The Cytoskeletal and Contractile Apparatus of Smooth Muscle: Contraction Bands and Segmentation of the Contractile Elements

A. Draeger,* W. B. Amos,‡ M. Ikebe,§ and J. V. Small*

*Institute of Molecular Biology of the Austrian Academy of Sciences, 5020 Salzburg, Austria; ‡Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom; and §Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106

Abstract. Confocal laser scanning microscopy of isolated and antibody-labeled avian gizzard smooth muscle cells has revealed the global organization of the contractile and cytoskeletal elements. The cytoskeleton, marked by antibodies to desmin and filamin is composed of a mainly longitudinal, meandering and branched system of fibrils that contrasts with the plait-like, interdigitating arrangement of linear fibrils of the contractile apparatus, labeled with antibodies to myosin and tropomyosin. Although desmin and filamin were colocalized in the body of the cell, filamin antibodies labeled additionally the vinculin-containing surface plaques. In confocal optical sections the contractile fibrils showed a continuous label for myosin for at least 5 μm along their length: there was no obvious or regular interruption of label as might be expected for registered myosin filaments. The cytoplasmic dense bodies, labeled with antibodies to α-actinin exhibited a regular, diagonal arrangement in both extended cells and in cells shortened in solution to one-fifth of their extended length: after the same shortening, the fibrils of the cytoskeleton that showed colocalization with the dense bodies in extended cells became crumpled and disordered. It is concluded that the dense bodies serve as coupling elements between the cytoskeletal and contractile systems.

After extraction with Triton X-100, isolated cells bound so firmly to a glass substrate that they were unable to shorten as a whole when exposed to exogenous Mg ATP. Instead, they contracted internally, producing ~10 regularly spaced contraction nodes along their length. On the basis of differences of actin distribution two types of nodes could be distinguished: actin-positive nodes, in which actin straddled the node, and actin-negative nodes, characterized by an actin-free center flanked by actin fringes of 4.5 μm minimum length on either side. Myosin was concentrated in the center of the node in both cases. The differences in node morphology could be correlated with different degrees of coupling of the contractile with the cytoskeletal elements, effected by a preparation-dependent variability of proteolysis of the cells. The nodes were shown to be closely related to the super-contracted cell fragments shown in the accompanying paper (Small et al., 1990) and furnished further evidence for long actin filaments in smooth muscle. Further, the segmentation of the contractile elements pointed to a hierarchial organization of the myofilaments governed by as yet undetected elements.

SPECULATION about the three-dimensional order of the myofilaments in vertebrate smooth muscle has spawned a number of conflicting models (Bagby, 1983, 1986). At issue is the nature of the basic contractile unit, its conformation, size, and associations with the cytoskeletal apparatus. It is generally agreed that the cytoplasmic dense bodies, that contain α-actinin (Geiger et al., 1981) are associated with the intermediate filament cytoskeleton and one report (Bond and Somlyo, 1982) has demonstrated bipolar actin arrays emanating from these structures and entering neighboring regions containing myosin thick filaments. Accordingly, it has been tacitly assumed (e.g., Bond and Somlyo, 1982; Kargacin et al., 1989) that sarcomere-like structures spanning adjacent dense bodies constitute the contractile units in smooth muscle. A periodic arrangement of myosin and actin in subcellular fibrils has, however, not yet been demonstrated. Further, the specific colocalization of filamin with the intermediate filament cytoskeleton (Small et al., 1986) has pointed to the possible existence of a noncontractile actin component in the vicinity of the dense bodies. Actin filaments have been presumed to transmit tension to the cell surface via anchorage to the subplasmalemmal dense plaques (Pease and Molinari, 1960) that contain both vinculin and talin (Geiger et al., 1981; Small, 1985; Volberg et al., 1986; Drenckhahn et al., 1988; Draeger et al., 1989) and that show a geometrically regular but tissue-specific surface organization (Draeger et al., 1989).

Although any semblance of three-dimensional order in the

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contractile machinery has been generally obscured by the homogeneity of the cytoplasm in the light microscope and of the myofilament distribution in the electron microscope, indications of a hierarchical organization were already evident in the very earliest histological studies of smooth muscle. Thus Kölliker (1849), on establishing the unicellular nature of smooth muscle cells, also noted in some of them regularly spaced contraction bands, or "Knötchen". These contraction bands were described later in some detail by McGill (1909) who drew attention to the "striated" appearance of cells bearing them. Subsequently, little attention was paid to these structures which were apparently dismissed as artefacts of limited interest. More recently, suggestive evidence for long or semilong range order in the contractile apparatus of smooth muscle has reemerged from two studies. In the first (Bennett et al., 1988) an approximately periodic arrangement of staining for phosphorylated myosin was observed in guinea pig *Taenia coli* cells fixed during contraction. And in the second (Kargacin et al., 1989) motion analysis of dense body distributions in contracting toad stomach cells was interpreted as indicating the existence of coupled groups of dense bodies separated axially by $\sim$6 μm.

In the accompanying paper (Small et al., 1990) we have demonstrated a supercontracted state of isolated chicken gizzard cell fragments that furnished the first evidence for the length of actin filaments in smooth muscle. We here show that the mode of supercontraction of cell fragments is related to the formation of contraction bands in whole cells and demonstrate a clear and reproducible segmentation of the contractile apparatus under certain conditions. Further, from parallel confocal microscope studies on the organization of the contractile and cytoskeletal elements in extended and shortened cells we provide further insights into the interrelationships between the contractile and cytoskeletal systems.

Materials and Methods

Isolated Cells

Chicken gizzard smooth muscle cells were prepared and stored as described in the accompanying paper (Small et al., 1990) and by Draeger et al. (1989). For some cell preparations, Triton X-100 (0.05–0.2%) was included during digestion of the muscle strips in collagenase to effect mild proteolysis of the contractile and cytoskeletal apparatus (see Results).

Immunocytochemistry

Extended, uncontracted cells were centrifuged onto coverslips as described by Draeger et al. (1989). Fixation was performed for 30 min at room temperature using a mixture of 3% paraformaldehyde/0.3% Triton X-100 or 0.25% glutaraldehyde/0.5% Triton in solution 1 as described in the accompanying paper (Small et al., 1990). Cells fixed in glutaraldehyde were treated with sodium borohydride (0.5 mg/ml) twice for 5 min in ice-cold solution 1. Cells were labeled as described by Small et al. (1990) with polyclonal antibodies to filamin, desmin, α-actinin, tropomyosin and myosin subfragment-S1 and mAbs to the L_{17} myosin light chain (MM10) and desmin (DU10; kindly provided by Dr. M. Osborn, Max-Planck-Institut, Göttingen, FRG). Actin filaments were labeled with fluorescent phalloidin (kindly provided by Prof. H. Faulstich, Max-Planck-Institut, Heidelberg, FRG). Secondary antibodies were conjugated with FITC (Sigma GmBH, Munich, FRG) or Texas Red- (Vector Laboratories, Burlingame, CA) conjugated antibodies. In some cases the biotin-streptavidin Texas Red system supplied by Amersham International (Amersham, UK) was used. Final mounting was in Gelvatol (20-30; Monsanto Corp., New York) with 5 mg/ml *n*-propyl gallate (Giloh and Sedat, 1982) added as an antibleach agent.

For contraction experiments, the extended cells were labeled, unfixed in suspension with fluorescent phalloidin and antibodies to desmin, filamin,

and α-actinin, followed by the appropriate second antibody. These antibodies penetrated easily but occasionally a patchy staining was observed. We therefore included a brief treatment with 0.2% Triton X-100 to allow even antibody penetration. Staining of unfixed cells with antibodies to myosin and tropomyosin failed to produce uniform and complete penetration in our hands, even after skinning and extensive incubation periods.

Contraction Conditions

As indicated (Small et al., 1990) isolated chicken gizzard cells were spontaneously permeable to phalloidin and readily contracted in solutions containing Mg ATP. The contraction solution (see Small et al., 1990) contained in millimolar: 60, KCl; 3, MgCl$_2$; 4, EGTA; 0.5, DTT; 1, NaN$_3$; 10, Pipes; 1, ATP; supplemented with dextran (M_r 500,000) 2.5–5% (wt/vol) and with the pH adjusted to 6.5. Cells prelabeled with different combinations of the probes described above were contracted by mixing an aliquot of a cell suspension with an equal volume of the contraction medium either in solution or on a glass slide (see also Small et al., 1990).

Microscopy

Conventional fluorescence microscopy was carried out on a Zeiss Photomicroscope III equipped with epifluorescence, phase-contrast, and Nomarski interference optics. Confocal microscopy was performed as described (White et al., 1987) on an MRC 500 instrument (Bio-Rad Laboratories, Abingdon, UK) equipped with high reflectance mirrors and attached to a Nikon Optiphot fluorescence microscope. Images were viewed with a 60× planapo or 60× phase-contrast objective and recorded on Ilford FP4 film.

Results

The Cytoskeletal and Contractile Apparatus in Extended Cells

Due to the loss of membrane integrity during preparation, the cells isolated from chicken gizzard were in a state of rigor. For this reason many maintained their linear form and were well suited to immunocytochemical studies. The fibrillar structures were, however, so closely packed in these cells that overlapping effects often led to a loss of detail in the conventional fluorescence microscope. Accordingly, we took advantage of the confocal laser scanning microscope (White et al., 1987) to probe the organization of the cytoskeletal and contractile elements.

Staining of cells with antibodies to desmin revealed the overall organization of the intermediate filament cytoskeleton (Fig. 1, *a* and *b*). As shown in these two optical sections (vertical separation 0.8 μm) the intermediate filaments follow a mainly longitudinal, but rather tortuous path through the cell. There is no obvious association of fluorescent label with the cell membrane. In enlarged images (Fig. 1 *g*) fine transverse branches between the longitudinal filament bundles may be recognized. Consistent with earlier data (Small et al., 1986) antibodies to filamin demonstrated a colocalization of this protein both with the intermediate filaments (Fig. 1, *c* and *d*) and with the surface plaques associated with the cell membrane (Fig. 1 *c*). In gizzard cells, the surface plaques are enriched in the protein vinculin and talin and show a characteristic, periodic banding pattern (Draeger et al., 1989) that is also revealed by filamin antibodies, both polyclonal (Fig. 1, *a* and *c*) and monoclonal (supplied by Drs. Otey and Burridge, University of North Carolina at Chapel Hill; data not shown).

Alpha-actinin was formerly shown to reside in the dense bodies of smooth muscle and was reported to be associated with the dense membrane-associated plaques (Geiger et al., 1981; Fay et al., 1983). In our preparations the dense bodies
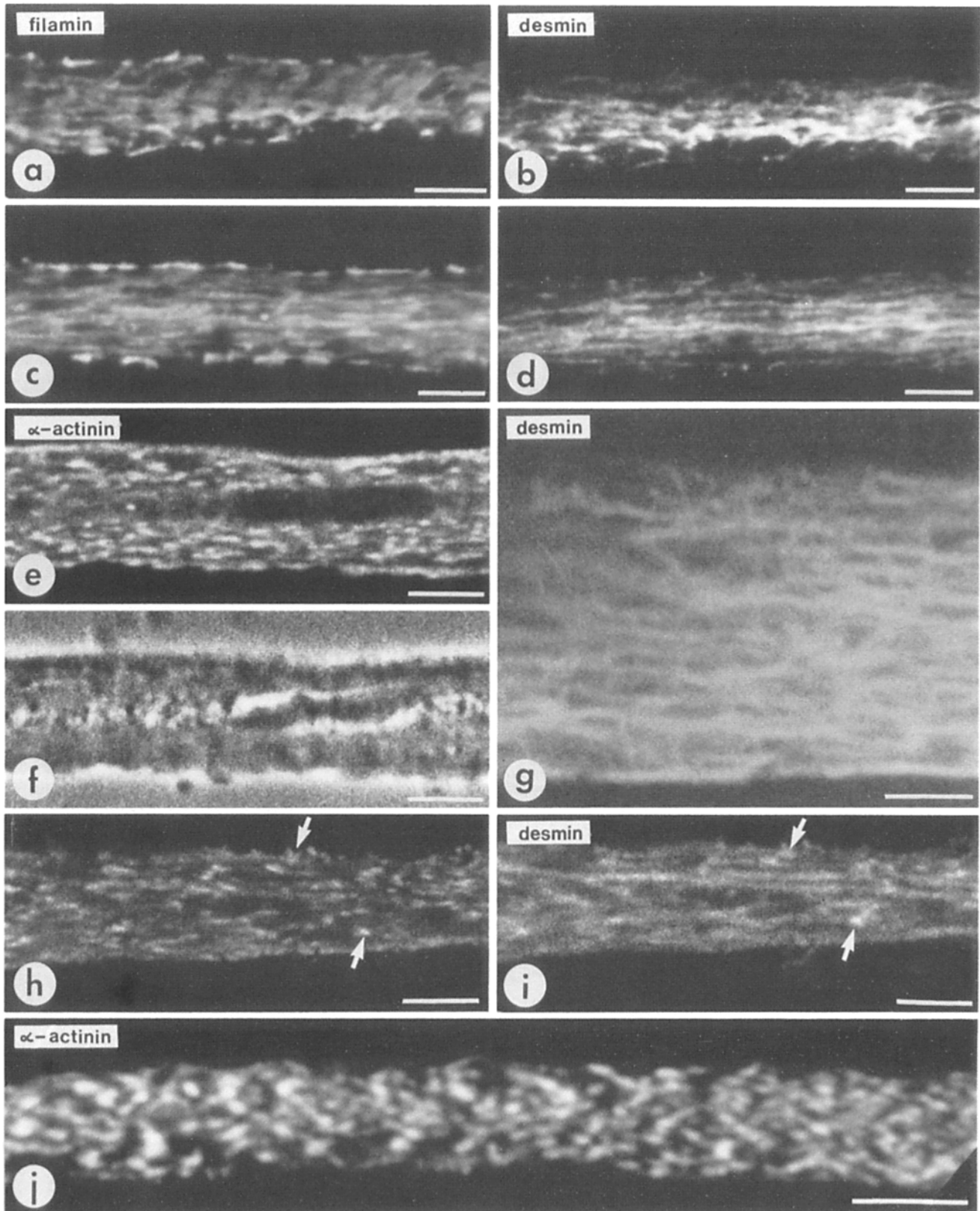
Figure 1. Confocal microscope optical sections through isolated chicken gizzard cells labeled with the antibodies indicated. *a* and *b* and *c* and *d* are double-label pairs, taken in the fluorescein and Texas red channels at the same optical level; *a* and *b* show a level close to the cell surface and *c* and *d,* a level 0.8 μm deeper into the cell. Filamin and desmin show a similar intracellular distribution, but filamin is additionally localized at the cell membrane. (*e*) Fluorescence and (*f*) phase-contrast image of a cell stained with an antibody to α-actinin. (*g*) Enlarged image of a desmin-labeled cell showing fine lateral projections of the intermediate filaments. (*h* and *i*) Colocalization of dense bodies (α-actinin) within the desmin fibrils (*arrows*). (*j*) Regular, oblique pattern of α-actinin-labeled dense bodies in an extended cell. Bars: (*a–f*) 5 μm; (*g*) 2 μm; (*h–j*) 5 μm.
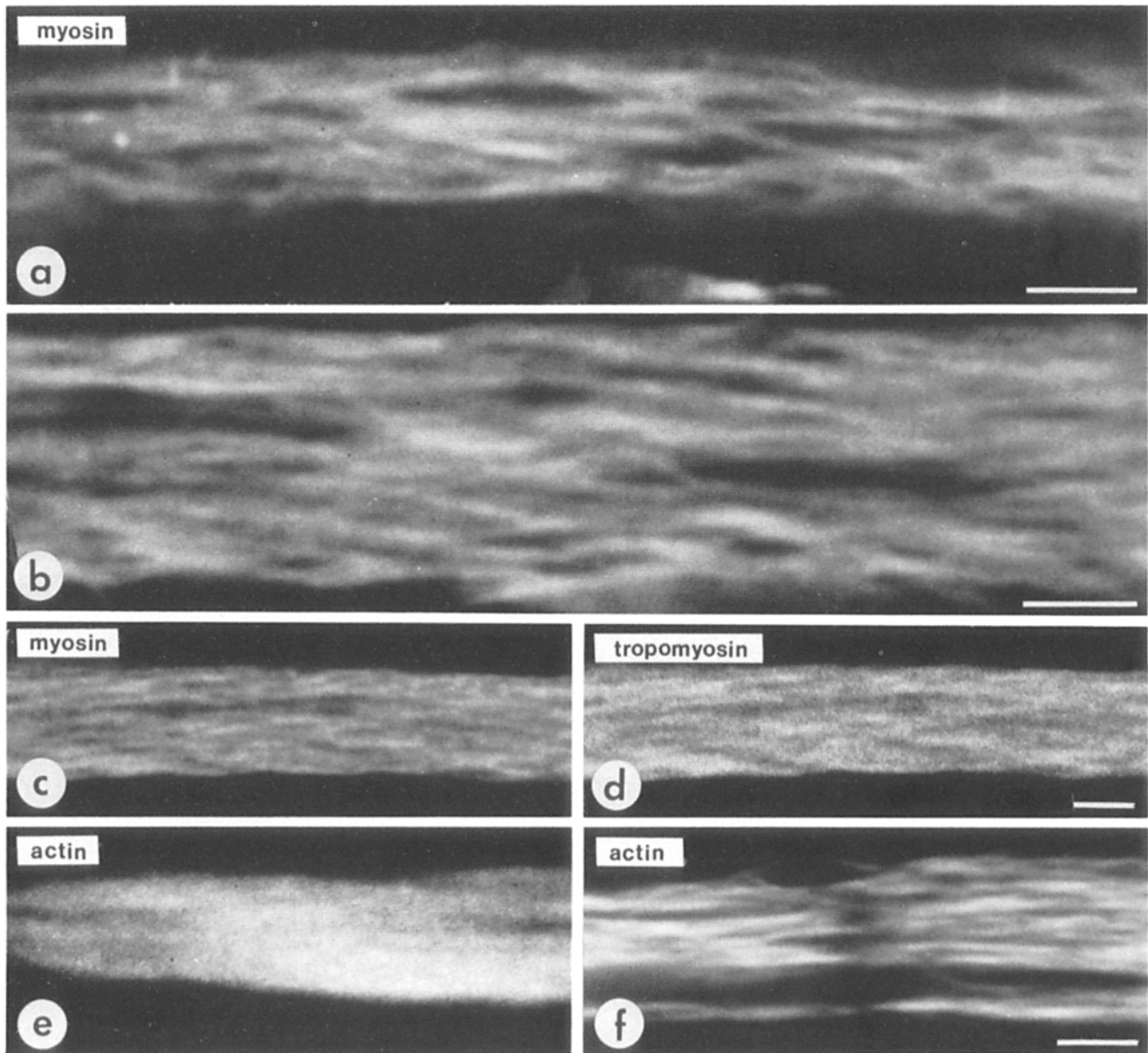
Figure 2. Confocal microscopy of contractile proteins in chicken gizzard cells (glutaraldehyde-Triton fixation). (*a* and *b*) Two optical sections (vertical separation 1 μm) of cell labeled for myosin: note a fibrillar, plait-like pattern. (*c* and *d*) Double labeling for myosin and tropomyosin showing colocalization in the same fibrils. (*e* and *f*) Selected images of cells labeled with fluorescent phalloidin: homogeneous or fibrillar patterns were observed. Bars: (*a–f*) 3 μm.

were strongly labeled with α-actinin antibodies (Fig. 1, *e, h,* and *j*) but rather little label was found at the cell surface and, when present, did not clearly show the periodic banding pattern characteristic for vinculin, talin, and filamin. Immunoelectron microscopy performed with polyvinylalcohol-embedded gizzard muscle (see Small et al., 1986) showed that our α-actinin antibody stained exclusively the cytoplasmic dense bodies (not shown). Kargacin et al. (1989) recently showed punctate structures in phase contrast images that they took as dense bodies; we resolved no such structures in similar micrographs of the smaller gizzard cells (Fig. 1, *e* and *f*). Confocal microscope images of cells double-labeled for desmin and α-actinin (Fig. 1, *h* and *i*) revealed a colocalization of the dense bodies within the desmin fibrils, as would be expected from the common presence of

intermediate filaments at the periphery of dense bodies, seen in the electron microscope (see Bagby, 1986). A notable feature of the α-actinin-labeled dense bodies, that showed up both with normal through focusing and in the confocal images was their apparently regular, geometric arrangement. This was most evident in very straight regions of cells (Fig. 1 *j*) where the punctate, elongated structures formed an oblique, diamond-like pattern with their long axis at a slight angle to the cells long axis. We shall return to this feature in the following section.

Myosin polyclonal and monoclonal antibodies labeled a distinctive set of subcellular fibrils that gave rise to a plait-like pattern in single optical sections (Fig. 2, *a* and *b*). This was apparently due to the interdigitation of slightly oblique fibrils of myosin that could be seen in these sections to be
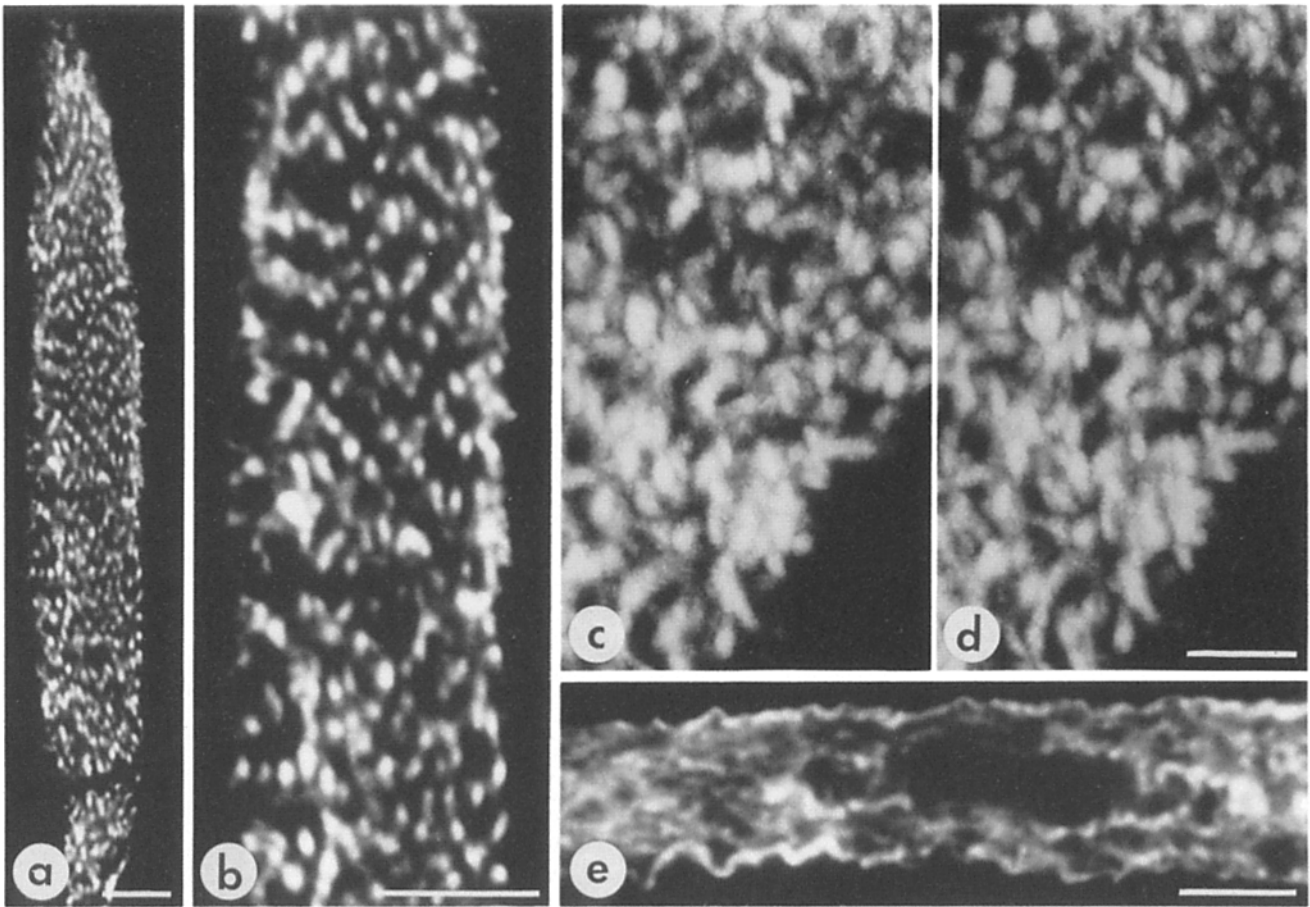
Figure 3. Confocal images of isolated chicken gizzard cells contracted in suspension after prelabeling with antibodies to α-actinin (*a–d*) and desmin (*e*). The dense bodies retain an ordered geometrical arrangement, whereas the intermediate filaments assume a folded appearance. (*c* and *d*) α-actinin stereo pair. The angle of the dense bodies to the cell's long axis increases with shortening. Bars: (*a*, *b*, and *e*) 5 μm; (*c* and *d*) 3 μm.

labeled continuously over lengths of up to at least 5 μm. Double-labeling with myosin and tropomyosin antibodies showed more or less colocalization of these two proteins in the same fibrils (Fig. 2, *c* and *d*). Fluorescent phalloidin, which revealed the distribution of filamentous actin stained cells either homogeneously (Fig. 2 *e*) or in a fibrillar pattern (Fig. 2 *f*) that in double labels (not shown) was coincident with that obtained for myosin.

Structural Rearrangements in Cells Contracted in Solution

When induced to contract freely in solution gizzard cells typically shortened to ∿50 μm (mean 54 +/− 6 μm) corresponding to approximately one-fifth of their extended length. In practice we found that the penetration of antibodies into shortened cells was often limited to the peripheral regions. More complete penetration with some antibodies was achieved by labeling the extended cells and then exposing these to contraction solution (see Materials and Methods).

Fig. 3 shows shortened cells stained in the above manner with antibodies to α-actinin (Fig. 3, *a–d*) and desmin (Fig. 3 *e*) and then shortened in solution with ATP. As shown, the arrangement of dense bodies, revealed by the α-actinin label

was highly geometric in shortened cells (Fig. 4, *a* and *b*) and this contrasted with the concertina-like arrangement adopted by the intermediate filaments (Fig. 3 *e*). Cell shortening also resulted in a foreshortening of the dense bodies themselves so that they appeared more spherical in shape. However, in stereo views (Fig. 3, *c* and *d*) the dense body orientation was still evident and their long axes could be seen to be oriented at high angles to the cell axis. Image processing methods have yet to be applied to track similarly oriented dense bodies through the cell.

With the myosin and tropomyosin antibodies we obtained only peripheral staining of unfixed, extended cells and this precluded studies of shortened cells stained for these proteins. Cells labeled with fluorescent phalloidin either before or after contraction showed only a homogeneous label in the confocal microscope. However, some cells showed a layer of fine radiating actin bundles at their surface (see also Small et al., 1990) consistent with a marked reorientation of the thin filaments on shortening.

Contraction under the Coverslip: Segmentation of the Contractile Apparatus

After treatment with Triton X-100 (0.1–0.2%) gizzard cells adhered rather firmly to a glass surface, so firmly that they
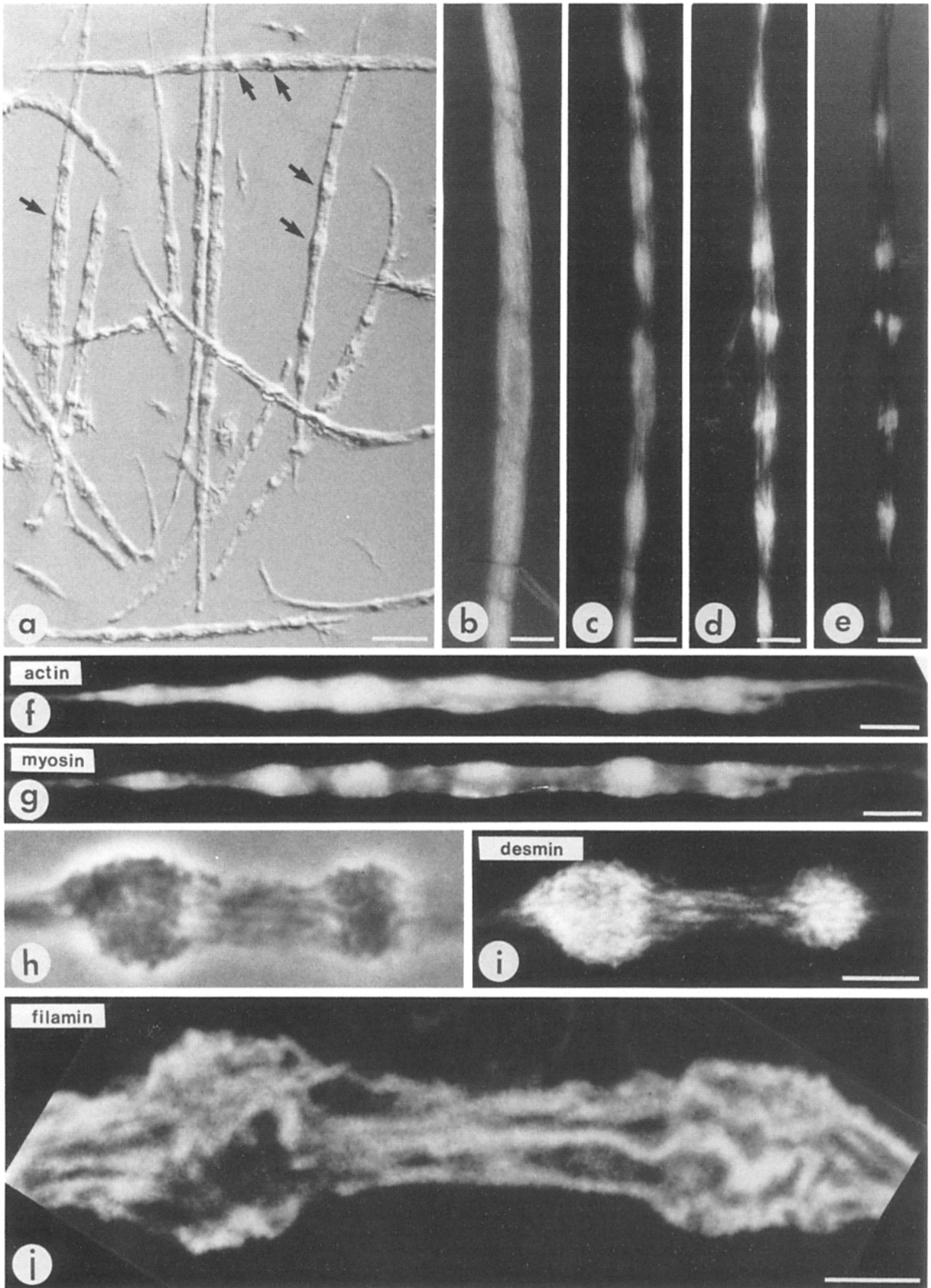
then resisted shortening as a whole in the presence of ATP. When the ATP solution was drained under the coverslip many of them contracted in a segmented fashion in such a way that after an extended period of time (up to many hours) they developed prominent nodes of contraction at regular intervals along their length (Fig. 4 *a*). In preparations of extended cells (average length 250 +/− 60 μm) the distance between adjacent contraction bands was rather constant and averaged ~20 μm (mean 22 +/− 4 μm). The number of contraction bands per cell ranged from 7 to 14.

In Fig. 4, *b* and *c* the early phase in formation of the contraction bands, as observed under polarization optics is illustrated. The same cell is shown before contraction (Fig. 4 *b*) and several minutes after application of the contraction solution (Fig. 4 *c*). The initially homogeneous positive birefringence became segregated on contraction into several equally spaced segments. After a much longer period of time (one or more hours) the birefringence was concentrated into the contraction bands (Fig. 4 *d*) (different cell from *b* and *c*). These contraction bands also showed birefringence on rotation of the cell by 90° about the optical axis (Fig. 4 *e*), indicating a considerable range of orientation of the myofilaments within them (see below).

Staining with fluorescent phalloidin revealed two distinct patterns of actin organization in cells exhibiting contraction bands. In the first, actin straddled the band (Fig. 4 *f*) and in the second, actin was mainly excluded from the center of the band and formed either a broad fringe on either side of it or completely filled the gap between adjacent contraction bands (Fig. 5). We shall refer to these two contraction band forms as actin-positive bands and -negative bands, respectively. In both forms myosin was concentrated at the center of the bands (Fig. 4 *g* and below). The two forms could further be distinguished from the shape of the contraction bands themselves as seen under phase contrast: in the actin-positive bands the contraction node was rather bulbous, whereas in the actin-negative bands no significant bulging at the band was evident. Actin-negative contraction bands were more common in cells that were isolated with Triton X-100 in the collagenase digestion solution (see Materials and Methods) and that from immunoblotting data (not shown here) exhibited some proteolysis of their contractile and cytoskeletal proteins (myosin, myosin light chain kinase, caldesmon, filamin, and desmin). Measurements made from those cells where a clear phalloidin-labeled fringe flanked the actin-negative bands yielded a minimum actin fringe width of close to 4.5 μm (Fig. 5, *c*, *g*, and *h*). Cell fragments were also found in the preparations that were visibly torn apart to various degrees after contraction (Fig. 5 *h*). In these cases the actin fringes around the contraction bands were com-

posed of radiating actin bundles and resembled the star structures formed by contraction of cell fragments in solution (Small et al., 1990). Double labeling with antibodies to myosin showed that myosin was concentrated at the center of the contraction bands in a distribution essentially coincident with the dense nodes recognized in the phase contrast images (Fig. 5, *i–k*).
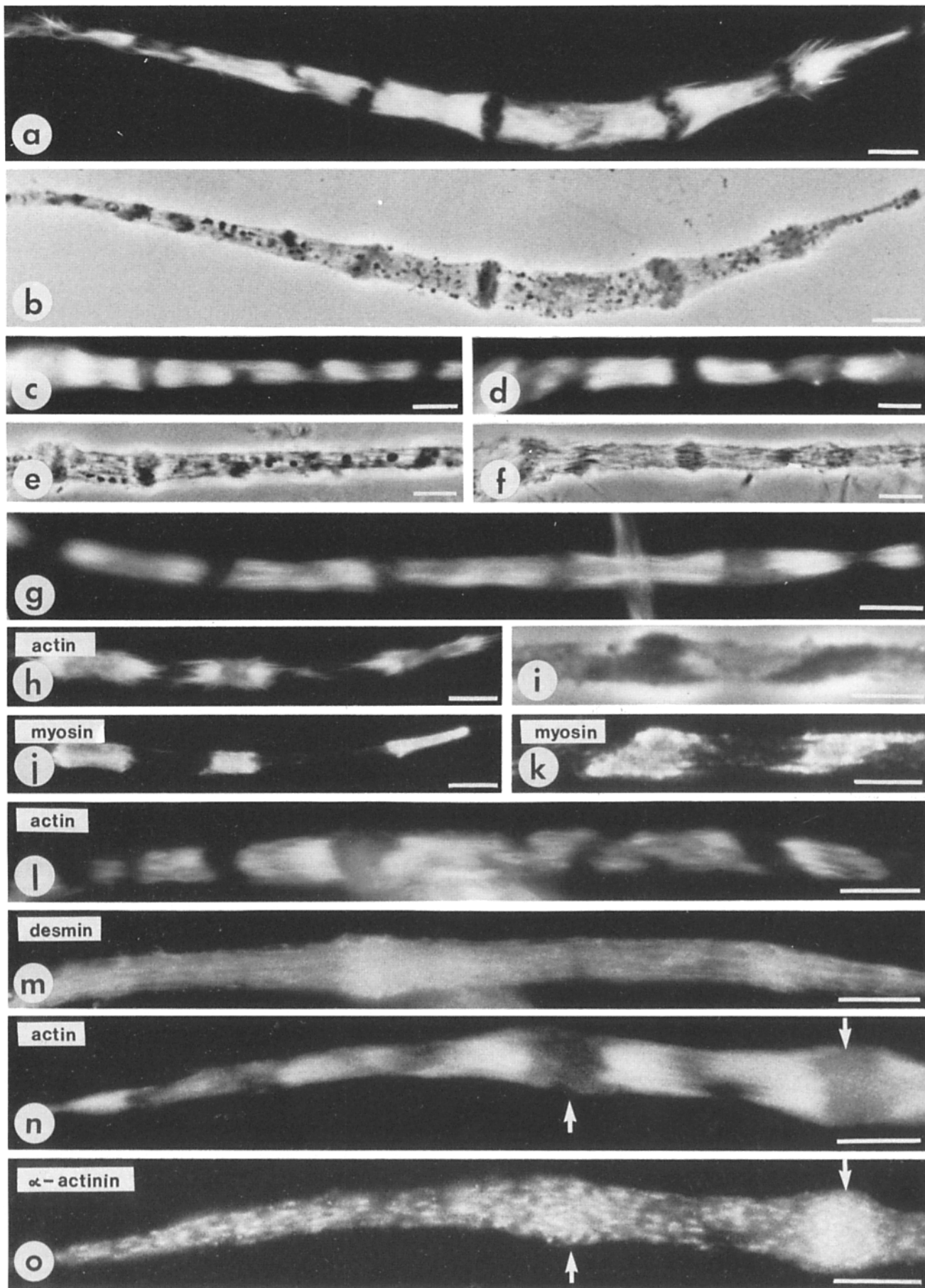
Interesting redistributions of the cytoskeletal elements were also noted in the contraction band cells. For cells showing bulging (actin-positive) contraction bands both desmin and filamin were concentrated in crumpled filament arrays in and around the bands (Fig. 4, *h–j*), indicating that the cytoskeleton had been forcibly drawn into the bands by the contractile apparatus. Cells with actin-negative bands showed, in contrast a distribution of filamin and desmin that was essentially unchanged as compared with uncontracted cells; in these cases the antibody label was not concentrated in the bands (Fig. 5, *l* and *m*). The same was not true for α-actinin. In both forms of contraction bands described α-actinin was drawn into the bands. Noteworthy in this regard was the presence of a regular, geometric arrangement of dense bodies in the center of the actin-negative contraction bands, that is, where actin was essentially absent (Fig. 5, *n* and *o*).

Discussion

Using confocal microscopy we have demonstrated the overall spatial arrangement of the cytoskeletal and the contractile systems of smooth muscle cells. As is shown the two systems are quite distinct. The cytoskeleton, delineated by antibodies to desmin and filamin, is composed of a mainly longitudinal meandering and branching filament network. Stromer and Bendayan (1988) have noted a central bundle of intermediate filaments in electron micrographs of gizzard cells: we have also observed such bundles but they were the exception rather than the rule in desmin-labeled preparations. In the isolated gizzard cells, desmin showed no obvious association with the membrane plaques whereas filamin antibodies marked these surface structures in a manner identical to that shown for antibodies to vinculin and talin (Draeger et al., 1989). A possible explanation for the dual labeling by filamin antibodies of the surface plaques and the intermediate filament network came from the demonstration of two filamin isoforms in gizzard, antibodies to one showing specificity for focal contacts in cultured cells (Pavalko et al., 1989). As indicated, however, this latter antibody did not label exclusively surface plaques of gizzard cells. Further work is necessary to explain this differential localization of filamin as well as its characteristic abundance in smooth muscle.

Whereas our images confirmed the association of the α-ac-

Figure 4. (*a*) Overview of smooth muscle cells contracted while fixed to a substrate (Nomarski interference optics). Nodes of contraction (*arrows*) can be seen at regular intervals. (*b–e*) Polarization optics. The same cell before (*b*) and after (*c*) addition of contraction solution: note a gradual shift of birefringence into the areas of contraction. (*d* and *e*) Another cell photographed a few hours after addition of contraction solution: increased birefringence is evident in the contraction bands (*d*) and some birefringence remains on rotation of the cell by 90° (*e*), indicating gross reorientation of the contractile elements. (*f* and *g*) Cell contracted after prelabeling with fluorescent phalloidin and antimyosin antibodies. Actin and myosin are both concentrated in the contraction bands. (*i*) Confocal image of a cell prelabeled with desmin antibodies and then contracted under the coverglass; (*h*) corresponding phase-contrast picture. *j*, As in *i* but cell prelabeled with filamin antibodies. Note withdrawal of desmin and filamin positive fibrils into the contraction bands. Bars: (*a*) 25 μm; (*b–g*) 10 μm; (*h* and *i*) 5 μm; (*j*) 3 μm.

In panels, labels read: **actin** (h), **myosin** (j), **myosin** (k), **actin** (l), **desmin** (m), **actin** (n), **α−actinin** (o)
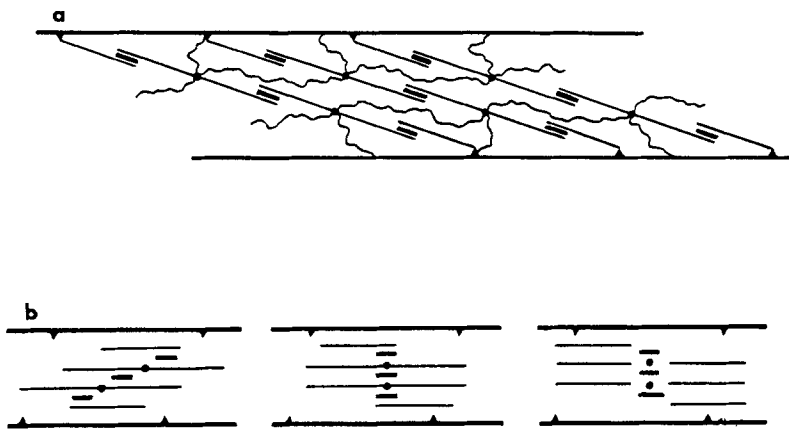
Figure 6. (*a*) Schematic illustration of suggested arrangement of cytoskeletal and contractile elements in an intact cell. Only a few elements are considered. The actin filaments of the contractile units are linked to the cell membrane at the attachment plaques (*triangles*) and within the cell to the cytoplasmic dense bodies. Myosin filaments are presumed to be side polar and are indicated by short thick lines. The dense bodies are also linked to the intermediate filament cytoskeleton (*wavy lines*) that forms longitudinal and transverse connections. (*b*) Proposed stages in the formation of contraction bands in substrate bound cells. Due to partial degradation of the cytoskeleton (not depicted) the contractile units become uncoupled from the cytoskeleton and move unhindered. Myosin filaments move to the center of the contraction bands (*center and right*) with actin either straddling the band (*center*) or sheared out on both sides (*right*). In the latter case the dense bodies remain in the vicinity of the myosin filaments.

tinin-containing dense bodies with the intermediate filament network it was striking how regular an arrangement these structures exhibited in extended cells. This difference in apparent order between the intermediate filament system and the dense bodies was even more pronounced in the contracted cells, where the geometrical arrangement of the dense bodies was maintained despite an obvious crumpling of the cytoskeletal elements. We can only conclude that the regular dense body order is maintained by other structural or contractile elements. In this connection the reorientation of the dense bodies in concert with the reorientation of the actin filaments in shortened cells (see also Small et al., 1990) provided a compelling argument for a link between these two systems, in line with earlier suggestions (Bond and Somlyo, 1982). But in this case it is difficult to explain why, even in contraction bands of gizzard cells that apparently lack actin filaments, the dense bodies are equally well ordered (see Fig. 5 *o*). We shall return to this problem again below.

One goal of this work was to identify individual contractile units in the contractile apparatus. This was achieved, in as much as we could demonstrate fine myosin-containing fibrils that interdigitated in three dimensions in an oblique arrangement throughout the cell. However, we encountered difficulties in tracking single fibrils through different confocal levels, apparently because these structures fuse and branch along their length (see also Cooke, 1983): further image analysis is required to demonstrate this feature adequately. It was possible nevertheless, to establish that myosin filaments, which are ∼1.6 μm long in gizzard (see Small et al., 1990) do not form registered A-band like groups within the contractile fibrils (see Ashton et al., 1975) but must be stag-

gered along the fibril to give rise to the continuous myosin staining (over at least 5 μm) that is observed. We analyzed cells labeled with both myosin and α-actinin antibodies, but due to the close packing of the myosin-positive fibrils it was not possible, even in the confocal images to establish whether or not the two were linked in the same fibrils. Birefringent fibrils of around the same diameter and oblique orientation as the myosin-positive fibrils were earlier detected by polarization microscopy (Small, 1977*a,b*): the present results confirm that they belong to the contractile apparatus.

In the majority of extended, isolated smooth muscle cells antibodies against cytoskeletal and contractile proteins showed a more or less unbroken staining pattern throughout the cell length. And as we have shown elsewhere the surface attachment plaques, positive for vinculin antibodies form a regular barrel-like array of bands with a longitudinal spacing of ∼5 μm (Draeger et al., 1989) that we have suggested marks the attachment sites for the actin component of the contractile apparatus. The rediscovery of the contraction bands of Kölliker and McGill (Kölliker, 1849; McGill, 1909) colored with the modern labels for contractile and cytoskeletal proteins now raises new and testing questions about the global organization of the contractile apparatus.

Images such as shown in Fig. 5 *h* clearly illustrated the close relation of the contraction bands with the star-like structures produced by cell fragments when contracted either in solution or when weakly bound to glass (Small et al., 1990). The only difference was that when strongly bound to glass (by skinning) cell fragments produced up to several nodes on contraction, whereas in solution only single nodes

Figure 5. Actin-negative contraction bands (see text). Conventional fluorescence images of chicken gizzard cells that were prelabeled with phalloidin and the antibodies indicated before contraction under the coverslip. Comparison of the phase contrast (*b*, *e*, and *f*) with the fluorescent images (*a*, *c*, and *d*) shows that actin is excluded from the contraction bands of these cells. Fine, radiating spikes can be seen in the actin fringes bordering the contraction bands formed in cell fragments (*h*). Myosin is concentrated at the center of contraction bands (*j* and *k*): *i* shows phase contrast image of cell in *k*. The distribution of intermediate filaments remains essentially unchanged in cells showing actin-negative contraction bands (*l* and *m*), whereas α-actinin is drawn into the actin-free center of the bands (*n* and *o*; *arrows*). Bars: (*a–g*, *h*, *j*, *l*, *m*) 10 μm; (*i*, *k*, *n*, *o*) 5 μm.

(i.e., stars) were observed. This behavior indicates that, despite its apparent segmentation the contractile apparatus must be coupled between adjacent segments. Following on from the conclusion made from the star structures in the accompanying work, we propose that the contraction bands are formed as indicated in Fig. 6. What type of band is formed appears to be dependent on the degree of interaction between the contractile and the cytoskeletal systems or on the general intactness of the cytoskeleton. An early stage in the formation of the bands is characterized by a visible splitting into separate domains (Fig. 4 c): we conclude that this early phase was detected in *Taenia coli* cells in the studies of Bennett et al. (1988). For the present purposes we have assumed (Fig. 6 b) that the thin filaments of the contractile apparatus are connected to the dense bodies (Bond and Somlyo, 1982), that they are about four times longer than the myosin filaments (Small et al., 1990) and are anchored to the cell surface in the intact cell (Fig. 6), either directly or indirectly to the surface plaques that contain vinculin, talin, and filamin (Draeger et al., 1989 and references therein; Small et al., 1986 and this study).

As we have indicated, two types of terminal contraction bands are formed, both with myosin at the center but with actin showing one of the two distributions: either straddling the band (Fig. 4 f) or in two fringes on either side (Fig. 5), of minimum length ∼4–5 µm. We presume that the second pattern arises by a crossing of actin filaments from one side of the band to the other by a mechanism analogous to that deduced for the bipolar actin dimers isolated from the supercontracted star structures (Small et al., 1990). This mode of sliding was indicated in the latter study by the demonstrated outward direction of arrowheads formed after decoration with myosin head subfragments.

As we show, the actin-positive bands are bulbous and the cytoskeletal components are drawn into these bands with the contractile elements. It follows that the two systems are either mechanically coupled or their mutual interdigitation and association with the cell surface inevitably results in the dragging of the cytoskeleton along with the contractile elements. Deliberate proteolysis of the cells (by inclusion of Triton X-100 in the collagenase) appears to break down this interaction: many cells are then found with actin-negative bands in which the intermediate filaments are not concentrated. Of particular interest was the finding that the dense bodies are still drawn into the actin-negative bands and, remarkably, retain their geometric order in the myosin-rich core. It would appear then that the dense bodies remain ordered when uncoupled from both the intermediate filaments and the actin filaments. If, as we tentatively assume (Fig. 6), the dense bodies are linkage structures in the contractile fibrils (Bond and Somlyo, 1982) we must invoke a further structural element for their regular organization. In this respect we risk calling on the results from skeletal muscle which have shown that the molecule titin (see review by Wang, 1985 and Maruyama, 1986) extends from the Z-line to the M-band (Fürst et al., 1988). Although titin has not yet been isolated from smooth muscle a protein with similar elastic properties but with an even more extravagant length (Nave et al., 1989) could explain these data. If such a structure does exist it must however be more resistant to proteolysis than skeletal muscle titin to be mechanically functional in these isolated smooth muscle preparations. Kargacin et al.

(1989) in their recent study proposed that the dense bodies were laterally linked but made no suggestions as to what elements may be involved.

In conclusion, the contractile apparatus of smooth muscle, while coupled along the entire cell length, shows the ability to segregate into macroscopic segments under certain conditions. Although this behavior appears artefactual, it points to the presence of a hierarchical order of the contractile apparatus and in turn to a segmental registration of the contractile elements. Evidence from contracted cells suggests that the cytoskeleton is mechanically linked to the contractile apparatus and the dense bodies have been implicated as the linkage elements. Open questions remain, among others, about the structural basis of the segmentation process and how it can be reconciled with the fine period of attachment sites for actin at the cell surface (Draeger et al., 1989).

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