Arrangement and Possible Function of Actin Filament Bundles in Ectoplasmic Specializations of Ground Squirrel Sertoli Cells

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ABSTRACT We have investigated the arrangement and function of actin filament bundles in Sertoli cell ectoplasmic specializations found adjacent to junctional networks and in areas of adhesion to spermatogenic cells. Tissue was collected, from ground squirrel (*Spermophilus* spp.) testes, in three ways: (*a*) seminiferous tubules were fragmented mechanically; (*b*) segments of intact epithelium and denuded tubule walls were isolated by using EDTA in a phosphate-buffered salt solution; and (*c*) isolated epithelia and denuded tubule walls were extracted in glycerol. To determine the arrangement of actin bundles, the tissue was fixed, mounted on slides, treated with cold acetone (−20°C), and then exposed to nitrobenzoxadiazole-phallacidin. Myosin was localized using immunofluorescence. To investigate the hypothesis that ectoplasmic specializations are contractile, glycerinated models were exposed to exogenous ATP and Ca^{++}; then contraction was assessed qualitatively by using nitrobenzoxadiazole-phallacidin as a marker.

Actin bundles in ectoplasmic specializations adjacent to junctional networks circumscribe the bases of Sertoli cells. When intact epithelia are viewed from an angle perpendicular to the epithelial base, honeycomb staining patterns are observed.

Filament bundles in Sertoli cell regions adjacent to spermatogenic cells dramatically change organization during spermatogenesis. Initially, the bundles circle the region of contact between the developing acrosome and nucleus. They then expand to cover the entire head. As the spermatid flattens, filaments on one side of the now saucer-shaped head orient themselves parallel to the germ cell axis while those on the other align perpendicularly to it. Before sperm release, all filaments course parallel to the rim of the head.

Contrary to the results we obtained with myoid cells, we could not convincingly demonstrate myosin in ectoplasmic specializations or induce contraction of glycerinated models. Our data are consistent with the hypothesis that actin in ectoplasmic specializations of Sertoli cells may be more skeletal than contractile.

Sertoli cells of the mammalian seminiferous epithelium are model systems in which to study cytoskeletal structure and function. Not only do these cells undergo remarkable shape changes during spermatogenesis, they also determine the position of spermatogenic cells within the epithelium, phagocytose cytoplasm shed by developing spermatids, release spermatozoa into the duct system, and may even influence sperm cell shape. Perhaps it is not surprising that Sertoli cells contain an elaborate and very dynamic cytoskeleton with some elements organized into specialized structures not seen in other cells.

One such group of structures, termed ectoplasmic specializations (22), occur in submembrane regions usually adjacent to the basally situated Sertoli–Sertoli junctions and in regions of adhesion to spermatogenic cells. These structures consist of a dense mat of actin filaments sandwiched between the plasma membrane and a cistern of endoplasmic reticulum (1, 3, 6, 7, 14, 27). Because they are best developed in regions of

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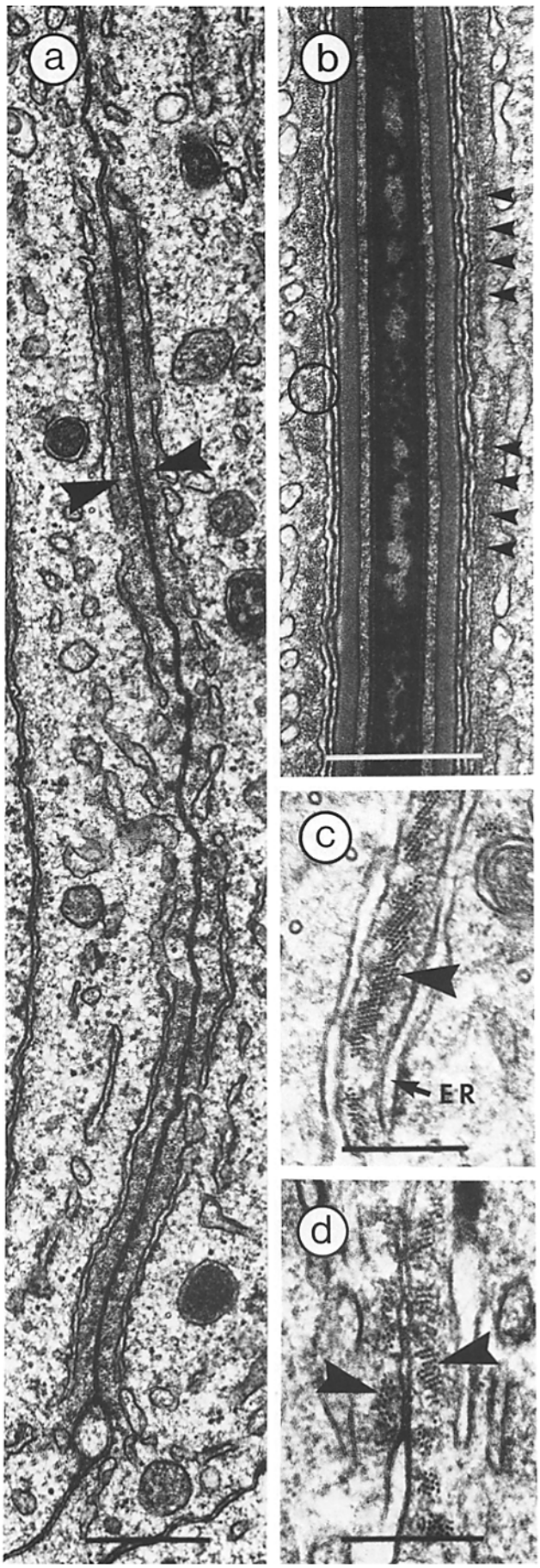


attachment to adjacent cells, they are thought to facilitate intercellular adhesion in some way—possibly by rigidifying specific areas of the Sertoli cell plasma membrane (22, 24). They are also thought to play a role in sperm release and in the movement of spermatogenic cells through the epithelium (3, 18, 24). Although the mechanism underlying these proposed functions is not known, it has been suggested that ectoplasmic specializations may be contractile (8, 10, 27).

If ectoplasmic specializations are contractile, one would expect both myosin to be present and actin filament bundles to be arranged in a pattern consistent with force generation that has some significant function during spermatogenesis. These predictions have not been adequately investigated, partly because of difficulties encountered in working with the system.

The seminiferous epithelium is one of the most complex tissues in the body. It is composed of multiple clones of small spermatogenic cells distributed among highly branched Sertoli cells. Beneath this fairly thick epithelium is a wall composed of extracellular matrix and contractile peritubular or myoid cells. The epithelium and wall together form small seminiferous tubules that in turn are surrounded, at least in rodents, by large lymphatic sinuses (5).

It previously has been shown that the seminiferous epithelium of the ground squirrel is an ideal system for studying spermatogenesis (4, 28). Compared with seminiferous epithelia in other mammals, it is thin and has few spermatogenic cells. Moreover, all cells are large. In addition, morphogenetic events in Sertoli cells and involving spermatogenic cells are exaggerated when compared with those in similar cells from other species. This model system would be further improved if segments of intact epithelium could be separated from tubule walls, thereby allowing Sertoli and spermatogenic cells to be visualized directly without having to revert to sectioning techniques.

In this study we introduce a method for isolating sheets of intact epithelium from seminiferous tubules of the ground squirrel and use this technique to expand our knowledge of the structure and function of Sertoli cell ectoplasmic specializations. We describe the three-dimensional arrangement of actin bundles adjacent to spermatogenic cells and show, for the first time, that the organization of these bundles dramatically changes during spermatogenesis. Further, we present evidence that ectoplasmic specializations may not be contractile. In using immunofluorescence techniques, we could not convincingly demonstrate myosin at these sites. Also, glycer-

FIGURE 1 Electron micrographs of Sertoli cell ectoplasmic specializations in perfusion-fixed testes. (*a*) Ectoplasmic specializations (arrowheads) at the junctional site between two adjacent Sertoli cells. The structures are characterized by a densely stained layer of filaments sandwiched between a cistern of endoplasmic reticulum and the plasma membrane. Bar, 0.5 μm. × 39,200. (*b*) Ectoplasmic specializations adjacent to the head of an elongate spermatid. Notice that filaments on one side of the head are visualized in cross section (circle) whereas those on the other side are seen in longitudinal section (arrowheads). Bar, 0.5 μm. × 49,000. (*c*) Shown here is an ectoplasmic specialization that lies adjacent to another Sertoli cell, but in a nonjunctional region. Notice that the filaments are packed in hexagonal arrays (arrowhead) between the plasma membrane and a cistern of endoplasmic reticulum (*ER*). Bar, 0.25 μm. × 78,120. (*d*) Sertoli–Sertoli cell junction similar to that in *a* but at a higher magnification. The arrangement of filaments adjacent to the junctional site is obvious. Bar, 0.25 μm. × 89,000.

inated models do not visibly contract in the presence of exogenous ATP and Ca^{++}. These results are contrary to those we observed in myoid cells in which myosin is co-distributed with actin and glycerinated models do contract. Our data are consistent with the hypothesis that ectoplasmic specializations of Sertoli cells may be more skeletal than contractile.

MATERIALS AND METHODS

Animals: Ground squirrels used in this study were of two species—golden-mantled (*Spermophilus lateralis*) and Richardsons (*Spermophilus richardsonii*). They were housed and maintained generally as described elsewhere (28), and each animal was reproductively active when used for study.

Isolated Epithelia and Tubule Walls: Seminiferous epithelia were separated from extracellular matrix and myoid cells of seminiferous tubule walls by using EDTA in a phosphate-buffered saline (PBS) solution. Testes were excised from squirrels anesthetized with sodium pentabarbitone administered intraperitoneally. The spermatic arteries were cannulated and the organs were perfused for 5 min with PBS (150 mM NaCl, 5 mM KCl, 1.7 mM Na_2HPO_4, 0.8 mM KH_2PO_4, buffered to pH 7.3 with 0.1 N NaOH) containing 18 mM EDTA. Each testis was removed from the cannula and decapsulated in PBS containing 4.5 mM EDTA (isolation buffer). With two scalpels used in a scissor-like fashion, the decapsulated tissue was cut into small segments (1 mm^3). During this procedure, seminiferous tubules separated from interstitial tissue and numerous sheets of epithelia separated from tubule walls. Over the course of 10–20 min and by using polypropylene pipettes, epithelial sheets, tubule walls, and intact tubules were collected for further processing.

Glycerinated Models: Intact seminiferous tubules, epithelial sheets, and tubule walls were centrifuged at low speed and the supernatant was replaced with 50% (wt/vol) glycerol in either 150 mM KCl, 1.7 mM Na_2HPO_4, 0.8 mM KH_2PO_4, and 4.5 mM EDTA buffered to pH 7.3 with 0.1 N NaOH or 100 mM KCl, 1.7 mM Na_2HPO_4, 0.8 mM KH_2PO_4, and 4.5 mM EDTA, pH 7.0. Also present were protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 1 μg/ml soybean trypsin inhibitor). Extraction was carried out at 4°C overnight.

Electron Microscopy: Tissue from intact testes was fixed, processed, and photographed as described elsewhere (27). Isolated epithelia were fixed for 2 h with 3.7% paraformaldehyde, or 1.5% paraformaldehyde and 1.5% glutaraldehyde, in the isolation buffer, washed with 0.1 M sodium cacodylate (pH 7.3), then postfixed in buffered 1% OsO_4 on ice. Samples were stained en bloc with aqueous uranyl acetate, dehydrated through a graded series of ethyl alcohols, and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Sections were cut and stained by use of standard techniques for light and electron microscopy.

Fixation of Tissue for Fluorescence Microscopy: Tissue for use in determining the distribution of actin and myosin was processed in one of three ways. (*a*) Testes were removed from an anesthetized squirrel and decapsulated in PBS containing 3.7% paraformaldehyde. Seminiferous tubules were cut into small segments and, after 10 min, washed three times with PBS. Sheets of intact epithelia were obtained by squeezing individual tubules from one end to the other using pin probes. In addition, we fragmented seminiferous tubules by asperating them through an 18G then 22G needle. (*b*) Seminiferous epithelia, tubule walls, and intact tubules obtained by using the PBS-EDTA method were fixed for 10 min with 3.7% paraformaldehyde in PBS containing 4.5 mM EDTA, and then washed with PBS. (*c*) Glycerinated models were fixed for 10 min at room temperature with 3.7% paraformaldehyde or for 1 h on ice with 1% paraformaldehyde in the extraction buffer with and without glycerol, and then washed with the same buffer, but without glycerol or EDTA.

Tissue suspensions resulting from these three procedures and containing tubule fragments, epithelial sheets, tubules walls, and intact seminiferous tubules were further processed in the following manner. Drops of the suspensions were placed on polylysine-coated slides and allowed to stand for 15 min. Excess fluid was carefully removed with micropipettes; then the slides were plunged into cold (−20°C) acetone. After 5 min, the slides were removed and allowed to air-dry.

Localization of Actin by Using Nitrobenzoxadiazole-Phallacidin: Tissue on slides, prepared in the manner described above, was rehydrated with PBS for 10 min and then incubated, for 20 min and at room temperature, in one of the following: PBS; PBS and 1.65×10^{-6} M NBD-phallacidin[1]; PBS, 1.65×10^{-6} M NBD-phallacidin, and 1.04×10^{-4} M phalloidin; PBS and 1.04×10^{-4} M phalloidin. Slides were washed twice with PBS then wet-mounted for viewing.

Localization of Actin and Myosin by Using Antisera: Slides prepared in the manner discussed above were processed generally as described by Wolosewick and De May (29) for polyethylene glycol sections.

Primary antisera consisted of rabbit antiactin raised against chicken back muscle (Miles Laboratories Inc., Elkhart, IN; 65-096 Lot R826) and rabbit antimyosin raised against human platelet myosin (gift from Dr. K. Fujiwara,

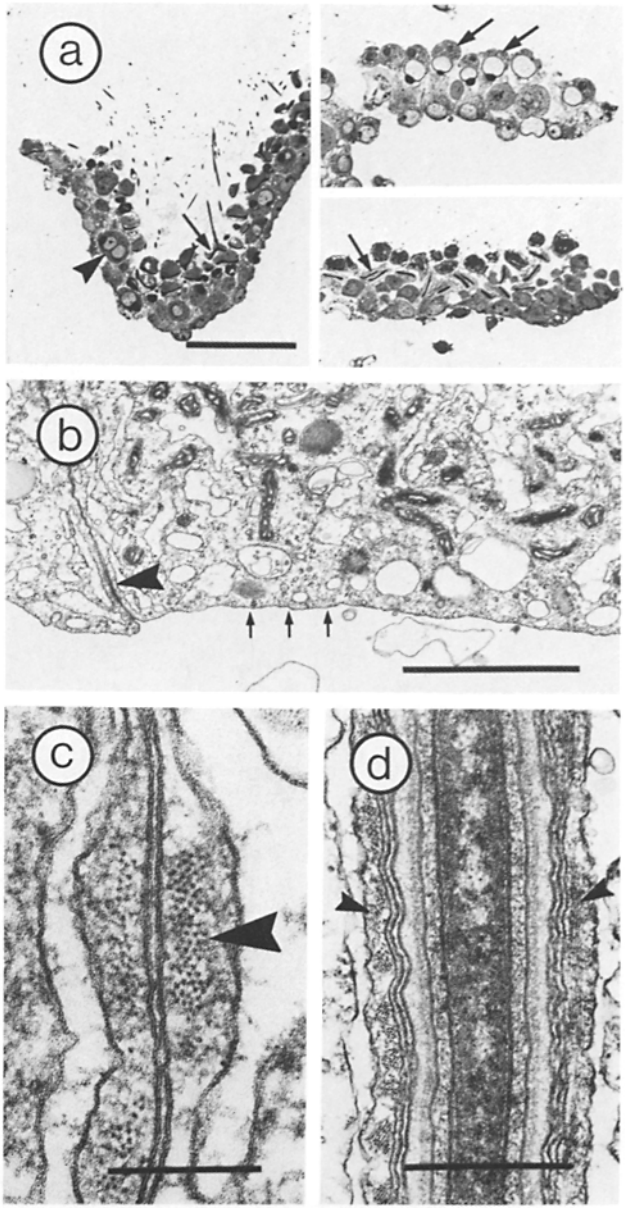


FIGURE 2 Light and electron micrographs of seminiferous epithelia isolated by use of the EDTA method described in the text. (*a*) Shown here are light micrographs of 1-μm sections of epithelial sheets. Spermatogenic cells (arrows) at different stages of development remain attached to the epithelium. Preservation is not ideal, as indicated by the occasional spermatogenic cell symplast (arrowhead). Bar, 100 μm. × 148. (*b*) Electron micrograph of the base of a Sertoli cell (arrows). Although a basal lamina is not present, Sertoli cell junctions (arrowhead) remain intact. Bar, 2 μm. × 11,914. (*c*) Sertoli-Sertoli cell junction and adjacent ectoplasmic specializations. Filaments are evident (arrowhead). Bar, 0.2 μm. × 107,000. (*d*) Sertoli cell regions adjacent to the head of an elongate spermatid. Ectoplasmic specializations are indicated by the arrowheads. Bar, 0.5 μm. × 45,200.

[1] *Abbreviation used in this paper:* NBD-phallacidin, nitrobenzoxadiazole-phallacidin.

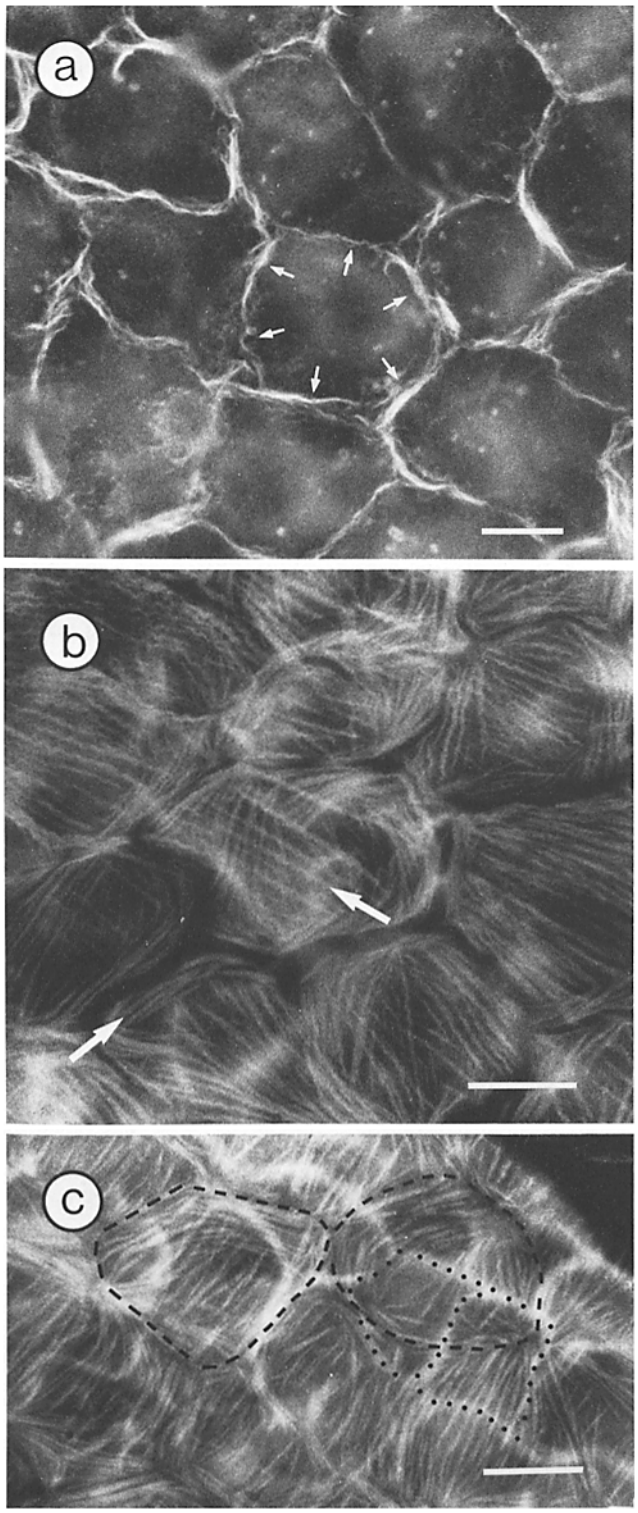


FIGURE 3 Micrographs showing the distribution of actin, as visualized with NBD-phallacidin, in seminiferous tubules of the ground squirrel testis. (*a*) Shown here is an isolated epithelial sheet viewed perpendicular to the base of the epithelium. The arrangement of actin in ectoplasmic specializations adjacent to Sertoli-Sertoli cell junctions at the base of the epithelium is clearly evident. The base of a single Sertoli cell is indicated by the arrows. Bar, 20 μm. × 505. (*b*) Shown here is a group of flat myoid cells within the wall of a seminiferous tubule that has been denuded of epithelium. The arrangement of actin is strikingly different from that seen in Sertoli cells. Linear bundles of actin line the cell surfaces and are oriented in predominantly two directions, as indicated by the arrows. By changing the plane of focus, one can show that filament bundles on one surface of a cell are generally oriented perpendicular to those on the other. Bar, 20 μm. × 700. (c) In intact seminiferous tubules, actin at the bases of Sertoli cells and within myoid cells can be visualized simultaneously, as documented in this micrograph. Two myoid cells are indicated by the broken line, and the bases of two Sertoli cells are indicated by the dots. Notice that Sertoli cell bases are smaller than myoid cells. Bar, 30 μm. × 460.

Department of Anatomy, Harvard Medical School). Antibodies were used at a dilution of 1:50 (antimyosin) and 1:10 (antiactin).

Secondary antibodies (goat anti-rabbit IgG conjugated to rhodamine or fluorescein; Miles Laboratories Inc.) were used at a dilution of 1:40. Incubation with both primary and secondary antibodies was for 1 h at 37°C.

Controls consisted of the following: (*a*) preimmune serum (myosin) or pooled rabbit serum (actin) instead of the primary antisera, (*b*) no primary antiserum, and (*c*) no primary or secondary antiserum.

In some experiments, NBD-phallicidin was mixed with the antiserum to myosin. This resulted in labeling of both actin and myosin on the same slide.

Contraction Experiments: Contraction experiments were modeled after those by Hirokawa and co-workers (13) on brush borders isolated from intestinal epithelial cells. Glycerinated tissue was sedimented by using gentle centrifugation and then washed with 25 mM PIPES, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM EGTA at pH 6.9 (control buffer) on ice for 10 min. The wash was repeated and the tissue was again sedimented by using centrifugation. The supernatant was replaced with one of the following: control buffer; control buffer + 0.2 mM ATP; control buffer + 0.2 mM ATP + 0.9 mM $CaCl_2$ (free Ca^{++}, 10^{-6} M as calculated by Hirokawa and co-workers [13]); control buffer + 0.9 mM $CaCl_2$. All samples in the series were transferred together from an ice bath to a water bath and incubated for 10 min at 37°C. Contraction was stopped by adding an appropriate amount of 25% paraformaldehyde to yield a final concentration of 3.7% or by gently centrifuging the samples and replacing the supernatants with the appropriate media containing 3.7% paraformaldehyde. Samples were brought to room temperature and after 10 min washed twice with PBS. Samples were placed on polylysine-coated slides then processed with NBD-phallacidin as described above. Myoid cells and ectoplasmic specializations associated with Sertoli cell junctions and with adjacent germ cells were evaluated qualitatively for evidence of contraction.

RESULTS

Ectoplasmic Specialization Structure

Ectoplasmic specializations in the ground squirrel resemble those in other species. They exist on either side of Sertoli cell junctions and consist of filament bundles sandwiched between a cistern of endoplasmic reticulum and the plasma membrane (Fig. 1 *a*). Ectoplasmic specializations of the Sertoli cells in regions adjacent to spermatogenic cells are extensive, but have a similar ultrastructure to those adjacent to Sertoli cell tight junctions. Although the arrangement of filament bundles at these locations cannot easily be determined from the analysis of thin sections, there are indications that it is complex. For example, close inspection of the actin in Fig. 1 *b* reveals that on one side of the head of an elongate spermatid, the filaments course perpendicular to those on the other. Further, at both the Sertoli cell junctional sites and in regions adjacent to germ cells, filaments are hexagonally packed (Fig. 1, *c* and *d*).

Isolated Epithelia

Treatment with PBS-EDTA results in sheets of seminiferous epithelium separating from tubule walls. That spermatogenic cells remain attached to Sertoli cells in the epithelial sheets is obvious in 1-μm thick plastic sections (Fig. 2 *a*). Moreover, when viewed at the ultrastructural level, Sertoli cells are free of a basal lamina (Fig. 2 *b*), but retain their junctions with each other (Fig. 2 *c*) and with spermatogenic cells (Fig. 2 *d*). Ectoplasmic specializations remain intact.

Distribution of Actin in Seminiferous Tubules as Visualized with Fluorescence

In isolated seminiferous epithelia viewed from an angle perpendicular to the epithelial base, actin in ectoplasmic specializations adjacent to Sertoli cell junctions occurs as hoops that outline the bases of adjacent cells. Together these hoops form a honeycombed pattern as shown in Fig. 3*a*.

A somewhat more complex pattern occurs in myoid cells (Fig. 3*b*). These very flat and somewhat polygonal-shaped cells form a complete layer, one cell thick, beneath the basal lamina of intact tubules. In isolated walls, the cobblestone arrangement of these cells is clearly evident when viewed with phase or dark-field optics (not shown). Based on the fluorescence signal obtained with NBD-phallacidin, actin occurs in numerous linear tracts that line the cell surfaces. All of the filament bundles are oriented parallel to the base of the seminiferous epithelium. These bundles are oriented in two general directions—parallel (circular) and perpendicular (longitudinal) to the long axis of the tubule wall. Also present are some tracts that run in a more oblique direction.

In intact tubules, actin in both Sertoli and myoid cells could be visualized simultaneously (Fig. 3*c*). In these preparations, the bases of Sertoli cells are smaller than the outlines of myoid cells. In other words, the positions of Sertoli cell junctional complexes, as indicated by actin in ectoplasmic specializations, do not correspond to the positions of underlying myoid cells in the tubule wall.

Although actin in ectoplasmic specializations adjacent to spermatogenic cells can also be detected in intact tubules, it is more evident in fragmented epithelia, two examples of which are shown in Fig. 4, *a* and *b*. In these figures, well-organized tracts of actin occur adjacent to developing spermatid heads. Actin at sites similar to these is more easily studied in spermatids and adjacent Sertoli cell regions that together have separated from the epithelium.

It becomes increasingly obvious, by comparing spermatids at different stages of differentiation (Fig. 5), that actin in Sertoli cell ectoplasmic specializations adjacent to spermatogenic cells changes dramatically during spermiogenesis. Actin is not generally observed in association with isolated round spermatids (Fig. 5*a*), but does become evident as these cells polarize and elongate. It first appears adjacent to the region of contact between the nucleus and acrosome (Fig. 5*b*), then expands to surround most of the nuclear region being capped by the acrosome (Fig. 5, *c–e*). Actin in this region circles the spermatid perpendicular to the long axis of the cell. Also present are small bundles of filaments that extend out of the main actin mass and over that portion of the acrosome not associated with the nucleus. These bundles are oriented parallel to the long axis of the spermatogenic cell. As spermatids complete elongation and begin to flatten, differential staining of actin over nuclear and acrosomal regions disappears and the filaments assume a more spiral arrangement around the entire spermatid head (Fig. 5*f*). This spiral arrangement results in an apparent latticelike network becoming visible as filaments on one face of the now flattened head are seen in focus with those on the other (Fig. 5*g*). With continued spermatid differentiation, filaments adjacent to one face of the saucer-shaped head orient themselves perpendicularly to the long axis of the cells whereas those on other face orient themselves parallel to it (Fig. 5*h*). This arrangement results in a staining pattern resembling the ridges and color bands of a scallop shell. During spermatid maturation, all filaments assume a more circular distribution (Fig. 5*i*); that is, they tend to course parallel to the rim of the head. Moreover, actin becomes less abundant (Fig. 5*j*), and, as expected, is not present adjacent to spermatozoa released from the epithelium.

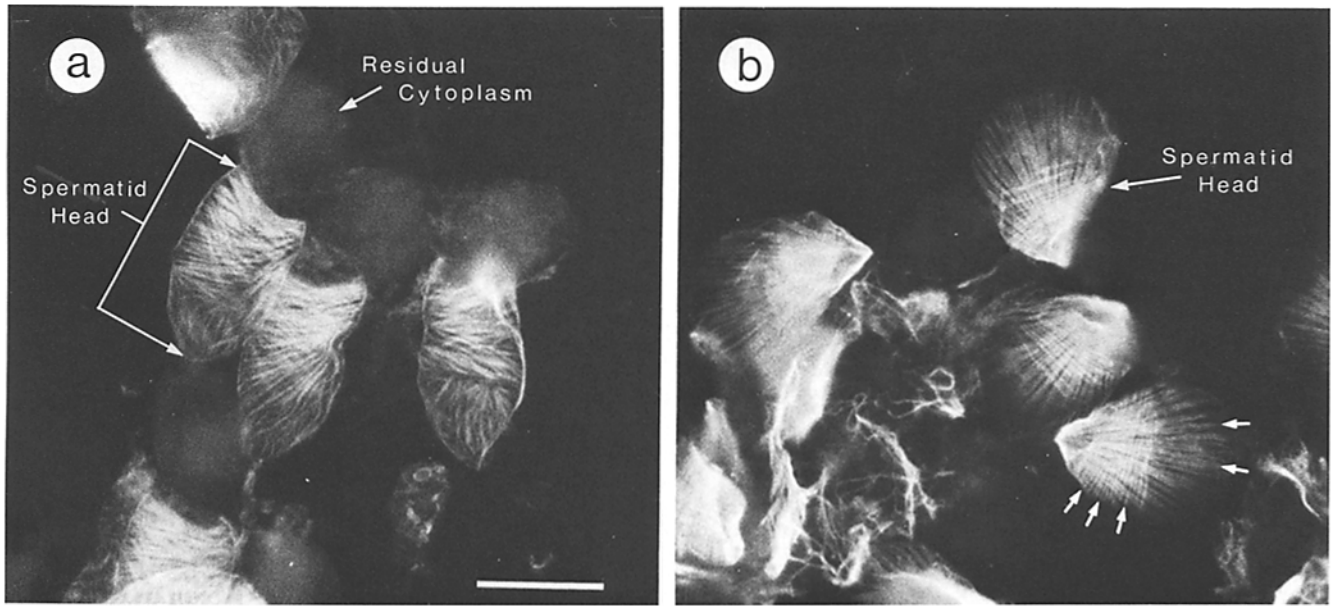
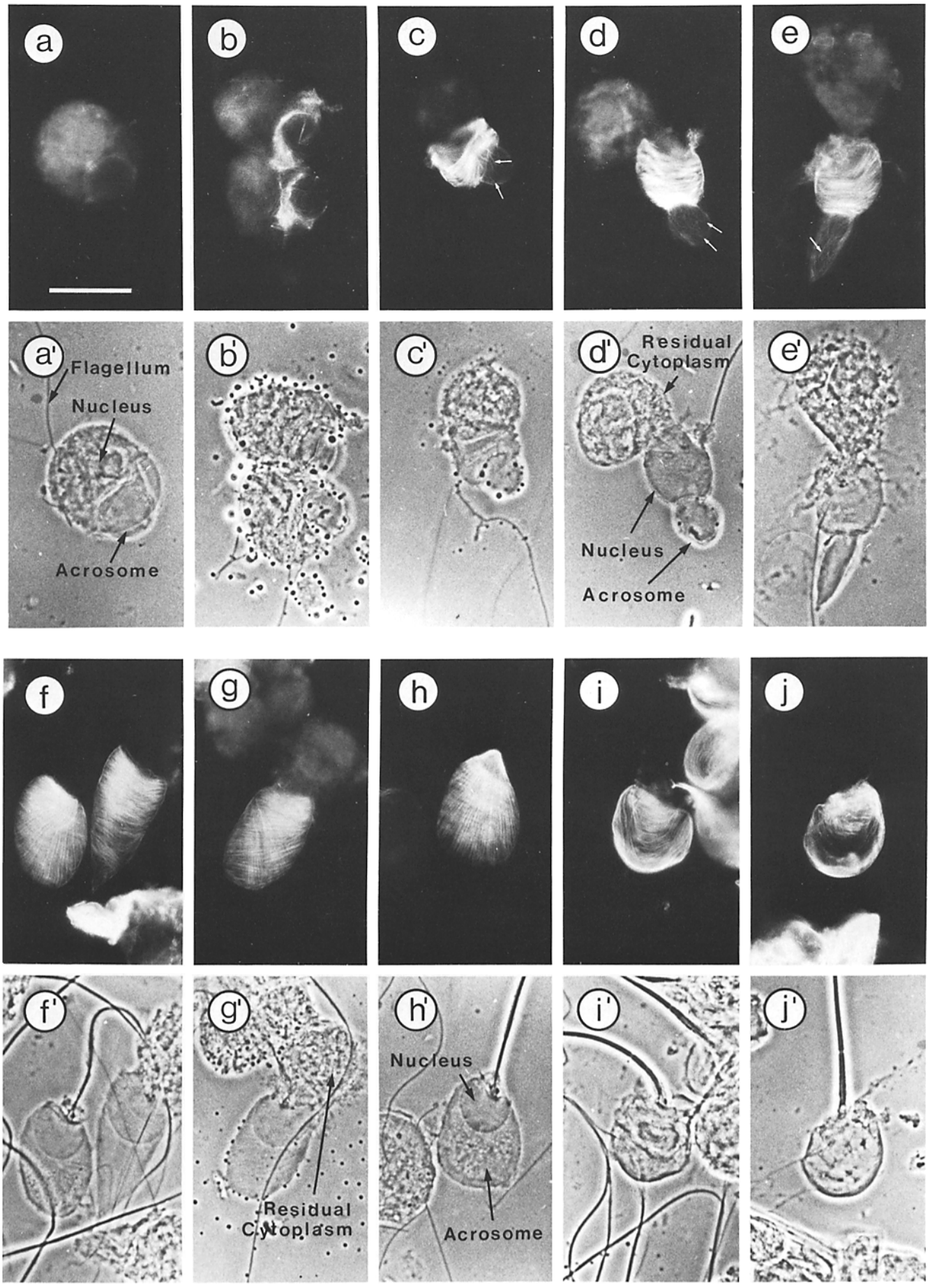


FIGURE 4 Distribution of actin in Sertoli cell ectoplasmic specializations adjacent to spermatids in isolated seminiferous epithelia. Spermatids in *a* are at an earlier stage of development than those in *b*. Notice that the actin bundles, as visualized with NBD-phallacidin, are closely associated with the spermatid heads. Also observe that actin bundles in *a* are organized differently than those in *b*. The orientation of actin bundles indicated by the arrows in *b* is consistent with the organization shown in Fig. 1*b*. By adjusting the focal plane we were able to show that filament bundles adjacent to one face of elongate spermatids are oriented parallel to the cell's axis, whereas those on the other are oriented perpendicular to it. In the spermatids shown here, both sets of bundles are visualized simultaneously. Bar, 20 μm. × 850.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The fluorescent signal obtained using antisera against actin was similar in all regions, to that obtained by using NBD-phallacidin (Fig. 6). However, a stronger signal with much less background was obtained using the latter compound. For these reasons, NBD-phallacidin was used for most experiments. No specific fluorescence was observed in any of the controls for either method.

Myosin Distribution

In material fixed before further treatment, in tissue isolated with calcium-free PBS, and in glycerinated models, a specific immunofluorescence signal similar to that seen with NBD-phallacidin occurs in cells of small interstitial blood vessels (Fig. 7, *a* and *b*), in myoid cells (Fig. 7, *c* and *d*), and in lymphatic endothelial cells (Fig. 7, *e* and *f*). A positive and fairly intense signal is also emitted from small unidentified cells found at the base of the seminiferous epithelium (Fig. 7*h*).

We observed no specific signal, comparable with that present in the cells listed above, at Sertoli cell junctional complexes (Fig. 7, *g–j*) or in regions adjacent to spermatogenic cells (Fig. 7, *k–n*). Occasionally, a very faint signal was detected at these sites, but it could not be differentiated from that obtained with the preimmune sera. In regions associated with spermatid heads, punctate staining was present in tissue treated both with the immune and preimmune sera.

Contraction Experiments

Because we were concerned that the antisera to human platelet myosin may not react with myosin in ectoplasmic specializations, that myosin in these structures may be masked by other proteins, or that our fixation protocol did not maintain the antigenicity of the myosin, we decided to take another approach. If myosin is present in ectoplasmic specializations and is part of an actomyosin contractile system, exposure to the appropriate conditions should induce contraction. Assuming ATP and perhaps Ca^{++} to be necessary, we attempted to induce contraction, in glycerinated models, by using methodology similar to that used in experiments on intestinal epithelium (13).

We were unable to generate contraction of ectoplasmic specializations adjacent to Sertoli cell junctions or spermatogenic cells with the concentrations of ATP (2 mM) and free Ca^{++} (10^{-6} M) reported here (Fig. 8). In marked contrast, myoid cells did contract and did so equally well with ATP alone, or with ATP in the presence of Ca^{++}. Moreover, results obtained by using tissue glycerinated in conditions suboptimal for myosin polymerization (150 mM KCl at pH 7.3) were the same as those obtained by using tissue extracted in more favorable conditions (100 mM KCl at pH 7) (see Pepe [16]).

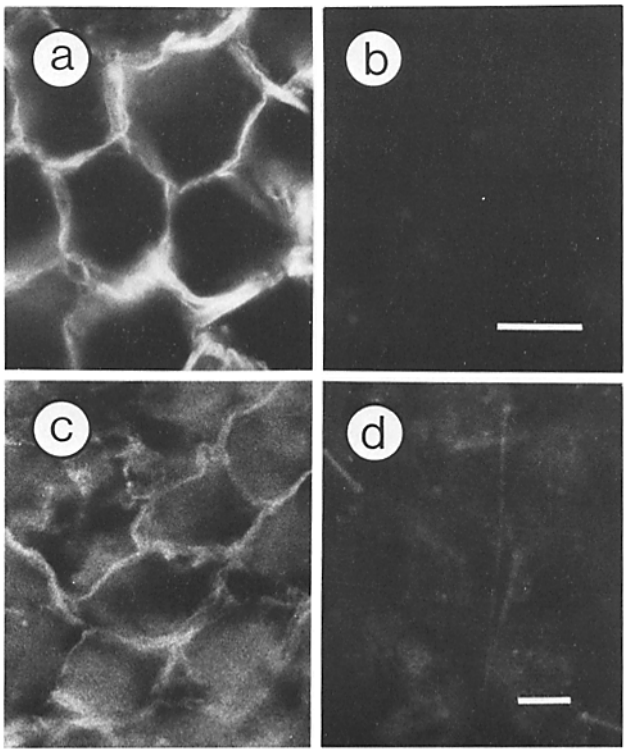

FIGURE 6 Fluorescence micrographs of actin distribution at Sertoli cell junctional sites in isolated and glycerinated epithelial sheets. The tissue shown in *a* was treated with NBD-phallacidin. That shown in *b* was also treated with NBD-phallacidin, but in the presence of phalloidin. Bar, 10 μm. × 1,100 (c) Epithelia treated first with rabbit antisera to actin then with goat anti-rabbit IgG conjugated to rhodamine emit a fluorescence signal similar to that in tissue treated with phalloidin. Shown in *d* is a sheet of epithelium treated with pooled rabbit serum then goat anti-rabbit IgG conjugated to rhodamine. Bar, 20 μm. × 675.

DISCUSSION

In this article we investigate the arrangement of actin filament bundles in specialized Sertoli cell regions, termed ectoplasmic specializations, found adjacent to tight junctions and sites of adhesion to spermatogenic cells. Actin bundles in these locations undergo profound organizational changes during spermatogenesis and do not appear to possess the characteristics of a contractile system. This is unlike the situation in myoid cells, found in the seminiferous tubule wall, where actin

FIGURE 5 A series of phase and fluorescence (NBD-phallacidin) micrographs showing the change in actin filament distribution in Sertoli cell ectoplasmic specializations adjacent to developing spermatids. The cells shown here are from *S. lateralis* and were fixed after isolation of intact epithelia by use of EDTA. Together with adjacent Sertoli cell ectoplasmic specializations, the spermatids have mechanically separated from the epithelium during the mounting procedure. Actin usually is not observed adjacent to round spermatids (*a*, *a'*) but does appear in the area of contact between the nucleus and acrosome as the germ cells begin to elongate (*b*, *b'*). As elongation continues, actin filaments become abundant adjacent to the nuclear region being capped by the acrosome (*c*, *c'*). Also, small bundles (arrows) appear over that portion of the acrosome not associated with the nucleus (*c–e*). As spermatids flatten, actin expands to cover the entire head (*f*, *f'*) and a latticelike arrangement becomes visible as filaments on one face of the spermatid are seen simultaneously with those on the other. The spermatid shown in (*h*, *h'*) has acquired a saucer shape. Filaments on one side are oriented parallel to cells axis and those on the other are oriented perpendicular to it. Both are evident in this micrograph (see also Fig. 4*b*). In mature spermatids (*i*, *i'*, *j*, *j'*) filaments are oriented parallel to the heads rim. Bar, 20 μm. × 756.

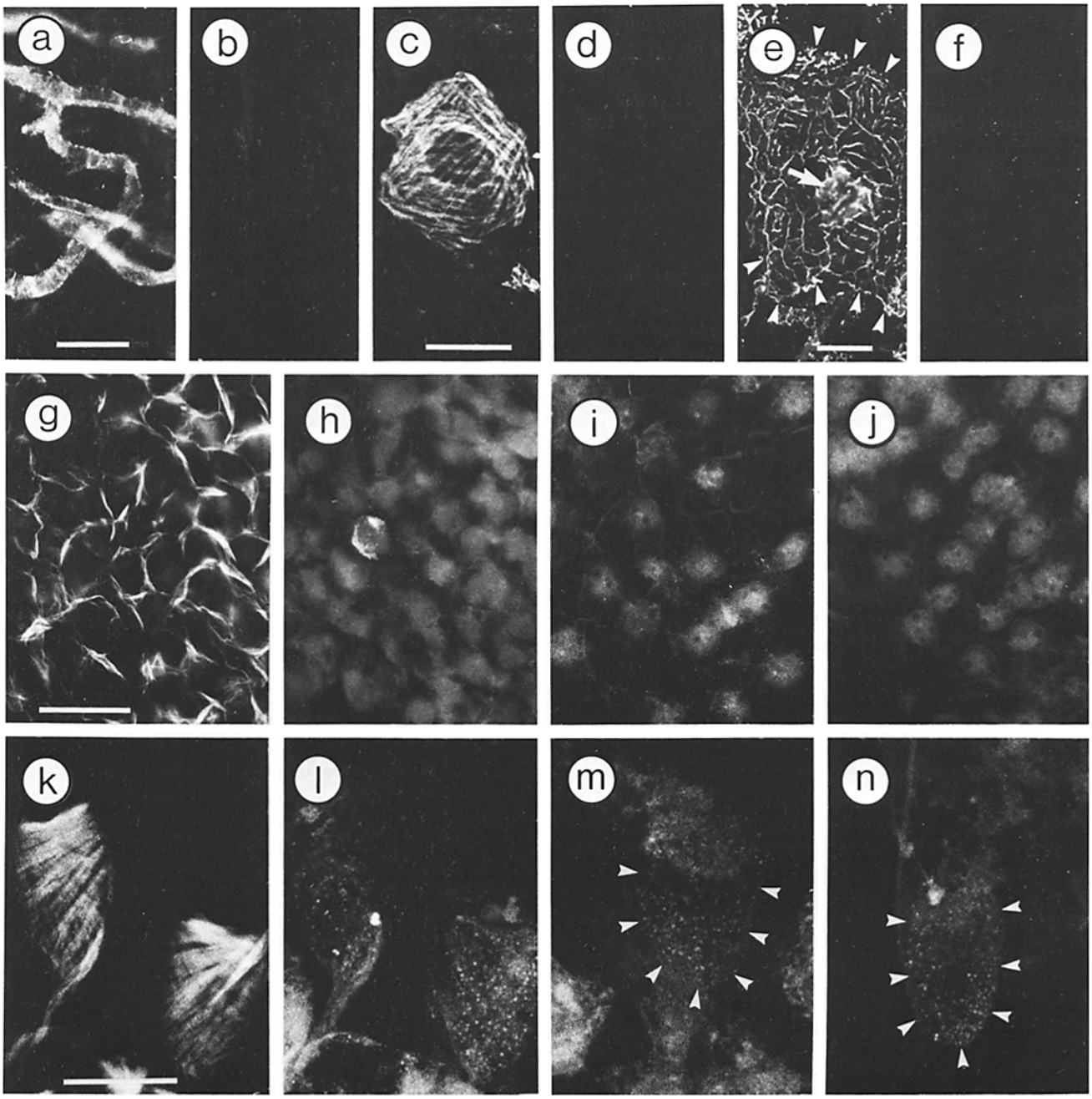

FIGURE 7 A series of micrographs illustrating the fluorescence signal obtained by treating testicular material first with rabbit antisera raised against human platelet myosin then with goat anti-rabbit IgG-RITC. (*a*) A small interstitial blood vessel, isolated with the EDTA buffer system, is strongly positive when treated with immune serum. (*b*) A vessel similar to that shown in *a*, but treated with preimmune serum rather than the primary antiserum, shows no specific fluorescence. Glycerol-extracted myoid cells treated with immune serum (*c*) and preimmune serum (*d*). Lymphatic endothelial cells, isolated with the EDTA buffer system, then treated with immune (*e*) and preimmune serum (*f*). Lymphatic endothelial cells are squamous, have irregular borders, and are larger than myoid cells. The periphery of the cell shown in *e* is outlined by the arrowheads. The nuclear region is indicated by the arrow. The fluorescence signal of this cell is similar to the pattern observed in similar cells treated with NBD-phallacidin. (*g* and *h*) A sheet of glycerol extracted seminiferous epithelium treated both with NBD-phallacidin and myosin antiserum. Ectoplasmic specializations are clearly labeled with the NBD-phallacidin (*g*) but not with the myosin antiserum (*h*). (*i*) An epithelial sheet, isolated with the EDTA buffer system, and treated with immune serum. (*j*) An epithelial sheet similar to that in *i* but treated with preimmune serum. (*k* and *l*) Glycerol extracted spermatids and adjacent Sertoli cell regions treated both with NBD-phallacidin and immune (myosin) serum. Filament bundles are clearly visible around the spermatid heads (*k*). A similar pattern is not visible with the immune serum (*l*). Glycerol extracted spermatid heads treated with immune (*m*) and preimmune (*n*) sera exhibit a nonspecific punctate staining pattern. The spermatid heads are outlined by the arrowheads. Results similar to those shown here were consistently obtained by using tissue mechanically separated after fixation or with material fixed after isolation with PBS-EDTA or extracted with glycerol. (*a* and *b*) Bar, 30 μm. × 340. (c and *d*) Bar, 20 μm. × 680. (e and *f*) Bar, 20 μm. × 410. (*g–j*) Bar, 40 μm. × 370. (*k–n*) Bar, 20 μm. × 931.

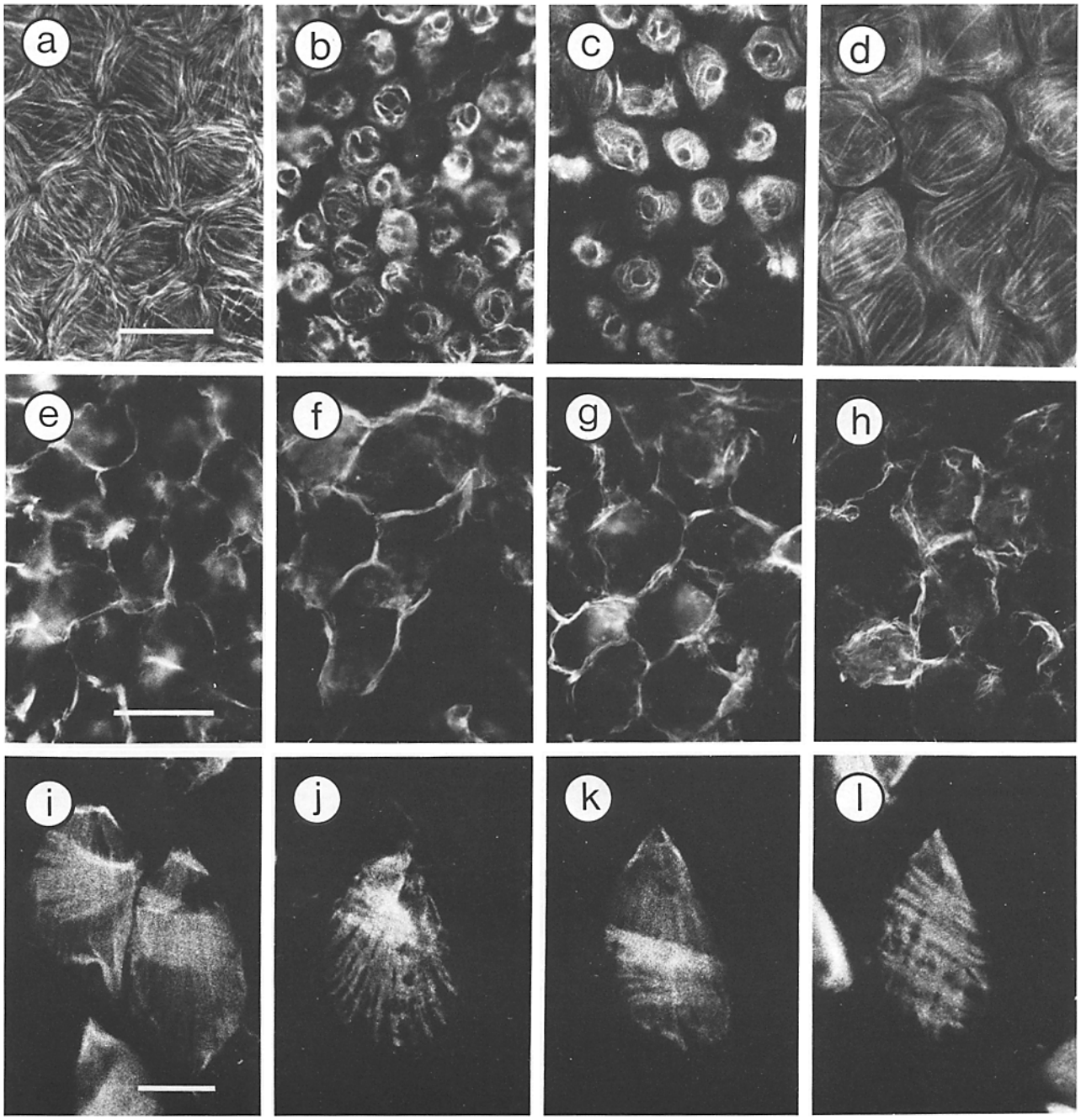

FIGURE 8 Shown here are the results of an experiment designed to test the hypothesis that ectoplasmic specializations are contractile. Isolated epithelia and denuded tubule walls were extracted overnight in 50% glycerol, 150 mM KCl, 1.7 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 4.5 mM EDTA, and adjusted to pH 7.3 with 0.1 N NaOH, then exposed to control buffer (*a*, *e*, and *i*), ATP (*b*, *f*, and *j*), ATP + Ca^{++} (*c*, *g*, and *k*), and Ca^{++} (*d*, *h*, and *l*), as described in the text. Myoid cells (*a–d*) contract equally well in ATP alone (*b*) or ATP in the presence of Ca^{+} (*c*). We could not induce contraction of ectoplasmic specializations associated with Sertoli cell junctions (*e–h*) or adjacent to spermatogenic cells (*i–l*). (*a*) Bar, 40 μm. × 380. (*e*) Bar, 40 μm. × 400. (*i*) Bar, 10 μm. × 1,230.

bundles do not change their arrangement during the spermatogenic cycle and do have contractile properties.

In myoid cells, actin bundles occur as linear tracts that line the cell surfaces. Interestingly, bundles on one surface lie predominantly perpendicular to those on the other. This arrangement enables a single layer of cells to generate contractile forces both in circular and longitudinal directions relative to the seminiferous tubule wall. The distribution of actin bundles in myoid cells and in Sertoli cells is summarized in Fig. 9.

Our images of actin in ectoplasmic specializations adjacent to Sertoli–Sertoli cell junctions are consistent with the arrangement predicted on the basis of ultrastructural data (26). Filaments circle the periphery of each Sertoli cell and are juxtaposed to junctional networks which, unlike in other epithelial cells, occur near the base of the cell and not at the apex. Moreover, the bundles of actin filaments remain cortical and do not extend deeper into the cell.

The function of ectoplasmic specializations at these junctional sites is not known, although, it has been suggested that

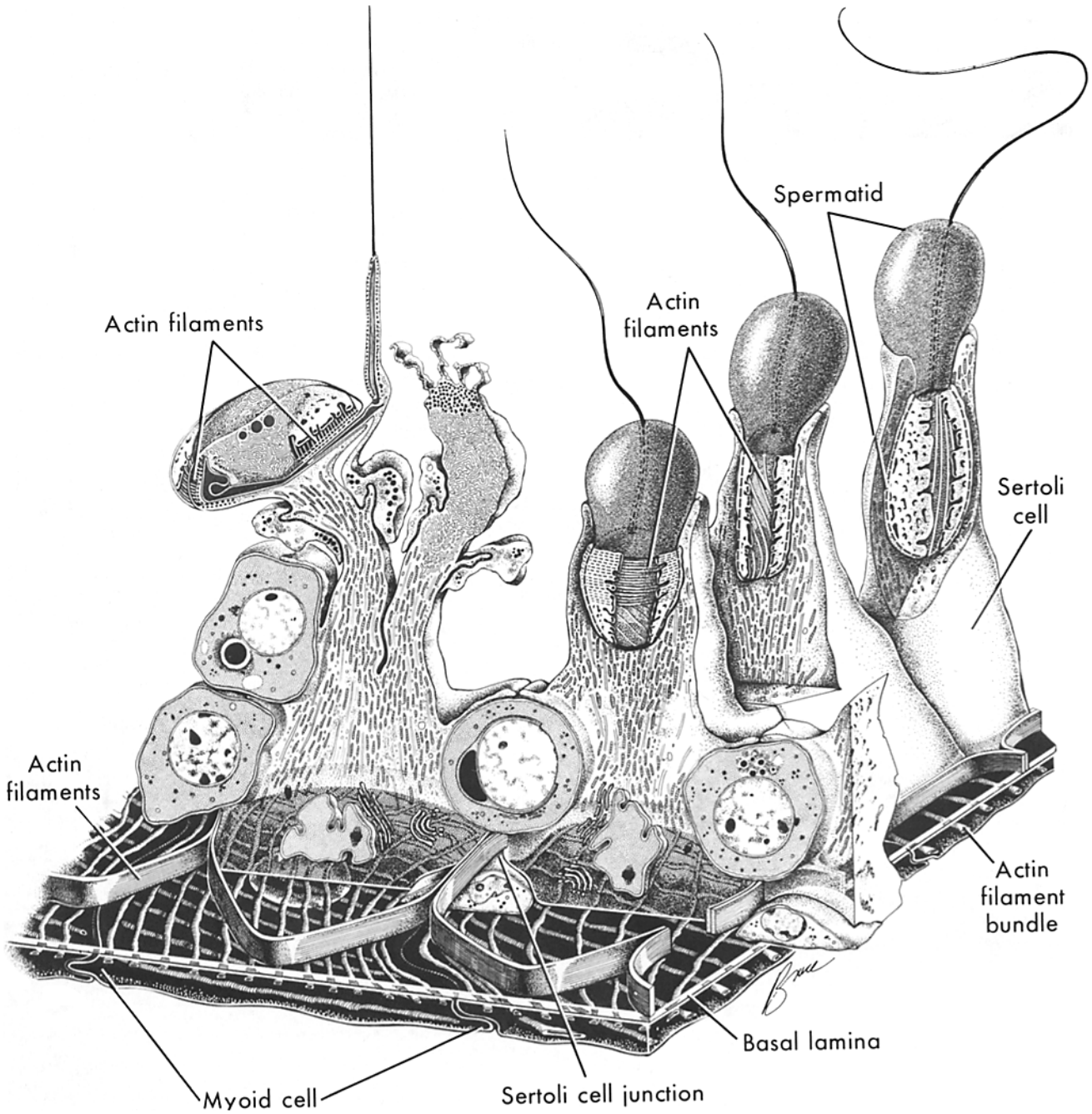

FIGURE 9 Schematic diagram of the ground squirrel seminiferous epithelium and adjacent tubule wall. No attempt has been made to represent one epithelial stage of spermatogenesis. Rather, Sertoli and spermatogenic cells are shown at different stages of the spermatogenic cycle. Myoid cells form a single pavementlike layer within the tubule wall and are situated external to the basal lamina of the seminiferous epithelium. Bundles of actin filaments, diagramed as cables, line the surfaces of these cells. In Sertoli cells, filament bundles are concentrated in ectoplasmic specializations that lie adjacent to Sertoli cell junctions and in regions of known adhesion to spermatogenic cells. Filaments adjacent to Sertoli cell junctions circumscribe the bases of the Sertoli cells. Those adjacent to spermatogenic cells occur mainly in association with developing spermatids. Shown on the right in the diagram are three spermatids each embedded in the apex of a Sertoli cell. The one on the left and in the foreground of this group is at an early stage of differentiation whereas the other two are at progressively later stages. Actin filaments in Sertoli cell ectoplasmic specializations initially circle the spermatid in the region where the acrosome caps the nucleus. In addition, small bundles extend into regions adjacent to the body of the acrosome. As the spermatid continues to differentiate, filament bundles assume a more spiral arrangement around the entire spermatid head. Eventually, and as the spermatid acquires a more flattened form, filament bundles on one side of the head orient parallel to the cell axis while those or the other align perpendicular to it. Filaments adjacent to mature spermatids, as shown on the far left of the diagram, are all oriented parallel to the rim of the head.

they may play a role in changing the "form and interrelations" of Sertoli cells to facilitate the movement of spermatogenic cells through the blood–testis barrier (3). Sertoli cell junctional complexes (junctions and the associated ectoplasmic specializations) are known to be dynamic structures because spermatogenic cells have to move through this barrier to differentiate fully into spermatozoa. Ultrastructural studies reveal that junctional complexes dissassemble above migrating sper-

matocytes while simultaneously forming below, thereby maintaining the integrity of the blood–testis barrier (23).

Although ectoplasmic specializations are also known to form adjacent to each new generation of spermatogenic cells and to disappear from these sites during spermiation, changes in the distribution of actin bundles within these cortical structures during spermatogenesis has not been appreciated previously. Our results clearly demonstrate that, in the ground squirrel, ectoplasmic actin bundles are not static features, but undergo distinct organizational changes that correlate with specific stages of spermatogenic cell differentiation.

Actin bundles associated with spermatids first encircle the region of contact between the acrosome and nucleus. They then expand to cover the entire spermatid head. Later, as the nucleus and acrosome flatten, ectoplasmic filaments orient in linear tracts that course parallel to the germ cell's axis on one side of the head and perpendicular to it on the other. Adjacent to late spermatids, actin is less abundant and assumes a more circular distribution.

The function of ectoplasmic specializations adjacent to differentiating spermatids is not known. Their association with sites of adhesion to spermatogenic cells and their eventual loss from these sites during sperm release has led to the conclusion that they are in some way involved with intercellular attachment and hence with spermiation (9–11, 18–21). A role in determining spermatid shape has also been put forward (4, 19), as has the concept that they actually grip elongate spermatids (7, 27). More recently, Russell (22, 24) has suggested that ectoplasmic specializations may "rigidify" or maintain the shape of Sertoli cell apical crypts in which spermatids mature.

Although the function of ectoplasmic specializations is not known, the structures are generally thought to be contractile, mainly because they contain actin (7, 27) and possess an ATPase activity (12).

There are now at least four pieces of information supporting the argument that ectoplasmic specializations may, in fact, not be contractile. First, in good-quality electron micrographs (Fig. 13 in reference 3, Figs. c and e in reference 17, Fig. 10 in reference 25, and Fig. 6 in reference 28), the actin filaments are closely packed in hexagonal arrays. Moreover, filaments within each bundle appear to have the same polarity (27, 28) and are laterally attached to both the plasma membrane and adjacent cistern of endoplasmic reticulum (7, 22).

Second, myosin has not yet been demonstrated amongst the ectoplasmic actin bundles. Thick filaments are not visible in electron micrographs of these cortical regions nor have we, in this study, been able to demonstrate conclusively the presence of myosin using immunofluorescence techniques. Although we feel our data do not allow us to rule out the presence of myosin in ectoplasmic specializations, they indicate to us that a protein reactive to antisera raised against human platelet myosin is at best present in low quantities relative to that found in myoid cells, lymphatic endothelial cells, and cells of small interstitial blood vessels.

Third, glycerinated models of ectoplasmic specializations do not visibly contract when treated with exogenous ATP and Ca^{++}, unlike models of myoid cells (as in this article) or filament bundles in other epithelia (13, 15). Our finding that myoid cells can be induced to contract is consistent with the suggested contractile function of these cells (2). In addition, the fact that extracted myoid cell models contract equally well in the presence or absence of Ca^{++} is consistent with findings in other systems that glycerinated models lack Ca^{++} sensitivity (13).

Fourth, changes in actin distribution adjacent to developing spermatids are not those indicative of a contractile system. For example, it is difficult to visualize how the pattern shown in Fig. 5 *h* could be derived solely by contraction of the pattern in Fig. 5 *e*, or how that in Fig. 5 *i* could be derived from Fig. 5 *h*.

If ectoplasmic specializations are not contractile and hence do not actively pull Sertoli cell cytoplasm away from spermatids or separate intercellular junctions, then how do they function? At present, the data are consistent with some form of structural role. Perhaps the filaments maintain the shape of Sertoli cell crypts in which spermatids are embedded, as previously suggested (22). They might also facilitate the development of acrosome shape, or in some way stabilize Sertoli–spermatogenic cell junctions. At the base of the epithelium, filament bundles may add support or rigidity to Sertoli cell junctions. As yet, mechanisms of controlling the synthesis and degradation of ectoplasmic specializations and the possible relationship of these mechanisms to sperm release and the stability of Sertoli–Sertoli junctions remains to be determined.

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