observed to indicate that they could be attributed, in any cell, to a specific cause such as the rotation of the cell about its axis during shortening.

Because of the diameter increase that accompanies cell shortening, one would expect dense bodies to have both an axial and a radial component to their motion. Unlike the predicted trajectory, the actual trajectory of the dense body

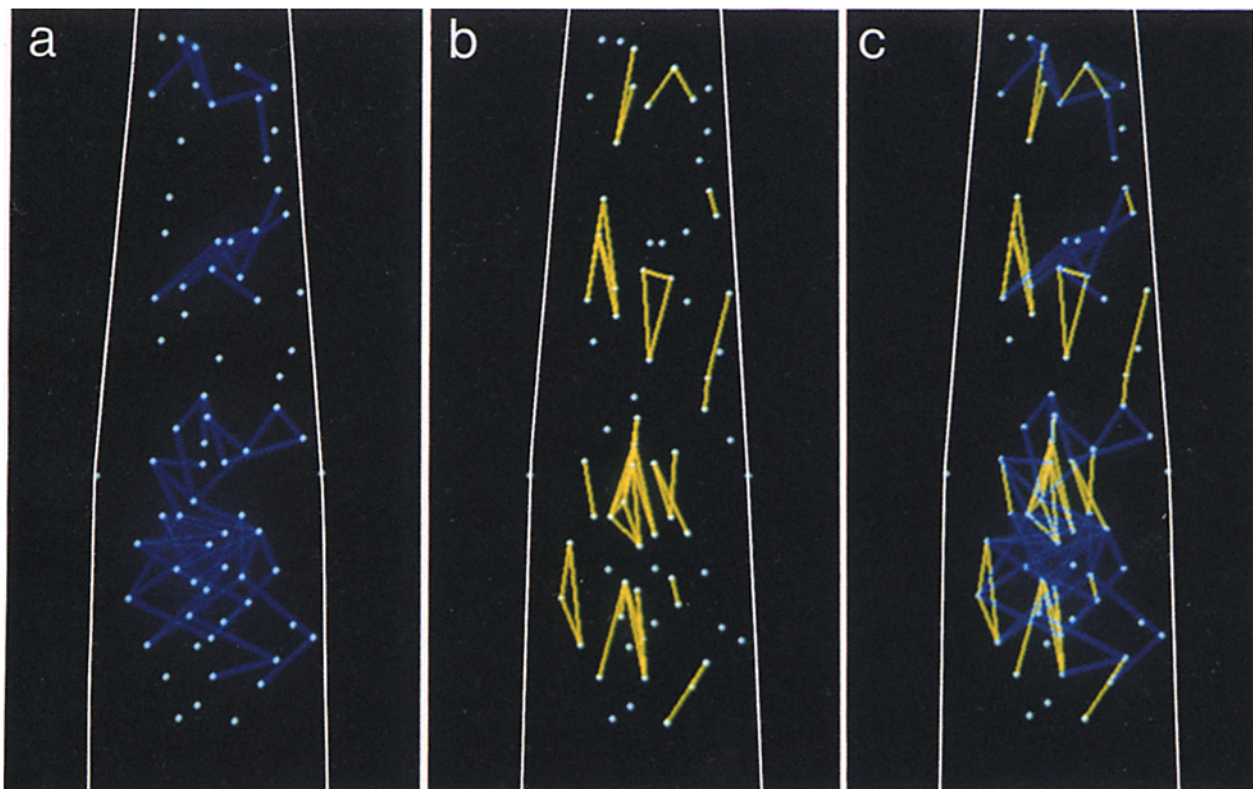


Figure 7. Dense body maps of a cell showing bodies that moved relatively little with respect to one another during shortening connected by blue lines (a and c) and those that move rapidly toward one another during contraction connected by yellow lines (b and c). The lines from a and b are overlaid in c. The white lines in the figure mark the approximate position of the cell edges.

in Fig. 6 *c* deviated little from the axial (horizontal) direction. This indicates that the body moved less radially than predicted by the extent of the diameter increase of the cell. This constrained radial motion appeared to be a general feature of the motion and was evident when the separation of pairs of bodies, located at the same axial positions within cells, was examined before and after shortening. The results of this analysis (not shown) indicated that, although some dense bodies did separate radially to the extent expected from the observed increase in cell diameter, in general, this was not the case. Most bodies moved less than expected radially and a few separated more than expected.

When the trajectories of single bodies or small groups of bodies were examined alone or compared to one another, two other features of the motion became apparent. First, the rate at which bodies moved was not uniform throughout the shortening (Fig. 6 *c*, *arrow heads*). Second, although the trajectories of some nearby bodies were similar to one another, other trajectories were markedly different from one another. These results further indicated that dense body motion could not be described by the model we suggested above and motivated a more detailed study of motion. The complexity of the information contained in trajectory maps such as the one shown in Fig. 6 *b* indicated that more quantitative methods of analysis were necessary for such a study. The approach described in the next section was therefore developed.

Movement between Dense Bodies

The observation that some dense bodies had trajectories that were similar to one another but different from those of other bodies suggested that some bodies might be structurally linked or constrained in such a way that they moved together. If this was true one might expect such bodies to be near one another and the distance between them to remain relatively constant as cells contracted.

To examine the relative motion of dense bodies, the distance between each pair of bodies was determined at the beginning and end of a contractile sequence. From this information the ratio of the final separation of each pair of bodies to their initial separation was computed. Thus, this separation ratio would be less than one if two bodies moved toward one another, greater than one if they moved apart and equal to or near one for bodies that were constrained or linked to one another in some manner so that they remained the same distance apart as they moved. To display the ratio information, the points on a dense body map that had a separation ratio that fell within any chosen range were connected by lines. When analysis of the relative motion was carried out and pairs of bodies with ratios approximately equal to one were connected, it was found that there were groups of nearby bodies in cells that remained at more or less fixed distances from one another during shortening. The groups found in one cell are shown in Fig. 7 *a*. In this case bodies were connected by blue lines if their separation ratio fell between 0.96 and 1.04. The range of ratios near one was chosen to compensate for errors in the marked positions of the bodies. The maximum length of the lines drawn in this figure was 5 μm . This distance was slightly greater than twice the average distance between dense bodies in cells (see Fay et al., 1983). Thus, in general, the relative motion of nearest neighbors and next nearest neighbors was analyzed. The con-

nected bodies in the cell shown in Fig. 7 *a* appear to fall into more or less lateral arrays separated from one another by regions without connections.

Groups of bodies such as those shown in Fig. 7 *a* were found when separation ratios were computed from dense body positions at the beginning and end of a contractile sequence. It is possible that bodies that appeared not to move relative to one another actually did so but fortuitously ended up the same distances apart at the beginning and end of the sequence. To test this possibility, separation ratios were computed for all of the 2- or 3-s sub-intervals during the shortening. When this was done, it was found that bodies that were the same distance apart at the beginning and end of a shortening sequence also did not move much relative to one another during the shortening. For example, in the cell shown in Fig. 7 *a*, 75% of the connected bodies maintained a separation ratio between 0.96 and 1.04 for most (seven out of nine) of the 3-s sub intervals in the shortening sequence (91% maintained a ratio between 0.93 and 1.07 for seven out of nine sub-intervals).

When lines were drawn on dense body maps connecting bodies that moved the most toward one another during shortening (separation ratios <0.7) these connections were seen to generally fall between the lateral arrays of more rigidly connected bodies. This is shown in Fig. 7, *b* and *c* where the bodies that moved closest toward one another (ratio between 0 and 0.7) are connected by yellow lines (the portion of the cell studied shortened to 0.76 its initial length over the time interval).

The axial separation of the lateral, semirigid groups of bodies appeared to be about the same in three of the cells analyzed and was $\sim 6 \mu\text{m}$ (the average center to center separation was $6.2 \pm 0.8 \mu\text{m}$ SD; nine measurements in three cells). Analysis of a fourth cell also revealed the presence of semirigid groups of bodies although the axial separation of the groups was somewhat less apparent. Similarly, regions of the cell where a lot of shortening occurred between pairs of dense bodies were also found at intervals along the cell axis. The average separation between such regions in all four of the cells studied was $6.4 \pm 1.0 \mu\text{m}$ SD (10 measurements). This is illustrated in Fig. 8 where images such as that shown in Fig. 7 *b* were overlaid so that the centers of the upper most regions of maximum shortening were aligned. The arrows in the figure are drawn at 6.4- μm intervals.

As cells contracted, the semirigid dense body groups changed shape even though the relative separation of the bodies within them remained more or less constant. This is illustrated in Fig. 9 where the same group of bodies is shown at the beginning of a contractile sequence and 24 s later at the end of the sequence. The final configuration of the group is not a simple geometric transformation of the initial configuration suggesting that the group was distorted by unequal forces acting on different parts of it during the contraction.

Discussion

The changes in the optical properties of smooth muscle cells that result from saponin treatment have provided us with an opportunity to directly study the movement of specific contractile structures within the cells during shortening without the introduction of fluorescent labels. We have previously discussed possible explanations for these optical changes and

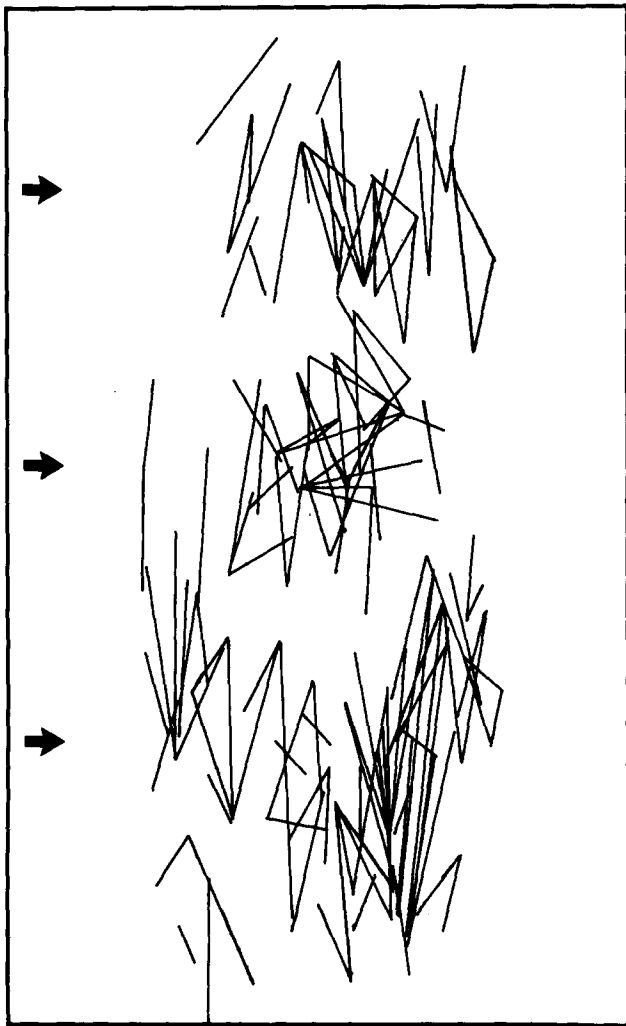


Figure 8. Overlay of areas of maximum shortening between dense bodies in four cells. The upper most areas from the four cells were aligned so that their centers coincided. The arrows are drawn at $6.4\text{-}\mu\text{m}$ intervals along the long axes of the cells. Because the four cells studied varied in diameter, the cell diameter was normalized before the images were overlaid.

have examined, in detail, the effects of saponin treatment on the physiological properties of the cells. This work (Kargacin and Fay, 1987) indicated that the major contractile proteins were retained by the cells and that the contractile and regulatory mechanisms of the skinned cells were similar to those of intact cells. As a result of this we believe that the findings we have presented in this paper are applicable to both skinned and intact smooth muscle cells.

The coincidence of the phase contrast and fluorescent images (Fig. 3) leaves little doubt that the punctate phase dense structures are dense bodies. One of the findings of this study, that some bodies remain at more or less fixed distances from one another during contraction, suggests that such bodies are linked or constrained by noncontractile elements. It is also possible, however, that the seemingly rigid groups represent areas where shortening had not yet started or had already finished. This seems unlikely for two reasons. First, these areas were not arranged in a pattern that would be consistent with a wave of activation spreading through a cell. Second,

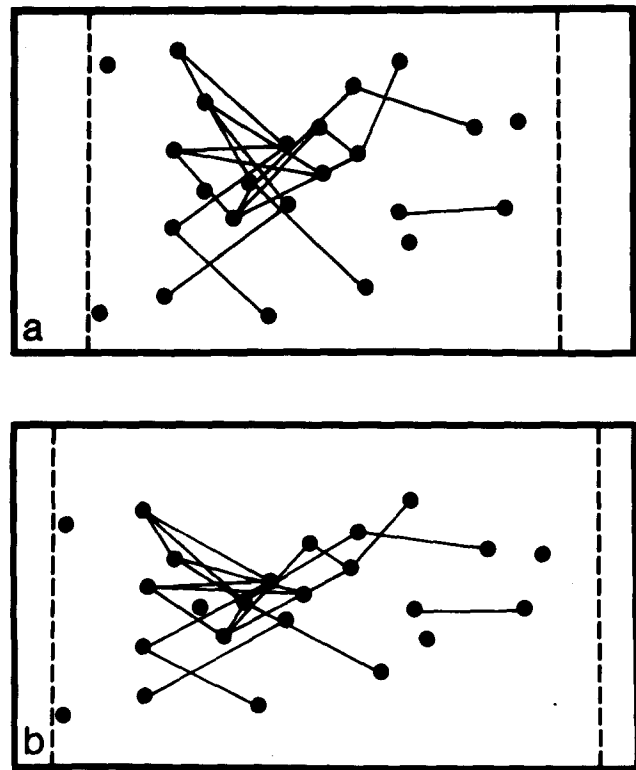


Figure 9. Distortion of a semirigid group of dense bodies during cell contraction. The bodies in one group (lines connect dense bodies whose separation ratio was between 0.98 and 1.02) are shown at the beginning (a) and end (b) of a 24-s contractile sequence. The long axis of the cell is vertical. An $8.7\text{-}\mu\text{m}$ portion of the cell is enclosed between the top and bottom of the sections. The approximate positions of the sides of the cell are indicated by the dashed lines. As the cell shortened, the axial (vertical) dimension of the group decreased and the radial (horizontal) dimension increased. A simple geometric transformation (vertical compression and horizontal extension) was not sufficient to transform the positions in a into those in b.

the rigidity was present for at least 30 s, longer than the time required for ATP to diffuse into skinned cells (see Kargacin and Fay, 1987). It thus seems more likely that the connections or constraining structures are noncontractile. Our results also suggest that shortening may take place between these networks of interconnected bodies (Fig. 7 c). These possibilities raise a number of interesting questions about the dense body groups and the links between the elements within them. (a) Is there other evidence for the existence of such groups? (b) What molecules are involved in the linkage? (c) How rigidly connected are the elements? (d) What is the three-dimensional structure of the groups?

There is some structural evidence that dense bodies may be found in groups in smooth muscle cells. Although, in general, under phase contrast, dense bodies appeared to be distributed uniformly in any image plane (Fig. 1 c), in some cells there were regions with relatively few bodies. Bagby (1980) and Fay et al. (1983) have also noted lateral groupings of dense bodies in cells labeled with fluorescent antibodies to alpha actinin.

Although, as we have described, there is an axially oriented fine fibrillar structure visible within skinned cells in

phase contrast images, we have not seen evidence for more laterally directed structural ties nor are such connections apparent in electron micrographs of the cells (Fig. 2 *b*). Cooke and Fay (1972), Cooke (1976), Cooke (1983), Bond and Somlyo (1982), and Tsukita et al. (1983) have proposed that intermediate filaments link dense bodies in smooth muscle cells but the limited field that can be examined with the electron microscope prevents visualization of more than a few bodies at any one time. It is of interest to note, however, that Cooke (1976) and Tsukita et al. (1983) found that when isolated, dense bodies appeared to be found in groups suggesting that some linkage may have survived the isolation procedure. The possibility also exists that some actin filaments may be part of the noncontractile network. Small et al. (1986) have suggested that some actin in smooth muscle may have a structural rather than a contractile function. The question of the existence of such connections will perhaps be resolved by studies with fluorescent probes. There are a number of actin binding proteins (see Craig and Pollard, 1982) and proteins that are associated with the Z line structures in skeletal and cardiac muscle (see Gard and Lazarides, 1980; Tokuyasu et al., 1984; Fishman, 1970) that have been found in smooth muscle. Among these, desmin and vimentin have been implicated in the assembly of Z disks and in the alignment of these structures in developing skeletal muscle (Gard and Lazarides, 1980; Tokuyasu et al., 1984), and vinculin (Geiger et al., 1980; Pardo et al., 1983) has been associated with the attachment between skeletal muscle myofibrils and the sarcolemma. Desmin has been found at periphery of the dense bodies within smooth muscle cells (Small et al., 1986) and is a constituent of 10-nm filaments (Hubbard and Lazarides, 1979). Vinculin is found in membrane-associated dense plaques (Geiger et al., 1981; Small, 1985). In developing skeletal muscle, alpha actinin aggregates into discrete structures which then become aligned during the formation of Z lines (Sanger et al., 1984; McKenna et al., 1986). In light of this and our results it is interesting to speculate that the same process may be involved in the formation of Z lines in skeletal muscle and dense bodies in smooth muscle but that the alpha actinin containing structures in skeletal muscle eventually become more tightly grouped.

Analysis of the relative motion of dense bodies was confined to bodies that were within 5 μm of one another. This eliminated from consideration, pairs of bodies that were far apart from one another radially but near one another axially. The separation ratio of such bodies would be dominated by the radial component of the distance between them. This would have introduced an artifact into the analysis since the separation ratio for such bodies could remain close to one even though the axial separation of the bodies changed. We also assumed that dense bodies were rigidly connected if their separation ratio for a contractile sequence fell within a range of values centered around one. This rather loose definition compensates for any error in marking the bodies from image to image but precludes, somewhat, any attempt to determine the true rigidity of the connections. With this in mind, however, examination of their overall morphology at the beginning and end of a shortening sequence (Fig. 9) indicates that the groups of bodies become distorted during contraction. This may be due in part to the fact that we were looking at a three-dimensional structure in two dimensions but also suggests that even though the distances between pairs of bod-

ies remain more or less constant the angles between the connections can change. The structure thus appears to be flexible to some extent rather than strictly rigid. This suggests that the bodies may be part of a noncontractile network that is distorted by non uniform forces, both active (contractile elements) and passive (cytoskeleton), acting on it during contraction.

A number of models describing the arrangement of the contractile structure in smooth muscle have been proposed (see Bagby, 1983). Our results are most consistent with those that suggest that both cytoplasmic and membrane dense bodies act as attachment sites for actin filaments and are an integral part of the contractile apparatus. We cannot, at the present time, say whether the groups of interconnected dense bodies that are suggested by our results include only cytoplasmic bodies or both cytoplasmic and membrane bodies. The contractile elements in the cell could link bodies that are members of separate groups and/or bodies within the groups to those on the cell surface. These possibilities have been incorporated into the model shown in Fig. 10.

It has been proposed (see Bagby, 1986) that the attachment sites for the contractile filaments in smooth muscle are helically arranged, and Fisher and Bagby (1977) and more recently Warsaw et al. (1987) have cited the "corkscrewing" of smooth muscle cells during contraction as evidence for this arrangement. The slight skewing of the areas of maximum shortening and the semirigid groups of dense bodies with respect to the long axis of the cell, evident in Fig. 7, could be an indication of a helical structure. However, although perhaps one-fourth of the cells we have observed do appear to corkscrew as they shorten we do not see this happen consistently nor is there a consistent handedness to the movement. The trajectories of dense bodies in most cells such as those shown in Fig. 6 *b* do not provide evidence of helical motion. If the contractile elements in smooth muscle are indeed attached to a helically arranged dense body structure our evidence would be more consistent with a pattern of contraction akin to the shortening of a stretched spring rather than a corkscrewing spring.

Our results indicate that analysis of the motion of elements within contractile cells can give clues to the structural organizations of these elements and to overall subcellular morphology. The work we have presented here raises a number of interesting questions about the contractile apparatus in smooth muscle that can be readily studied. It should be possible to combine, in the same cell, motion analysis with three-dimensional optical sectioning to resolve the three-dimensional structure of the semirigid groups. It should also be possible to use fluorescent probes to label other contractile elements in skinned cells, to determine their morphological relationship to the dense body structures, and to study the motion of these elements during shortening.

The methods we have developed and adapted for the analysis of the motion of dense bodies within smooth muscle should be directly applicable to a number of other studies of cellular motion. It should be possible to track the motion of structures and organelles within or on the surface of cells as we have done, and to objectively sort through this information to search for like patterns of behavior. This coupled with the analysis of the relative movement of such structures can provide information about the forces acting on these elements and demonstrate the existence of structural or func-

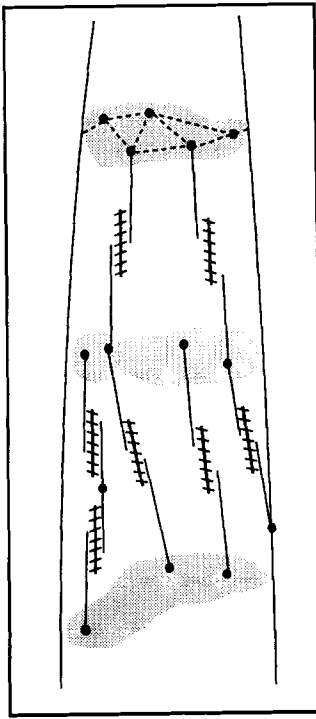


Figure 10. Proposed organization of the contractile apparatus in smooth muscle. Three dense body groups (shaded areas) in a cell are shown and the contractile structures linking them are indicated by solid lines. Myosin filaments are represented as thick lines with a side polar head configuration as suggested by Cooke et al. (1987, 1989). Actin filaments are represented by thin lines. Dense bodies within the groups are linked to one another or constrained by non-contractile elements (dashed lines).

tional ties that link them together. Similar methods of analysis could also be applied to the motion of cells themselves. This approach adds a temporal dimension to the study of cellular organization and should provide a more complete description of the dynamic processes occurring within cells.

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