

# Supercontracted State of Vertebrate Smooth Muscle Cell Fragments Reveals Myofilament Lengths

J. V. Small, M. Herzog, M. Barth, and A. Draeger

Institute of Molecular Biology of the Austrian Academy of Sciences, 5020 Salzburg, Austria

**Abstract.** Isolated cell preparations from chicken gizzard smooth muscle typically contain a mixture of cell fragments and whole cells. Both species are spontaneously permeable and may be preloaded with externally applied phalloidin and antibodies and then induced to contract with Mg ATP. Labeling with antibodies revealed that the cell fragments specifically lacked certain cytoskeletal proteins (vinculin, filamin) and were depleted to various degrees in others (desmin,  $\alpha$ -actinin). The cell fragments showed a unique mode of supercontraction that involved the protrusion of actin filaments through the cell surface during the terminal phase of shortening. In the presence of dextran, to minimize protein loss, the supercontracted products were star-like in form, comprising long actin bundles radiating in all directions from a central core containing myosin, desmin, and  $\alpha$ -actinin. It is concluded

that supercontraction is facilitated by an effective uncoupling of the contractile apparatus from the cytoskeleton, due to partial degradation of the latter, which allows unhindered sliding of actin over myosin. Homogenization of the cell fragments before or after supercontraction produced linear bipolar dimer structures composed of two oppositely polarized bundles of actin flanking a central bundle of myosin filaments. Actin filaments were shown to extend the whole length of the bundles and their length averaged  $\sim 4.5 \mu\text{m}$ . Myosin filaments in the supercontracted dimers averaged  $1.6 \mu\text{m}$  in length. The results, showing for the first time the high actin to myosin filament length ratio in smooth muscle are readily consistent with the slow speed of shortening of this tissue. Other implications of the results are also discussed.

ATTEMPTS to explain the special contractile properties of vertebrate smooth muscle, including slow and extensive shortening as well as economic maintenance of tension, have been hindered by a lack of information about the spatial organization of the contractile elements (see Murphy, 1979, for example). Several structural models have been proposed (reviewed in depth by Bagby, 1983, 1986) but no general consensus has been reached about the nature of the basic contractile unit, nor about the mode of association of the myofilaments with components of the cytoskeleton. One central problem has been to define the lengths of the actin and myosin filaments since the absence of obvious sarcomeres precluded their determination by the same direct means as used for striated muscle decades ago (Huxley, 1957). Measurements of myosin filament length have been made in sections of fixed material (Ashton et al., 1975) and from preparations of isolated smooth muscle cell homogenates (Small, 1977; Cooke et al., 1989) and have yielded values ranging from around two to several microns. But in view of the acknowledged lability of smooth muscle myosin filaments under physiological ionic conditions *in vitro* (e.g., Suzuki et al., 1978; Kendrick-Jones et al., 1983), the validity of these values as well as the idea of a constant thick filament length *in vivo* continues to be questioned (Gillis et al., 1988). Hitherto it has proved impossible to obtain any data

on actin filament lengths, either from fixed material or from cell homogenates.

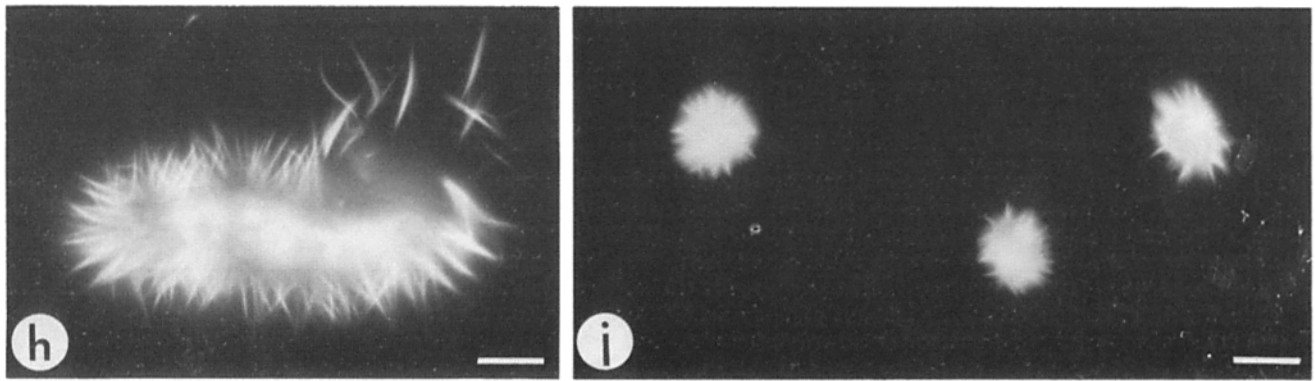
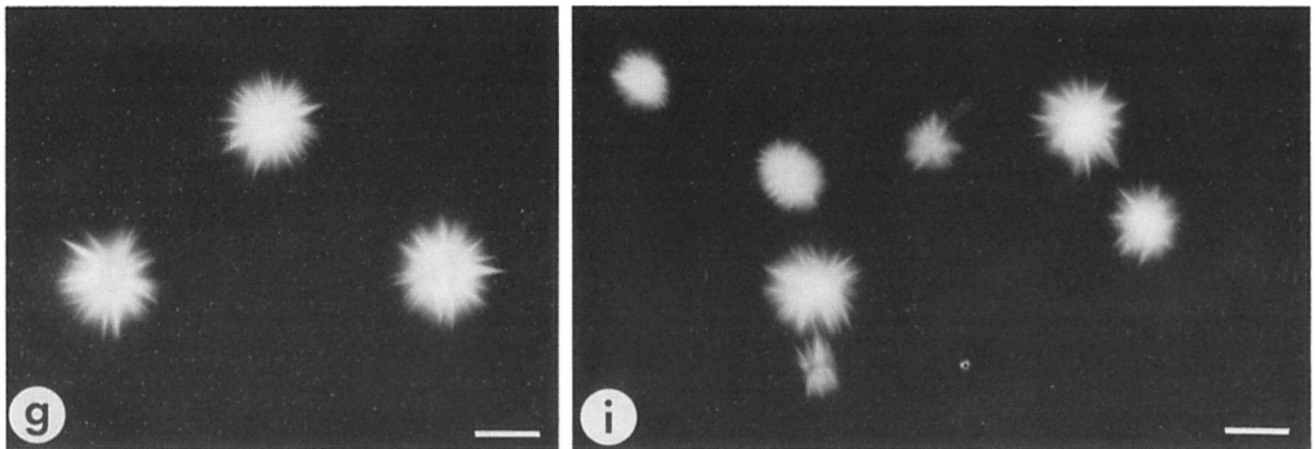
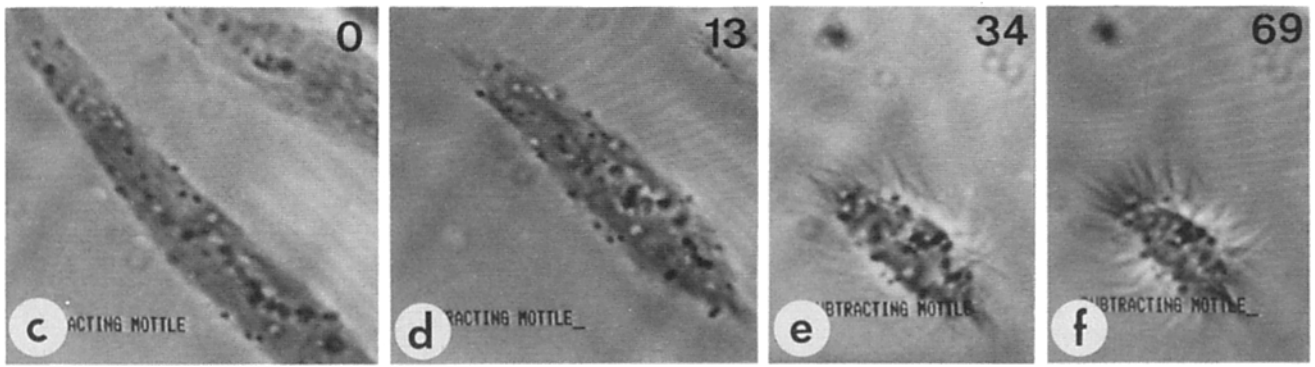
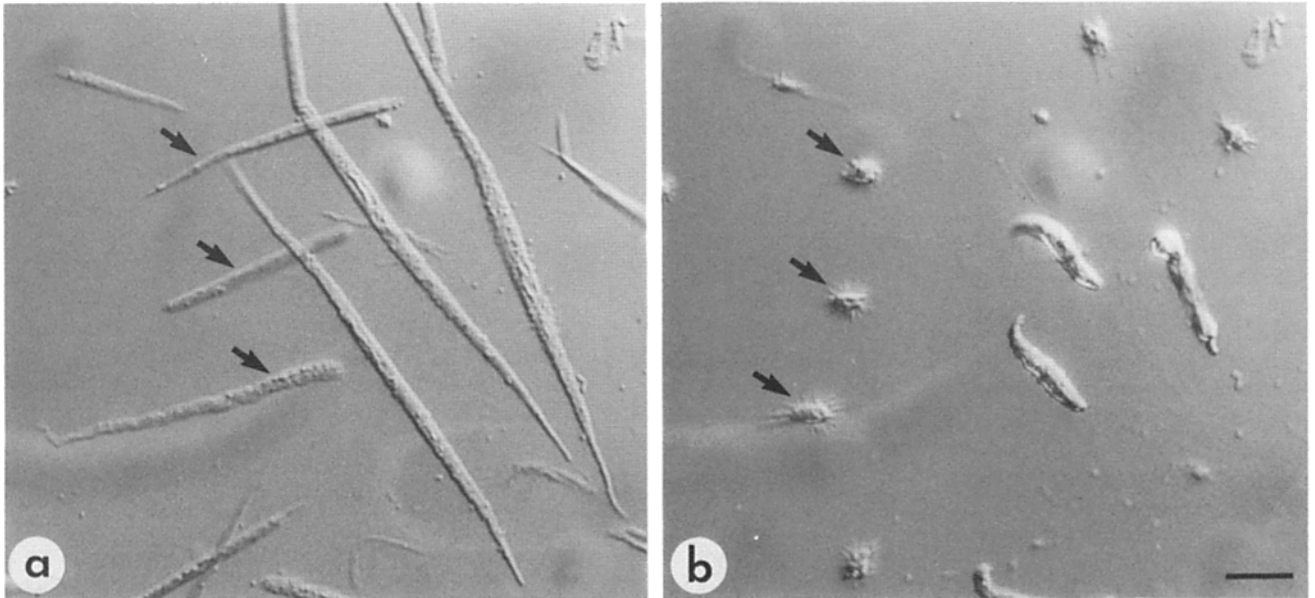
In the present study we describe a supercontracted state of isolated, permeabilized gizzard smooth muscle cell fragments that reveals both the lengths of the myofilaments after contraction as well as new features of the contractile apparatus. The slow shortening speed of smooth muscle, as well as the large range of shortening, are now readily explained by the existence of actin filaments that are several fold longer than the myosin filaments.

## Materials and Methods

### Isolation of Gizzard Smooth Muscle Cells and Cell Fragmentation

Cells were obtained from adult chicken gizzards essentially as described previously (Draeger et al., 1989) using the following buffer for digestion with collagenase: (in millimolar) 137, NaCl; 5, KCl; 4, NaHCO<sub>3</sub>; 5.5, glucose; 2, MgCl<sub>2</sub>; 2.5, CaCl<sub>2</sub>; 10, Pipes; pH 6.5. A single modification was the inclusion of the proteolytic inhibitor E 65 (Sigma Chemie GmbH, Munchen, FRG; 50  $\mu\text{g}/\text{ml}$ ) in the collagenase solution during digestion. Isolated cells were washed and stored in solution 1 (in millimolar): 137, NaCl; 5, KCl; 1.1, Na<sub>2</sub>PO<sub>4</sub>; 0.4, KH<sub>2</sub>PO<sub>4</sub>; 4, NaHCO<sub>3</sub>; 5.5, glucose; 2, MgCl<sub>2</sub>; 2, EGTA; and streptomycin 10 mg-liter<sup>-1</sup>, buffered additionally with 5 mM MES, pH 6.1.

Fragmentation of cells was carried out in solution 1 using as homog-



enizer, two 1-ml syringes mounted at the opposing ends of two mutually fitting needles, respectively, 0.4 and 0.65 mm o.d. and 30 mm long joined and sealed together with Super Glue. Cells were drawn up from the stock solution in one syringe and fragmentation was effected by passing the suspension back and forth through the needles 5–20 times, as required.

### Contraction Conditions

The contraction solution contained 2.5–5% dextran ( $M_r$  500,000; Sigma Chemie GmbH) to minimize the loss of myosin and other contractile and cytoskeletal proteins (see Results). The composition, based on that used for skinned smooth muscle (Arner, A., and P. Hellstrand, personal communication) was (in millimolar): 60, KCl; 3,  $MgCl_2$ ; 0.5, DTT; 1,  $NaN_3$ ; 20, imidazole; (pH 6.9) or Pipes 10 (pH 6.5); 1, ATP; supplemented with dextran, 2.5 or 5% (wt/vol).

Contraction was performed either in the test tube by resuspending a pellet of cells, obtained by sedimentation at low speed, in contraction solution, or directly on the microscope slide by flushing the contraction solution under a coverslip raised on two edges by a thin layer of silicone grease. In some cases free contraction of the cells was deliberately hindered by omitting the silicone grease and sandwiching the cells between slide and coverslip (24 × 24 mm) in a total volume of 4  $\mu$ l. Contraction was initiated at the mounting step by placing 2  $\mu$ l of the cell suspension on the slide and 2  $\mu$ l of the contraction solution on the coverslip, before bringing them together.

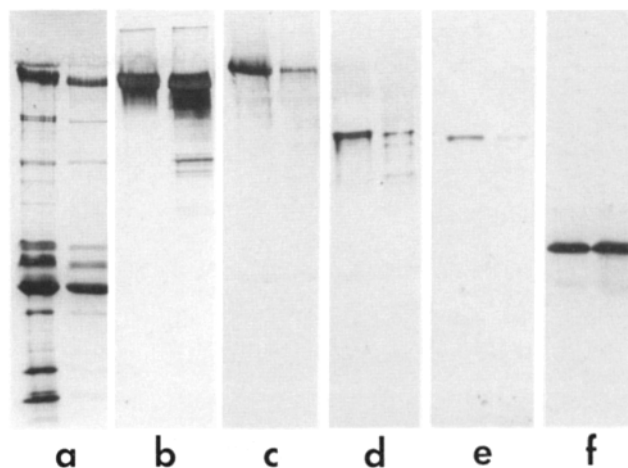
### Fluorescent Labeling

As discussed below, cells isolated from the chicken gizzard were found to be permeable to phalloidin as well as to antibodies, without the need for extra skinning procedures. Antibody labeling was thus performed as follows. The primary antibody was added at the appropriate dilution to the cell suspension in solution 1 and incubated for 45 min at room temperature. The cells were then sedimented in a table centrifuge, the supernatant was discarded, and the cells were washed once in solution 1. The second antibody was added, in the same solution, incubated again for 45 min and the washing step was repeated. As primary antibodies we used rabbit polyclonal antibodies against desmin, filamin, the phosphorylatable smooth muscle myosin light chain, light meromyosin, myosin subfragment -1, and tropomyosin (Small et al., 1986) and vinculin (supplied by Prof. B. Geiger, Weizmann Institute, Rehovot, Israel). The mAbs were directed against desmin (DU 10, supplied by Prof. M. Osborn, Max-Planck-Institut, Göttingen, FRG) and vinculin (vin 13/2, supplied by Prof. B. Geiger). Rhodamine-labeled secondary antibodies were prepared according to Brandtzaeg (1973) and fluorescein-labeled secondary antibodies were purchased from Sigma Chemie GmbH; in some cases the streptavidin-biotin system as supplied by Amersham International (Amersham, UK) was also used.

Fluorescent phalloidin (rhodamine, fluorescein or coumarine conjugated) was provided by Prof. H. Faulstich (Max-Planck-Institut, Heidelberg, FRG) and was diluted directly into the cell suspension to the appropriate concentration.

### Light Microscopy

Preparations were observed and photographed on a Zeiss photomicroscope III equipped with epifluorescence, differential interference, polarization, and phase-contrast optics and a video-enhanced Allen video-enhanced contrast (AVEC)-system (C1966) from Hamamatsu Corp. (Tokyo, Japan). Observation in the fluorescence mode was performed using either a 40× (n.a. 0.75) neofluor phase contrast lens or a 63× (n.a. 1.4) planapo objective with the specimen mounted in the contraction solution (with or without ATP) supplemented, if necessary, with 5 mg/ml *n*-propyl gallate as an antibleach agent (applied by flushing under the coverslip after mounting). Polarization microscopy was performed using a 100× planachromat objective (n.a. 1.3) in combination with the AVEC-system and with the analyzer slightly misad-



**Figure 2.** Immunoblots of gizzard cell preparations showing partial proteolysis of contractile and cytoskeletal proteins in the isolated cells. Two samples, shown on the Coomassie blue-stained gel (a) were used for immunoblotting and corresponded to: *first lane*, freshly homogenized gizzard muscle; *second lane*, a typical isolated cell preparation. The blots shown were obtained using polyclonal antibodies directed against the following gizzard proteins: *b*, myosin heavy chain; *c*, filamin; *d*, caldesmon; *e*, myosin light chain kinase; *f*, desmin.

justed to deliver sufficient light (from a 100-W rear-mounted mercury arc lamp) to the Chalnicon camera. Differential interference contrast microscopy was performed using 6.3, 16, or 40× plan objectives. Images were recorded on the microscope using black-and-white Agfapan 400 professional or Ektachrome 400 color film or taken directly from the television monitor using a Mamiya 70-mm camera loaded with Agfapan 25 film.

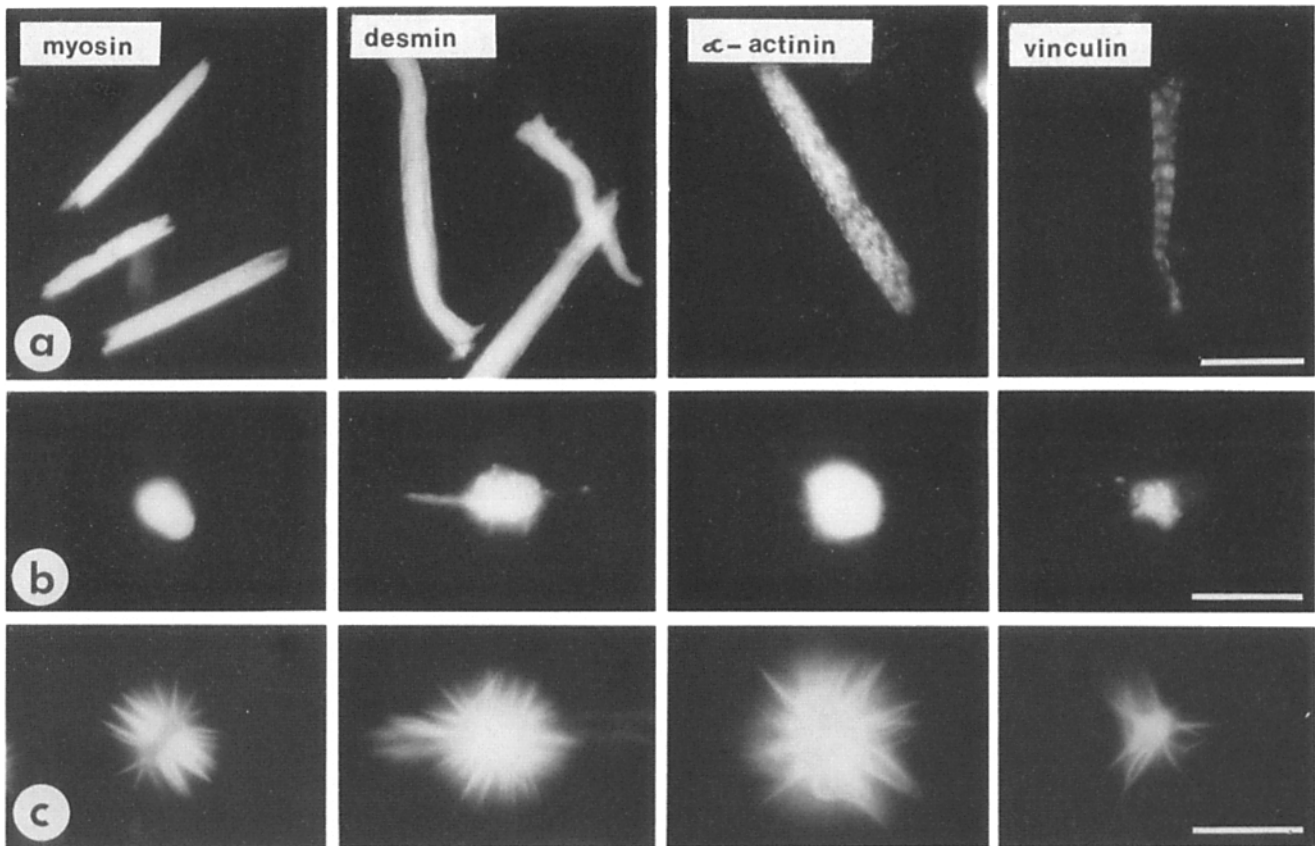
### Electron Microscopy

Supercontracted cells and cell fragments were analyzed in thin sections after conventional fixation and embedding as well as by the negative staining procedure. In all cases the starting cells were labeled with rhodamine-conjugated phalloidin and the various preparative steps were followed, where possible in the fluorescence microscope. Most steps were carried out at room temperature with interim storage of the cell preparations on ice.

For thin-section analysis the stock cells were contracted in the presence of 2.5 or 5% dextran, sedimented at 13,000 *g* for 15 min. in 500- $\mu$ l Eppendorf tubes and the pellets fixed for 1 h in 2.5% glutaraldehyde in the contraction solution (without ATP). After washing in solution 1 the pellets were postfixed in 0.5%  $OsO_4$  for 30 min at room temperature, dehydrated in ethanol and embedded in Araldite. Ultrathin sections, cut on a Reichert Ultracut E Microtome (Reichert Jung, Austria) were contrasted with 1% uranyl acetate in methanol or water, followed by aqueous lead citrate.

Small cell fragments were prepared for negative staining as follows. Cells in the stock solution were pelleted by low speed centrifugation and resuspended in the contraction medium without ATP and with added 2.5 or 5% dextran and 0.2% Triton X-100. The cells were sedimented again, resuspended in the same medium without Triton and homogenized in the double-syringe device (see above). 1 mM ATP was then added to the homogenate and the contraction products monitored in the fluorescence microscope. In some cases homogenization was performed also after the contraction step.

**Figure 1.** Supercontraction of smooth muscle cell fragments. (a and b) Low magnification field of a typical gizzard cell preparation before (a) and after contraction (b), induced by flushing with contraction solution under the coverslip. Only the cell fragments (arrows) and not the intact cells form star structures. Nomarski interference optics. (c–f) Contraction of a cell fragment as visualized using video enhanced polarization optics. The shift in position of the cell in e and f was due to a manual shift of the stage during contraction. Numbers indicate time in seconds after initiation of contraction. Note protrusion of bundles from the cell body in e and f. (g) Fluorescence images of star-like structures as in b and f labeled with rhodamine-conjugated phalloidin. (h) Example of supercontracted “whole” cell labeled with rhodamine-phalloidin and showing protruding actin bundles. (i and j) Supercontracted star-like structures observed in contracted, isolated cell preparations from the taenia coli (i) and vas deferens (j) of the guinea pig. Bars: (a and b) 25  $\mu$ m; (g–j) 10  $\mu$ m.



**Figure 3.** Relocalization of contractile and cytoskeletal proteins after supercontraction. Top row (*a*) shows cell fragments in the preparations as they appeared before contraction and after labeling with the antibodies indicated. Second and third rows (*b* and *c*) show stars in the same preparations, after contraction and double-labeled with the same antibodies (*b*: antibody as in *a* directly above; *c*) phalloidin. Bars: (*a*) 20  $\mu\text{m}$ ; (*b* and *c*) 10  $\mu\text{m}$ .

Simple application of a fragment suspension to a carbon-coated electron microscope grid, followed by negative staining invariably resulted in a very low yield of bound fragments. It was therefore necessary to sediment the fragments onto the grid surface, as follows: carbon-coated grids were mounted into the caps of small Eppendorf tubes and rendered hydrophilic by immersion in 100  $\mu\text{l}$  of a solution of 400  $\mu\text{g/ml}$  bacitracin (Sigma Chemie GmbH) and 0.1% amylalcohol in water; the bacitracin was drained with a pipette and replaced with 50  $\mu\text{l}$  of the fragment suspension and the tubes centrifuged in a swing-out rotor on a table centrifuge at 1,500  $g$  for 3–4 min. The grids were removed with forceps, rinsed with a few drops of 1% aqueous uranyl acetate followed by a few drops of bacitracin and then drained on one edge with filter paper. One drop of uranyl acetate was then applied to the sample side, left 5–10 s, drained with filter paper, and the grid allowed to dry. The use of this three step staining protocol gave the most reproducible result in the electron microscope.

Labeling of supercontracted cells with myosin subfragments was carried out using myosin subfragment-1 purified by gel filtration of a papain digest of chicken gizzard myosin (the myosin was provided by Dr. A. Sobieszek, Austrian Academy of Sciences, Salzburg). Dextran at concentrations  $> \sim 1.5\%$  inhibited the binding of S-1 to actin. S-1 labeling was therefore performed directly on the grid after sedimentation of the fragments, using as solvent the contraction solution lacking ATP and dextran. The myosin subfragment was added at a concentration of 0.5–1  $\text{mg/ml}$ , incubated for 10–20 s and then rinsed off with solvent before negative staining as above.

Electron microscopy was carried out using a Zeiss EM10 operating at 80 kV with a 50- $\mu\text{m}$  objective aperture.

### Immunoelectron Microscopy

Intact cells were incubated in the appropriately diluted first antibody for 45 min at room temperature in solution 1 or contraction solution (see above) without ATP. After centrifugation and washing in solution 1 they were la-

beled in suspension overnight at 4°C in gold buffer (in millimolar: 10, Tris; 155, NaCl; 2, EGTA; MgCl<sub>2</sub>; pH 7.6) containing 5 nm goat anti-rabbit Ig G gold (Janssen Pharmaceutica, Beerse, Belgium). After washing and resuspension in gold buffer the cells were pelleted at 13,000  $g$  and processed for EM as described above.

### Immunoblotting

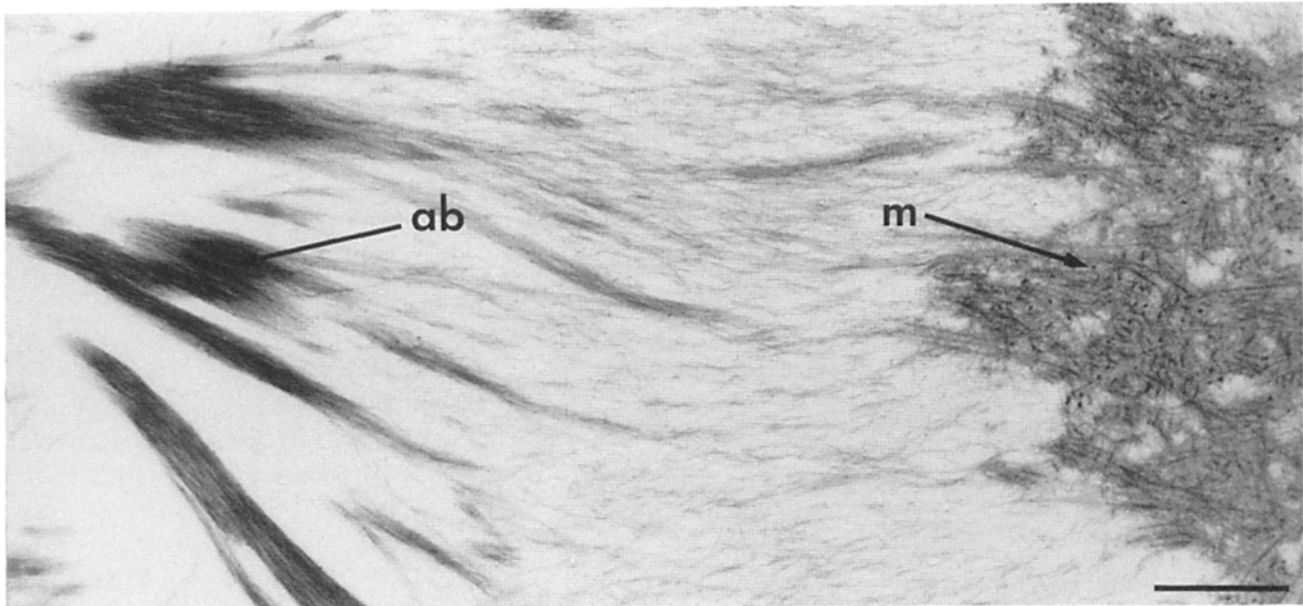
Immunoblotting was carried out essentially as described by Towbin et al. (1979) using 8–22% acrylamide minislab gels (Matsudaira and Burgess, 1978) and the silver enhanced immunogold staining procedure of Moermans et al. (1984).

## Results

### Isolated Cells: Characterization and Conditions

Before discussing the contractile properties of isolated gizzard cells, some aspects of the general experimental conditions and the integrity of the cells are worth mentioning.

When stored in isotonic saline for one to several days in the cold avian gizzard muscle strips gradually lose their reddish tint, attributable to a myoglobin-like protein Gröschel-Stewart et al., 1971) and become pale. This spontaneous loss of membrane integrity was accompanied by the depletion of ATP and entry of the contractile apparatus of the cells into rigor. Accordingly collagenase digestion, performed most suitably 24 h after death yielded mainly rigid, straight cells.



**Figure 4.** Electron micrograph of section of supercontracted star showing the general morphology along one radius, with the myosin filaments (*m*) in the central core and the actin bundles (*ab*) at the periphery. Cells were extracted with Triton X-100 before supercontraction. Bar, 0.5  $\mu$ m.

Typically, such gizzard cell preparations showed heterogeneous mixtures of "intact" cells and cell fragments (Fig. 1 *a*). (The adoption of various other buffers and conditions failed, in our hands, to produce cells with stable, intact membranes.)

In a former study (Kossmann et al., 1987), we demonstrated the loss and rearrangement of contractile proteins in skinned muscles exposed to ATP. Subsequently, it was established that the addition of dextran (*M<sub>r</sub>* 500,000) at 2.5–5% wt/vol as used in one skinned smooth muscle study (Arheden et al., 1987) inhibited the loss of myosin and other proteins into the bathing solution (Small, J. V., R. A. Cross, and P. Hellstrand, unpublished). Dextran was therefore routinely used as an additive in the contraction solution and in skinning solutions, where used.

Immunoblotting of the cell preparations with antibodies against contractile and cytoskeletal components revealed the existence of partial proteolysis, the extent of which varied somewhat from preparation to preparation. It was, however, clear that the presence of more cell fragments correlated with a higher degree of proteolysis, recognized by the appearance of breakdown products or a decreased band intensity of the proteins screened: filamin, caldesmon, desmin, myosin light chain kinase, and myosin (Fig. 2).

#### **Supercontraction of Cell Fragments**

Interesting differences were noted between the contractile properties of the intact cells and the cell fragments obtained routinely in the preparations (Fig. 1, *a* and *b*). Whereas the intact cells contracted into typical, short spindle-shaped forms, the fragments supercontracted into spherical structures with radiating bundles (Fig. 1 *b*, *arrows*). Video-enhanced microscopy with polarization optics revealed that the bundles arose by protrusion from the cell body during the terminal phase of shortening (Fig. 1, *c-f*). Labeling of

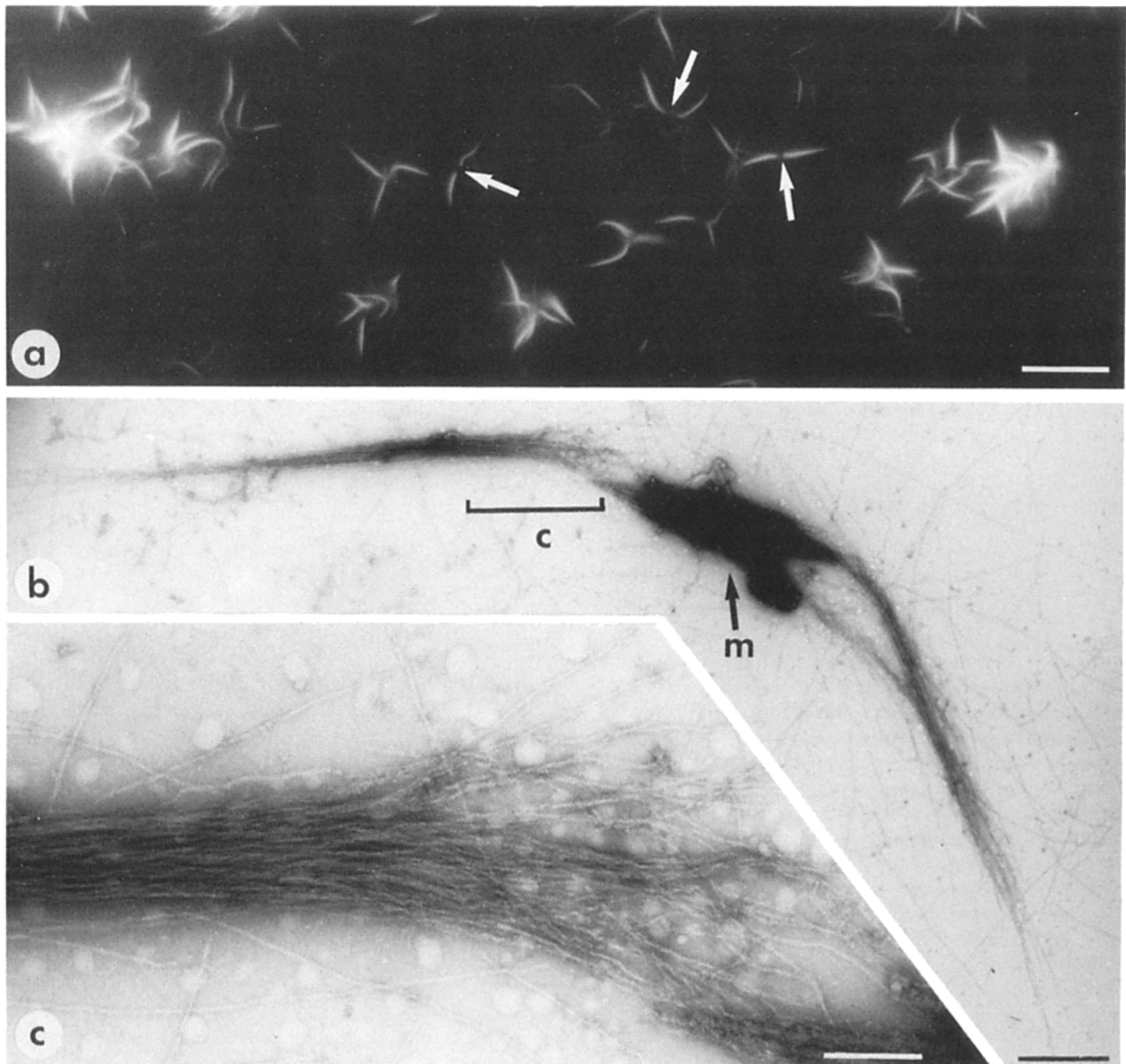
the cells before or after contraction with rhodamine-phalloidin showed that the bundles contained actin as a major component (Fig. 1 *g*).

In the absence of dextran the fragments contracted more slowly (as did the intact cells) and did not form the same visible bundles. If, however, dextran was added after contraction, bundles were revealed but these were more variable in length and more tortuous than those formed in dextran. In addition a general background of actin stain, in small fibers, was observed in these preparations, generally substantiating the loss of actin from the cells and cell fragments under these conditions.

The formation of radiating actin bundles was unaffected by the presence of cytochalasin D (30  $\mu$ g/ml) and it occurred, as indicated, with or without prelabeling of cells with phalloidin. Cell fragments extracted with Triton X-100 (0.2–0.5%) in the presence of dextran and then labeled with phalloidin likewise supercontracted into the same structures. It could therefore be concluded that the bundles were formed from preexisting actin filaments and not via polymerization of an actin monomer pool.

For the sake of brevity we shall from this point refer to the supercontracted cell fragments as "stars," a term prompted by their dramatic star-like appearance in the fluorescence microscope. We may also note that the integrity of the actin bundles was not dependent on the existence of a specific bundling protein but on the presence of dextran: when dextran was removed the star bundles were transformed into a diffuse fluorescent glow, a process that was reversible. Dextran thus served to reveal the presence of radiating actin filaments by causing them to bundle. The diameter of the stars ranged from 15 to 20  $\mu$ m and was essentially unaffected by changes in the concentration of dextran in the range of 2.5 to 7.5%.

The same stars were observed in contracted preparations of cells isolated from turkey and duck gizzard as well as from



**Figure 5.** Structure of bipolar dimers. (a) Fluorescent micrograph of contracted products obtained from homogenized cell fragments. The cells were labeled with fluorescent phalloidin before homogenization and contraction. Arrows indicate bipolar actin dimer bundles: dextran concentration 5%. (b and c) Electron micrographs, of a dimer structure. The myosin bundle (*m*), in *a* is flanked at its two ends by bundles of actin filaments. (c) Corresponding region marked in *b*. Negatively-stained with uranyl acetate. Bars: (a) 10  $\mu\text{m}$ ; (b) 1  $\mu\text{m}$ ; (c) 0.2  $\mu\text{m}$ .

the vas deferens and taenia coli of the guinea pig (Fig. 1, *i* and *j*). Whereas the intact cells did not form characteristic star-like forms some of them showed small bundles protruding from their surface in all directions, like bristles on a hedgehog; and intermediate forms were also seen (Fig. 1 *h*).

Contraction of the intact cells and of the fragments induced by exogenous Mg ATP was  $\text{Ca}^{2+}$  insensitive, indicating that the cells had lost their  $\text{Ca}^{2+}$  sensitivity during isolation, presumably via limited proteolysis effected by enzymes contaminating the collagenase. This was corroborated by the results of the immunoblots that showed in particular partial breakdown of the myosin light chain kinase (Fig. 2). A num-

ber of protease inhibitor cocktails were tried but without effect as far as Ca sensitivity was concerned.

#### **Relocation of Contractile and Cytoskeletal Proteins after Supercontraction**

Prelabeling of cell preparations with various primary and secondary antibodies had no effect on the supercontraction process. With antibodies other than those against myosin the cell fragments showed, however, a variable labeling intensity that was normally lower than that observed in the intact cells in the same preparation. The relocation of antibodies against

myosin,  $\alpha$ -actinin and desmin on star formation is shown in Fig. 3, *a-l*; as is seen all of the corresponding antigens condensed into the body of the supercontracted stars.

The relocation of myosin filaments and cytoskeletal proteins in the stars was also established by electron microscopy and immunoelectron microscopy. As shown in Fig. 4, the myosin filaments ended up in the core of the stars, oriented in all directions, with actin radiating outwards. This general arrangement of filaments was not dissimilar to that observed some time ago in contracted extracts of amoeba proteins (Pollard and Ito, 1970). Immunoelectron microscopy with  $\alpha$ -actinin antibodies identified flocculent structures as remnants of the cytoplasmic dense bodies, located in and around the myosin core (not shown).

### *The Actin Bundles, Filament Length, and Polarity*

Homogenization of cell preparations before or after exposure to contraction solution resulted in the production of a variety of supercontracted products ranging from normal stars to many small forms containing only a few or even single actin bundles (Fig. 5 *a*). Especially conspicuous, however, was a dimer species composed of a colinear bipolar pair of bundles bisected by a 1–2- $\mu\text{m}$  long actin-free gap (Fig. 5 *a*, *arrows*). Measurements of bundle lengths in phalloidin-labeled preparations yielded an average value of 4.4  $\pm$  0.8  $\mu\text{m}$ .

The morphology of the small dimer structures was revealed in more detail in the electron microscope (Figs. 5 and 6). Consistent with the data from thin sections, myosin filaments were commonly found at the centre of these fragments, although they were not always present. Their length was relatively constant, measurements from gizzard cells yielding a value of 1.6  $\pm$  0.3  $\mu\text{m}$ . The actin bundles showed tapered ends (Fig. 5 *b*) suggestive of a staggered filament arrangement: in the larger star structures containing multiple bundles the individual bundles often diverged into the basal core region. The diameter of the bundles was variable, ranging from 0.05 to 0.3  $\mu\text{m}$  and their length, from tip to the core center varied in the different star forms from 3 to 10  $\mu\text{m}$  (mean 4.8  $\pm$  1.8  $\mu\text{m}$ ). The ends of the bundles were more easily defined in the dimer structures and also in the single free bundles.

By rinsing the grids before staining in the contraction solution lacking dextran (and containing ATP) we found that the actin filaments could be splayed apart and measured individually (Fig. 6, *a* and *b*). This type of preparation showed that the actin filaments terminated close to tips of the myosin filaments in the dimers and extended over more or less the whole length of the star bundles from which they derived. Measurements of single filaments yielded length values of 3 to 6  $\mu\text{m}$ .

Labeling of the small star fragments with myosin subfragment-1 from smooth muscle showed that the actin filaments were polarized with their pointed ends (as defined from the decoration pattern) directed outwards (Fig. 6 *c*). Some examples were found of single actin bundles that exhibited a bundle of myosin filaments at one end (Fig. 6 *d*).

### *Partial Proteolysis Promotes Supercontraction of Cell Fragments*

It was notable that antibodies against vinculin and filamin

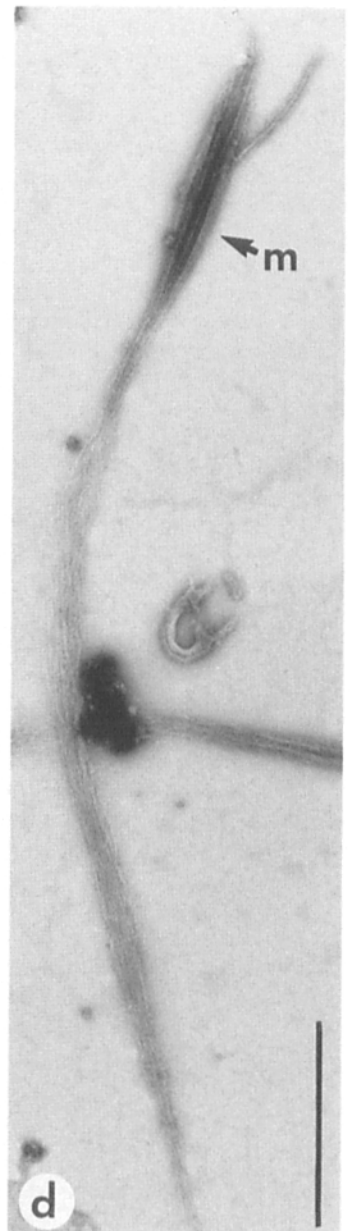
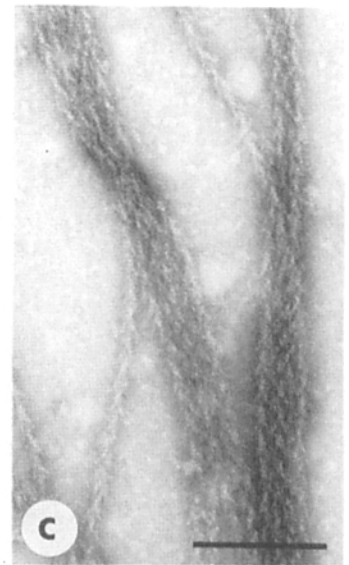
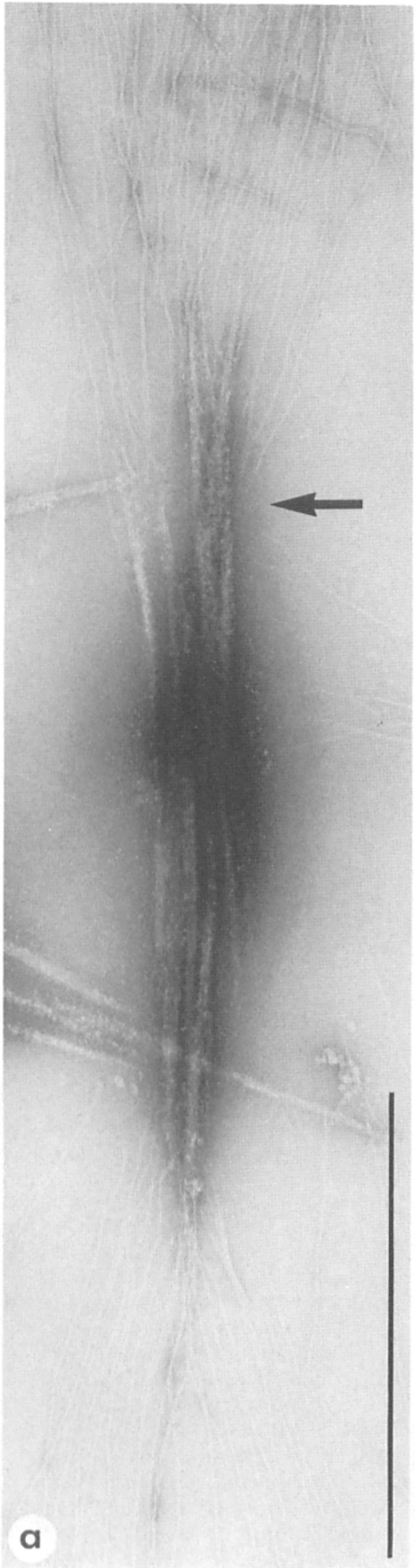
stained the cell fragments only very weakly or not at all, whereas the intact cells were strongly labeled (Fig. 7, *a* and *b*) and incapable of supercontraction (Fig. 7, *c* and *d*). This gave us the opportunity to test if fragments of intact cells that could be produced by homogenization were also capable of supercontraction. Fig. 7, *e-i* shows a fragment from an intact cell, identified from its vinculin-positive label, that was induced to contract under the microscope. Such fragments did not supercontract, as did the unlabeled fragments in the same preparations (Fig. 7 *h*, *arrow*) but shortened like part of an intact cell with the vinculin label remaining on the periphery (Fig. 7 *i*). After prolonged incubation in contraction solution (for 1 h or more) vinculin-positive star-like structures were however found (see Fig. 3, *k* and *l*; Fig. 4 *c*).

By including Triton X-100 (0.05–0.2%) in the collagenase digestion solution, proteolysis of the resulting cells, as judged from the immunoblots, could be deliberately enhanced (not shown). When induced to contract in solution these cells produced stars and short intact cells indistinguishable from those seen in normal preparations (e.g., Fig. 1 *a*).

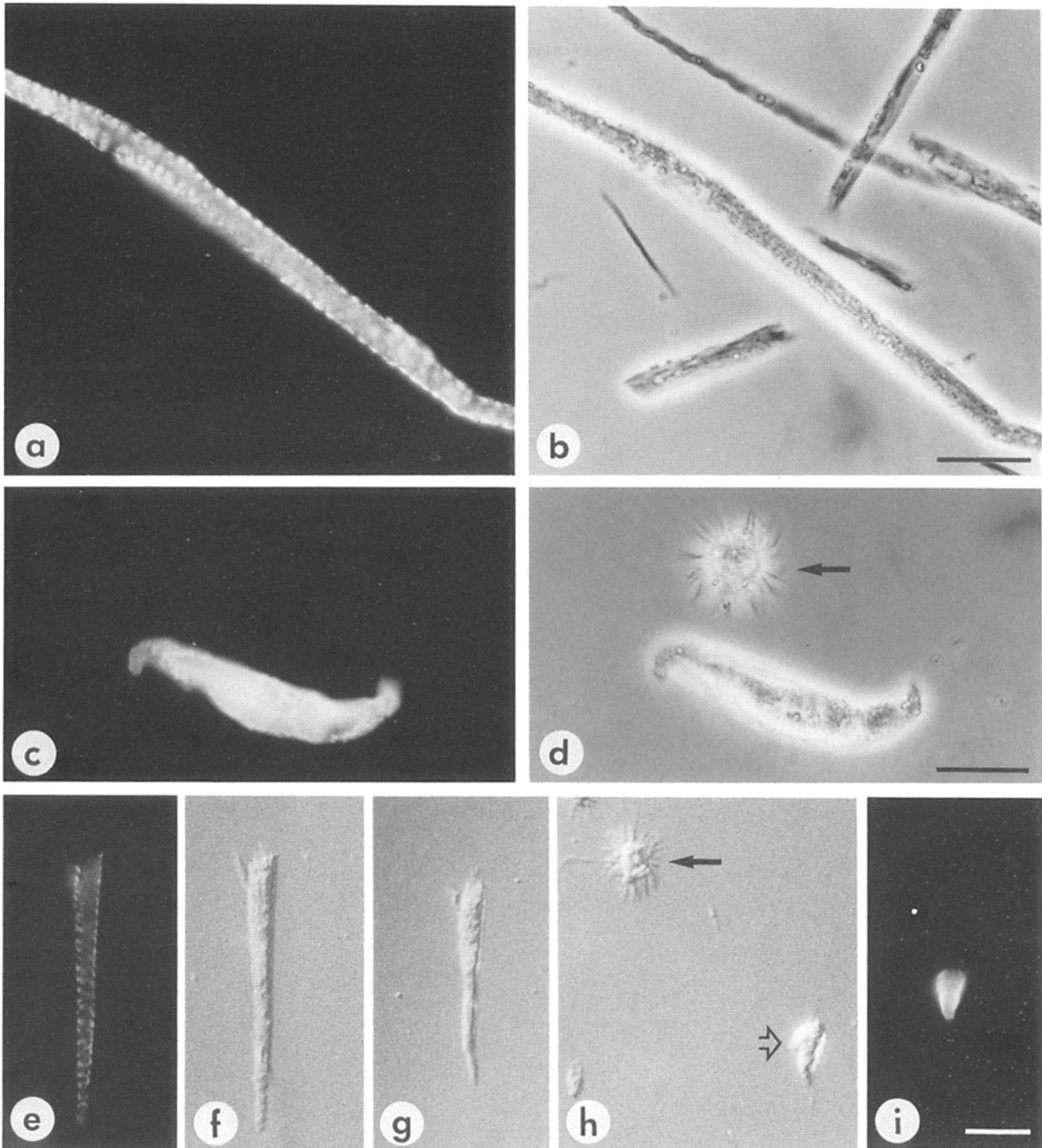
### *Discussion*

A schematic illustration of how we presume the star structures are formed is given in Fig. 8. It is assumed that the actin filaments are normally bound to specific anchorage sites associated with the intermediate filament cytoskeleton and the membrane skeleton. As a result of proteolytic breakdown of these sites or of the cytoskeleton itself free movement of actin filaments over myosin filaments proceeds unhindered, resulting in sliding far beyond the normal structurally imposed limits. A structural association of the cytoskeleton and membrane components with the contractile apparatus was directly indicated by the movement of desmin and vinculin label to the center of the supercontracted star structures. We presume that this label arises from remnants of the cytoskeleton still associated with the myofilament anchorage sites. In this scheme we further assume that the myosin filaments possess an arrangement of cross-bridges that allows sliding of thin filaments along the entire thick filament length as well as in opposite directions on the same filament (Small, 1977; Craig and Megerman, 1977; Hinssen et al., 1978); this type of polarity has been referred to as side polarity because of the apparent location of myosin heads on two opposite faces of the filaments (Craig and Megerman, 1977; Cooke et al., 1989), consistent with earlier observations on aggregated thick filament structures observed in thin sections (Small and Squire, 1972). As with thick filaments isolated from smooth muscle cells under relaxing conditions (Small, 1977; Cooke et al., 1989) we observed a continuous 14-nm cross-bridge repeat on the myosin filaments seen after supercontraction, with no central bare zone. To explain the bipolar dimers that contain an actin-free central bridge we must conclude (Fig. 8) that the junctions between oppositely polarized actin filaments, possibly maintained by  $\alpha$ -actinin-rich dense bodies (Bond and Somlyo, 1982) are disrupted at some stage of the contraction process; actin filaments would then be free to slide to the tips of the myosin filaments as is observed.

The finding of myosin filament bundles in the supercon-

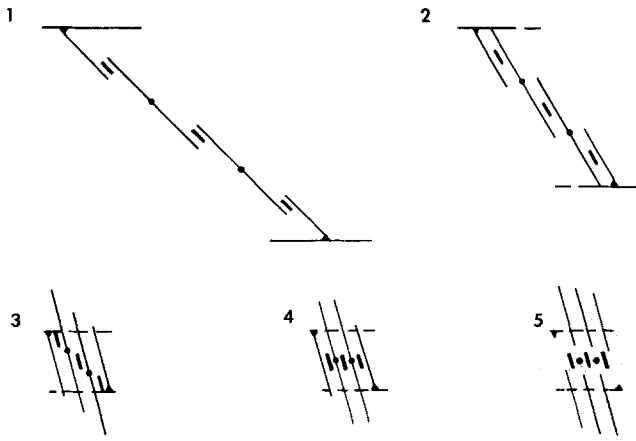






**Figure 7.** Intact cells and fragments of them do not readily form stars. (*a* and *b*) Antibodies to vinculin label only intact cells; (*a*) immunofluorescence, vinculin; (*b*) phase contrast. (*c* and *d*) Preparation as in *a* and *b* after contraction; vinculin-positive whole cells do not form stars (*c*), but unlabeled fragments (*d*, arrow) do. (*f*–*h*) Contraction series of a fragment of an intact cell. This fragment does not form a star (*h*, open arrow) but an adjacent vinculin negative fragment (*h*, solid arrows) does. (*e* and *i*) Fluorescence images of cell fragment before and after contraction. Bars: (*a*–*d*, *e*, *i*) 20  $\mu\text{m}$ .

**Figure 6.** Actin filament length and polarity. (*a* and *b*) Bipolar dimer structure treated additionally with ATP on the grid to splay out actin filaments. The ends of the thin filaments are marked by a large arrow at the myosin filament core and by small arrows at the far, splayed ends. (*c*) Part of actin bundle labelled with myosin subfragment. Arrowheads are unidirectional and are directed outwards towards the tip of the bundle (not shown). (*d*) hemidimer showing one actin bundle with myosin filaments (*m*) clustered at one end. Bars: (*a*, *b*, and *d*) 1  $\mu\text{m}$ ; (*c*) 0.2  $\mu\text{m}$ .



**Figure 8.** Suggested mode of formation of star structures. Actin filaments (a) are depicted as bipolar pairs attached to dense bodies (solid circles) in the body of the cell or as single filaments anchored to the membrane at sites containing vinculin (solid triangles). The myosin filaments (short solid lines) are depicted as side polar (see text) and are four times shorter than the actin filaments. Only one hypothetical contractile unit is shown (1). Under normal circumstances, the contractile apparatus is considered to be constrained by anchorages to the cytoskeleton, for example, via dense body-intermediate filament linkages. When these attachments are weakened or disrupted, actin and myosin slide unhindered. Finally, at stage 5, actin filaments of opposite polarity become segregated on either side of the central myosin core.

tracted structures provided an additional measure of the myosin filament length after contraction. As already indicated, the length of myosin filaments has previously been estimated from thin sections or from homogenates of relaxed cells with the uncertainties, in homogenates at least, about the extent of polymerization or depolymerization resulting from the choice of conditions. It has been shown that myosin phosphorylation, that precedes contraction, serves to stabilize the filamentous state of myosin (Suzuki et al., 1978; Kendrick-Jones et al., 1983; Cross et al., 1986; Trybus and Lowey, 1987) and from this we may presume that the lengths of filaments we observe after contraction, averaging  $1.6 \mu\text{m}$ , may represent a maximum value in gizzard cells. Although we have yet to obtain equivalent data from other smooth muscles, preliminary results with stars isolated from the guinea pig *Taenia coli* suggest the existence of filaments of similar length after contraction, that is somewhat shorter than the values obtained from "relaxed" cell homogenates (Small, 1977).

It could earlier only be intimated, from filament counts in thin cross sections that the thin filaments may be much longer than the thick filaments in vertebrate smooth muscle (Small and Squire, 1972; Murphy, 1979). That this is now demonstrated puts speculation about the structure-function relationships in smooth muscle on a firmer basis. The slower shortening velocity of smooth muscle, that is characteristically four or more fold lower than in fast skeletal muscle (Murphy, 1979; Hellstrand and Paul, 1982) can now be explained, at least in part, by the presence of longer sarcomere-like units than found in skeletal muscle (for a discussion about sarcomere length and shortening speed see Ruegg, 1971, and Murphy, 1979). Smooth muscle has characteristically a lower Mg-ATPase activity than skeletal muscle that

also must contribute to the slower shortening velocity: however, the reported ATPase values vary so widely—from 10 to 50% that of skeletal muscle (see review by Small and Sobieszek, 1983, for actomyosin)—that it would be unfit here to speculate in detail about the relative contributions of filament length and ATPase to this parameter. The range over which smooth muscle contracts and seen most dramatically from the shortening of single cells to less than one-fifth their maximum length, likewise fits with the existence of long actin filaments (Fig. 8). The type of filament arrangement depicted in Fig. 8 and characterized by face polar myosin filaments that are free to slide over long thin filaments is speculative since we are as yet unclear about the nature of the basic contractile unit and its cytoskeletal associations. But by defining the length of the myofilaments we have come one step further toward testing new ideas about the organization of the contractile apparatus.

A final remark should be made about the dramatic reorientation of the actin and myosin filaments that is expressed at its extreme in the star structures. Clearly, these structures are nonphysiological but their formation would appear to reflect the underlying geometry of the contractile apparatus. From previous data on isolated cells, an oblique arrangement of contractile elements has been postulated (Fay and Delise, 1973; Small, 1974, 1977; Fisher and Bagby, 1977) and the present data are consistent with these ideas. Notably, thin sections of the stars did not reveal discrete groups of myosin filaments similarly oriented that could be taken as belonging to individual contractile units. Rather, the thick filaments were organized at random. Likewise, bundles of actin filaments were not found in the body of the supercontracted stars; instead the thin filaments were splayed around the myosin core, and formed bundles only at the star periphery, through the influence of dextran. It is therefore premature to make the attractive suggestion that the dimer structures we observed correspond to a basic contractile unit in the cell. Using video-enhanced microscopy on small cell fragments we hope to shed more light on this problem.

We thank Dr. G. Rinnerthaler for assistance with video enhanced microscopy, Dr. M. Gimona for discussion, Dr. A. Sobieszek for myosin and Prof. B. Geiger, M. Osborn, and H. Faulstich for gifts of antibody and phalloidin probes. We also thank Ms. M. Klappacher and Miss K. Koppeltstatter for technical and photographic assistance and Mrs. C. Schöppel for typing.

This work was supported in part by a grant from the Muscular Dystrophy Association of America.

Received for publication 16 April 1990 and in revised form 1 August 1990.

## References

- Arheden, H., A. Arner, and P. Hellstrand. 1987. Force velocity relation and rate of ATP hydrolysis in osmotically compressed skinned smooth muscle of the guinea pig. *J. Muscle Res. Cell Motil.* 8:151-160.
- Ashton, F. T., A. V. Somlyo, and A. P. Somlyo. 1975. The contractile apparatus of vascular smooth muscle: intermediate high voltage stereo electron microscopy. *J. Mol. Biol.* 98:17-29.
- Bagby, R. M. 1983. Organization of the contractile/cytoskeletal elements. In *Biochemistry of Smooth Muscle*. N. L. Stephens, editor. CRC Press, Inc., Boca Raton, FL. 1-84.
- Bagby, R. M. 1986. Towards a comprehensive three-dimensional model of the contractile system of vertebrate smooth muscle cells. *Int. Rev. Cytol.* 105: 67-128.
- Bond, M., and A. V. Somlyo. 1982. Dense bodies and actin polarity in vertebrate smooth muscle. *J. Cell. Biol.* 95:403-413.
- Brandtzaeg, P. 1973. Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic exchange chromatography. *Scand.*

- J. Immunol.* 2:273-290.
- Cooke, P. H., F. S. Fay, and R. Craig. 1989. Myosin filaments isolated from skinned amphibian smooth muscle cells are side-polar. *J. Muscle Res. Cell Motil.* 10:206-220.
- Craig, R., and J. Megerman. 1977. Assembly of smooth muscle myosin into side-polar filaments. *J. Cell. Biol.* 75:990-996.
- Cross, R. A., K. E. Cross, and A. Sobieszek. 1986. ATP-linked monomer-polymer equilibrium of smooth muscle myosin: the free folded monomer traps ADP Pi. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2637-2641.
- Draeger, A., E. H. K. Stelzer, M. Herzog, and J. V. Small. 1989. Unique geometry of actin-membrane anchorage sites in avian gizzard smooth muscle cells. *J. Cell. Sci.* 94:703-711.
- Fay, F. S., and C. M. Delise. 1973. Contraction of isolated smooth muscle cells-structural changes. *Proc. Natl. Acad. Sci. USA.* 70:641-645.
- Fisher, B. A., and R. M. Bagby. 1977. Reorientation of myofilaments during contraction of a vertebrate smooth muscle. *Am. J. Physiol.* 233:C5-C14.
- Gillis, J. M., M. Linh Cao, and A. Godfraind-de Becker. 1988. Density of myosin filaments in the rat anococcygeus muscle, at rest and in contraction. II. *J. Muscle Res. Cell Motil.* 9:18-28.
- Gröschel-Stewart, U., U. Jaroschik, and H. Schwalm. 1971. Chicken gizzard, a myoglobin containing smooth muscle. *Experientia (Basel).* 27:512-513.
- Hellstrand, P., and R. J. Paul. 1982. Vascular smooth muscle: relations between energy metabolism and mechanics. In *Vascular Smooth Muscle: Metabolic, Ionic, and Contractile Mechanism*. M. F. Crass III and C. D. Barnes, editors. Academic Press, New York. 1-35.
- Hinssen, H., J. D'Haese, J. V. Small, and A. Sobieszek. 1978. Mode of filament assembly of myosins from muscle and non-muscle cells. *J. Ultrastruct. Res.* 64:282-302.
- Huxley, H. E. 1957. The double array of filaments in cross-striated muscle. *J. Biophys. Biochem. Cytol.* 3:631-647.
- Kendrick-Jones, J., W. Z. Cande, P. J. Tooth, R. C. Smith, and J. M. Scholey. 1983. Studies on the effect of phosphorylation on the 20,000 M<sub>r</sub> light chain of vertebrate smooth muscle myosin. *J. Mol. Biol.* 165:139-162.
- Kossmann, T., D. O. Fürst, and J. V. Small. 1987. Structural and biochemical analysis of skinned smooth muscle preparations. *J. Muscle Res. Cell Motil.* 8:135-144.
- Matsudaira, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386-396.
- Moeremans, M., G. Daniels, A. Van Dijck, G. Langanger, and J. DeMey. 1984. Sensitive visualization of antigen-antibody reactions in dot blot immune overlay assays with immunogold and immunogold/silver staining. *J. Immunol. Methods.* 74:353-360.
- Murphy, R. A. 1979. Filament organization and contractile function in vertebrate smooth muscle. *Am. Rev. Physiol.* 41:737-748.
- Pollard, T. D., and S. Ito. 1970. Cytoplasmic filaments of Amoeba proteins. I. The role of filaments in consistency changes and movements. *J. Cell Biol.* 46:267-289.
- Ruegg, J. C. 1971. Smooth muscle tone. *Physiol. Rev.* 51:201-248.
- Small, J. V. 1977. Studies on isolated smooth muscle cells: the contractile apparatus. *J. Cell Sci.* 24:327-349.
- Small, J. V. 1974. Contractile units in vertebrate smooth muscle cells. *Nature (Lond.)* 249:324-327.
- Small, J. V., and J. M. Squire. 1972. Structural basis of contraction in vertebrate smooth muscle. *J. Mol. Biol.* 67:117-149.
- Small, J. V., and A. Sobieszek. 1983. Contractile and structural proteins of smooth muscle. In *Biochemistry of Smooth Muscle*. N. L. Stephens, editor. CRC Press Inc., Boca Raton, FL. 85-140.
- Small, J. V., D. O. Fürst, and J. DeMey. 1986. Localization of filamin in smooth muscle. *J. Cell Biol.* 102:210-220.
- Suzuki, H., H. Onishi, K. Takahashi, and S. Watanabe. 1978. Structure and function of chicken gizzard myosin. *J. Biochem. (Tokyo).* 84:1529-1542.
- Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Trybus, K. M., and S. Lowey. 1987. Assembly of smooth muscle myosin minifilaments: effects of phosphorylation and nucleotide binding. *J. Cell Biol.* 105:3007-3019.