ACTIN ASSOCIATED INTERCELLULAR ADHESION JUNCTIONS IN THE MAMMALIAN TESTIS

By

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ABSTRACT

In the mammalian seminiferous epithelium, the cytoplasm of Sertoli cells adjacent to sites of intercellular attachment exhibits unique structural attributes. In each of these regions, a layer of hexagonally packed actin filaments lies situated between a cistern of endoplasmic reticulum and the plasma membrane. The filament layer together with the reticulum and adjacent plasma membrane are collectively termed an "ectoplasmic specialization". Ectoplasmic specializations occur in apical Sertoli cell regions at sites of attachment to spermatids and basally at sites of attachment to adjacent Sertoli cells.

Ectoplasmic specializations have been hypothesized to be actin associated intercellular adhesion junctions. If this is true, molecular components that characterize actin associated adhesion junctions in general should be present in ectoplasmic specializations. In this study, I tested this prediction in two ways. First, I investigated whether or not the protein vinculin is co-distributed with actin filament bundles in ectoplasmic specializations of the ground squirrel. Second, I immunologically probed ectoplasmic specializations for three cell adhesion molecules (CAMs) that are commonly found in regions of intercellular adhesion in other tissues. My results indicate that vinculin is co-distributed with actin in Sertoli cell regions attached to spermatids. These data are consistent with the conclusion that vinculin is a component of ectoplasmic specializations and, therefore, with the hypothesis that the latter structures are a form of actin associated adhesion junction. Experiments using probes for the CAMs indicate that E-cadherin, A-CAM and N-CAM are probably not present in ectoplasmic specializations. The adhesion molecule at these sites may be a different member of the known CAMs or an as yet unidentified CAM.

Based on data presented here and elsewhere indicating that ectoplasmic specializations are a form of actin associated adhesion junction, I describe the elaborate changes that occur in constituent filament bundles at sites of attachment to spermatids of the ground squirrel and interprete them in the context of the adhesion hypothesis.

During the course of the co-localization studies described above, I observed that vinculin and actin are co-distributed at certain sites of intercellular attachment between interstitial cells of Leydig in the ground squirrel testis. Moreover, at the ultrastructural level I found these sites correspond to microfilament rich junction regions. These observations are consistent with the conclusion that actin associated intercellular adhesion junctions exist between interstitial cells of Leydig in the ground squirrel testis.

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CHAPTER 1

General Introduction

In this thesis, I present evidence consistent with the hypothesis that actin associated adhesion junctions exist in at least two locations in the mammalian testis: (1) between Sertoli cells and adjacent cells; (2) and between neighboring Leydig cells.

Actin associated adhesion junctions play a fundamental role in the arrangement of cells into tissues. These junctions consist of adhesion domains in the plasma membrane that are related to actin filament concentrations within the cytoplasm.

In the mammalian testis, little is known about the mechanisms responsible for intercellular adhesion. Although concentrations of actin are associated with certain intercellular attachment regions in the seminiferous epithelium, these concentrations are morphologically distinct from those found at adhesion sites in any other tissue. In fact, they are so different that at least one author (Russell, 1977a) has questioned whether or not they are functionally associated with junctions at all. Other authors have suggested that these regions may be related to junctions (Flickinger and Fawcett, 1967; Nicander, 1967; Dym and Fawcett, 1970; Fawcett, 1975) and specifically to adhesion sites (Ross, 1977). However, it has only recently been proposed that these special regions may, in fact, be actin associated intercellular adhesion junctions homologous to those found in other tissues (Grove and Vogl, 1989; Grove, Pfeiffer, Allen, and Vogl, 1990). There is little evidence for actin associated adhesion junctions between other cell types specific to the testis.

Interstitial cells of Leydig exhibit a clustered arrangement indicative of the presence of some form of intercellular attachment. In this thesis I present data indicating that actin associated adhesion junctions occur between Leydig cells and, unlike those found in the seminiferous epithelium, appear to closely resemble those seen in other tissues.

Actin Associated Adhesion Junctions

One of the basic requirements of multicellular organization is the presence of a system by which intercellular and cell/substratum adhesion can be accomplished. A host of different mechanisms have evolved that meet this demand. One of the methods of adhesion most frequently seen involves the formation of specialized cortical regions in which adhesion molecules are concentrated in membrane domains that are associated with elements of the cytoskeleton.

Cytoskeletal associated adhesion sites are employed by cells in a diverse array of situations, ranging from contiguously organized cells of epithelia to isolated cultured cells in contact with surrounding components of the extracellular matrix. Based on the type of cytoskeletal filament associated with adhesion sites, two general categories of filament related adhesion sites can be distinguished; intermediate filament associated adhesion sites and actin associated adhesion sites. Each of these categories can be further subdivided into cell/cell and cell/substratum adhesion sites.

Intermediate filament associated adhesion sites consist mainly of desmosomes (cell/cell) and hemidesmosomes (cell/substratum). At both of these sites, intermediate filaments are related, via a series of linking molecules, to elements in the membrane responsible for adhesion (Franke et al., 1981; Mueller and Franke; 1983). These linking and adhesion molecules differ from those present at actin associated adhesion sites (Geiger et al., 1983).

Actin associated adhesion sites, the focus of this thesis, display considerable variety in cytoarchitecture. In polarized epithelial cells, the adhesion sites typically form zonular junctions, termed zonula adherens, that encircle cell apices (Farquhar and Palade, 1963). By contrast, actin associated adhesion junctions between cardiac muscle cells are sheet-like in nature (fascia adherens) (Tokuyasu et al., 1981). Still in other cells, such as ocular lens cells, the adhesion junctions are punctate (Lo, 1988). While the overall appearance of these junctions may differ, the manner through which adhesion is achieved follows a consistent plan. Actin filaments in the cortical cytoplasm are thought to be linked indirectly through a

membrane-bound plaque of proteins to adhesion mediating elements within the plasma membrane. The membrane adhesion proteins then function to physically bind adjacent plasma membranes together or link the plasma membrane to molecules of the extracellular matrix (Geiger et al., 1985a,b; Burridge et al., 1988; Takeichi, 1988; Geiger, 1989).

While the precise molecular organization of actin associated adhesion sites has not fully been determined, the puzzle is steadily being pieced together as knowledge of the identity and properties of the proteins that comprise these junctions increases. Certain proteins are present at both cell/cell and cell/substratum actin associated adhesion junctions. In addition to actin filaments, these include alpha-actinin and vinculin. The role of each of these latter two elements at adhesion sites is not entirely clear; however they both have been suggested to facilitate relating actin filaments to the membrane (Lazarides and Burridge, 1975: Geiger, 1982, 1983; Geiger et al., 1980, 1985a,b; Burridge et al., 1988). Alpha-actinin is an actin binding protein that can function to cross-link actin filaments into bundles (see Stossel et al., 1985). It has been identified at actin associated adhesion sites where its precise function is not presently known (see Burridge et al., 1988). Preliminary evidence does suggest that, at cell/substratum adhesion sites, alpha-actinin may bind directly to adhesion proteins (integrins) within the plasma membrane (Otey et al., 1990). This may indicate that, at this form of adhesion site, alpha-actinin is involved in the linking of actin filaments to the membrane. Vinculin (Geiger, 1979) is a 130 kD (recently revised to 116 kD - see Burridge, 1986) protein that, unlike alpha-actinin, is found exclusively at actin associated adhesion sites, and hence serves as a marker protein for this form of junction (Geiger, 1982; Geiger et al., 1983). It is a peripheral membrane protein that has been suggested either to link actin filaments to the membrane (Geiger, 1982, 1983; Geiger et al., 1980, 1985a,b; Burridge et al., 1988) or to nucleate actin polymerization (Geiger et al., 1984; Burridge et al., 1988) at adhesion junctions.

In addition to those elements that characterize actin associated adhesion sites in general, each of the subgroups (cell/cell, cell/substratum) contains its own unique set of proteins. At cell/substratum actin associated adhesion sites, the peripheral membrane protein talin has

been identified (Burridge and Connell, 1983; Burridge et al., 1988). Also at these sites, integral membrane molecules responsible for adhesion include the integrins (Buck and Horwitz, 1987; Geiger, 1989).

At cell/cell adhesion sites, unique peripheral membrane proteins include radixin and probably others (Tsukita et al., 1989; Tsukita and Tsukita, 1989). Interestingly, plakoglobin, a molecule present at desmosomes, has also been localized at these sites (Cowin et al., 1986). The molecules responsible for intercellular adhesion differ from those present at cell/substrate adhesion sites and appear to belong to the family of high molecular weight glycoproteins referred to as cell adhesion molecules (CAMs) (Hirano et al., 1987; Geiger, 1989; Kemler et al., 1989). Recently a group of three proteins, collectively termed the catenins, has been identified at intercellular junctions containing the CAM uvomorulin (Kemler and Ozawa, 1989; Ozawa et al., 1989). It has been suggested that these molecules may play a role in connecting the cytoskeleton to adhesion molecules.

As a result of the binding of actin filaments to surface adhesion proteins, the cytoskeleton becomes incorporated into the adhesion process. The fact that a broad spectrum of cell types exhibit actin associated adhesion junctions indicates that the linking of actin filaments to the adhesion site probably serves some fundamental role. The underlying functional purpose of this linking is still unclear. Among the proposed explanations, as summarized by Takeichi (1988), are:

(1) Actin filaments may serve to mechanically reinforce the junctional sites through a positioning and stabilizing of the surface adhesion proteins.

(2) The linking of actin filaments to surface adhesion proteins may represent a mechanism by which cells regulate the actions of their adhesion proteins, and thus a mechanism by which cells actively regulate their own adhesion.

(3) The linking may provide a means through which the surface adhesion proteins, upon cell-cell binding, may produce needed functional changes in the cortical actin and associated proteins.

(4) The actin filament-cell surface protein association may fulfill a combination of the above hypothesized roles.

Although actin associated adhesion junctions are widely distributed amongst cells and appear essential to tissue organization, these junctions are not well characterized in the mammalian testis - either between cells of the seminiferous epithelium or between interstitial cells.

Seminiferous Epithelium

In mammals the process by which spermatozoa form (spermatogenesis) takes place in epithelial lined tubes known as seminiferous tubules that are connected at both ends to the rete testis - an anastomotic series of ducts linked to the epididymis. The epithelium of these tubules (seminiferous epithelium), is structurally complex. It is composed of two cell types - numerous germ cells and a smaller population of Sertoli cells (Fawcett, 1975).

The germ cells proliferate and undergo dramatic structural transformations during spermatogenesis. The most immature cells (spermatogonia) are located basally within the epithelium. As they begin the process of differentiation, they gradually become more apically positioned. During this upward migration, the cells complete meiosis and become known as spermatids. Incomplete cytokinesis during spermatogenesis results in germ cells remaining attached to one another forming "clones" that move through the epithelium as units. Positioning of the spermatids in apical invaginations (crypts) of Sertoli cells occurs, and the cells develop the morphological features characteristic of mature spermatozoa. Events that occur during the morphological differentiation of spermatids (spermiogenesis) include nuclear condensation, development of an acrosome, reduction in cytoplasmic volume, and production of a flagellum or tail. Also occurring at this time is the acquisition of a species specific germ cell shape. This mainly involves changes in the contour of the nucleus and acrosome, which together form the spermatid head (de Kretser and Kerr, 1988; Junqueira et al., 1989). At one point during spermiogenesis, apical crypts of Sertoli cells invaginate deep into the epithelium, then later become positioned in Sertoli cell stalks that extend into the tubule lumen. From this site, mature spermatids are released as spermatozoa from the seminiferous epithelium, a process known as spermiation (for review see Russell, 1980).

Sertoli cells are, in the adult, a sessile and nondividing population of cells that constitutes the major structural element of the semininferous epithelium. They are situated on a basal lamina that separates the seminiferous epithelium from the underlying lamina propria

containing the contractile layer(s) of myoid cells. From this foundation the irregular columnar shaped Sertoli cells, each varying in contour, extend toward the tubule lumen. Cytoplasmic processes radiate asymmetrically from the lateral and apical surfaces of the cell bodies. Considerable changes in the shape and internal organization of Sertoli cells take place during spermatogenesis (Fawcett, 1975; de Kretser and Kerr, 1988).

Interposed between and attached to the Sertoli cells are the smaller and more numerous germ cells. Because there is a continual production and upward migration of germ cells through the epithelium, each Sertoli cell, at any given point in time, is in contact with numerous germ cells that are at different stages of differentiation (Fawcett, 1975; de Kretser and Kerr, 1988).

Sertoli cells are often referred to as "nurse" cells of the seminiferous epithelium and, indeed, this description is quite fitting. Sertoli cells are far from passive in their support of the germ cell population. They are actively involved in maintaining and regulating germ cell maturation and play an important role in mediating hormonal regulation of spermatogenesis (Tindall et al., 1985). In addition, Sertoli cells provide the germ cells with essential mechanical and nutritional support, and are likely responsible for germ cell translocation in and eventual release from the epithelium. Although the full realm of Sertoli cell functions has probably not yet been determined, this population of cells clearly plays an essential role in spermatogenesis (Fawcett, 1985; de Kretser and Kerr, 1988).

One significant role of Sertoli cells during spermatogenesis is to divide the seminiferous epithelium into two compartments (Dym and Fawcett, 1970). This partitioning arises from the fact that the junctional complexes of Sertoli cells, in contrast to those of cells in other epithelia, occur close to the base of the epithelium. Consequently, a small basal compartment, in which the most immature germ cells are housed, is formed below the junctions while a larger adluminal compartment, in which the more differentiated germ cells are situated, is created above them.

Sertoli cell junctional complexes consist of several intercellular junction types. Extensive occluding or "tight" junctions form the major structural elements of the blood-testis

barrier (Dym and Fawcett, 1970; Fawcett et al., 1970; Dym, 1973; Gilula et al., 1976). These occluding junctions form a continuous zone of intercellular contact throughout the seminiferous epithelium, creating a permeability barrier that effectively "seals off" the adluminal compartment from the basal one. Access of immune cells, or antibodies, to the adluminal compartment is thus prevented. These permeability junctions also allow the Sertoli cell to produce an adluminal microenvironment essential for normal germ cell maturation (Fawcett, 1975; de Kretser and Kerr, 1988).

Junction types other than occluding ones are present within basal Sertoli cell attachment complexes. Gap junctions occur interspersed between the tight junctions (Nicander, 1967; Dym and Fawcett, 1970; Fawcett et al., 1970; Gilula et al., 1976). In addition, desmosomes have been reported to occur between basal aspects of Sertoli cells (Russell, 1977b). Noticeably absent from Sertoli cell junctional complexes are typical zonula adherens that characterize junctional networks in most other epithelia. However, broad zones of close membrane apposition do occur within Sertoli cell junction complexes. These regions are associated with unique accumulations of cytoplasmic filaments and elements of the endoplasmic reticulum and are known as "ectoplasmic specializations".

Ectoplasmic Specializations

The plasma membrane of each Sertoli cell lies in close apposition to several different neighboring cells and follows a highly irregular course. Zones of intercellular adhesion with adjacent cells are present at sites along its contour. Some of these adhesion sites correspond to intermediate filament associated desmosomes (Russell, 1977b). At two notable locations, however, each Sertoli cell forms broad zones of strong intercellular adhesion from which desmosomes are generally absent. These zones are found basally at the level of Sertoli cell junctional complexes between adjoined Sertoli cells, and apically along the crypts housing elongating spermatids. The intercellular space at these sites is uniform and is reduced from 150-200 A to less than 100 A (Flickinger and Fawcett, 1967; Dym and Fawcett, 1970). Consistently found in association with these sites are elaborate arrays of actin filaments situated between the Sertoli cell plasma membrane and an underlying cistern of endoplasmic reticulum (Fig. 1.1). These complexes, consisting of the filament layer, the endoplasmic reticulum and regions of the plasma membrane involved with adhesion, are termed ectoplasmic specializations (ESs) (Russell, 1977a).

In ESs, the actin filaments exhibit a highly ordered arrangement in which they collectively assume a unipolar orientation (Toyama, 1976; Vogl et al., 1983, 1986). The filaments are grouped into bundles (Flickinger and Fawcett, 1967; Dym and Fawcett, 1970; Vogl and Soucy, 1985) in which the filaments are hexagonally packed (Dym and Fawcett, 1970; Franke et al., 1978), with an average interfilament spacing of 10-11 nm (Franke et al., 1978; Grove and Vogl, 1989).

Sertoli cell ESs are far from static complexes. The apical migration and dramatic shape changes of the developing germ cells during spermatogenesis are marked by the assembly and disassembly of ESs as well as dynamic changes in the patterns of ES actin filament bundles. As germ cells leave the basal compartment *en route* for the adluminal compartment, they pass through the blood-testis barrier. This is accompanied by the dissociation of junctions and ESs

above the germ cells and the simultaneous formation of new ones below (Russell, 1977c). Likewise, ESs located at the apical Sertoli cell/germ cell attachment sites undergo an assembly-disassembly process. While there appears to be interspecies variations with respect to the precise germ cell stage that ESs first associate with (see de Kretser and Kerr, 1988), well developed ESs are invariably found around elongating spermatid heads. This relationship is maintained throughout spermatid maturation during which considerable rearrangement of ES filaments occurs (Vogl and Soucy, 1985). An eventual ES dismantling takes place during spermiation at which time there is a corresponding loss of adhesiveness at the attachment sites (Ross, 1976; Russell, 1977a; Russell et al., 1980; Suarez-Quian and Dym, 1980; Vogl et al., 1983).

The precise functional relationship ESs assume with apical and basal Sertoli cell adhesion sites is unknown. Since their original description by Brokelmann (1963), numerous hyopotheses have been generated. That the filament type present at ES sites is actin has suggested to some authors that ESs may possess contractile properties since contractility is a common feature of many actin filament networks found in other cell types. Various hypotheses reflected this view including proposals that, through contraction, ESs may function to retain elongating spermatids within Sertoli cell crypts (Toyama et al., 1976; Russell, 1977a), or that ES contraction may play an active role in spermiation (Gravis, 1978), perhaps by pulling the plasma membrane away from the germ cells. More recent findings, however, indicate that, in mammalian species, ESs are not capable of contraction. Myosin II is absent from the structures and glycerinated models do not contract in the presence of appropriate buffers (Vogl and Soucy, 1985).

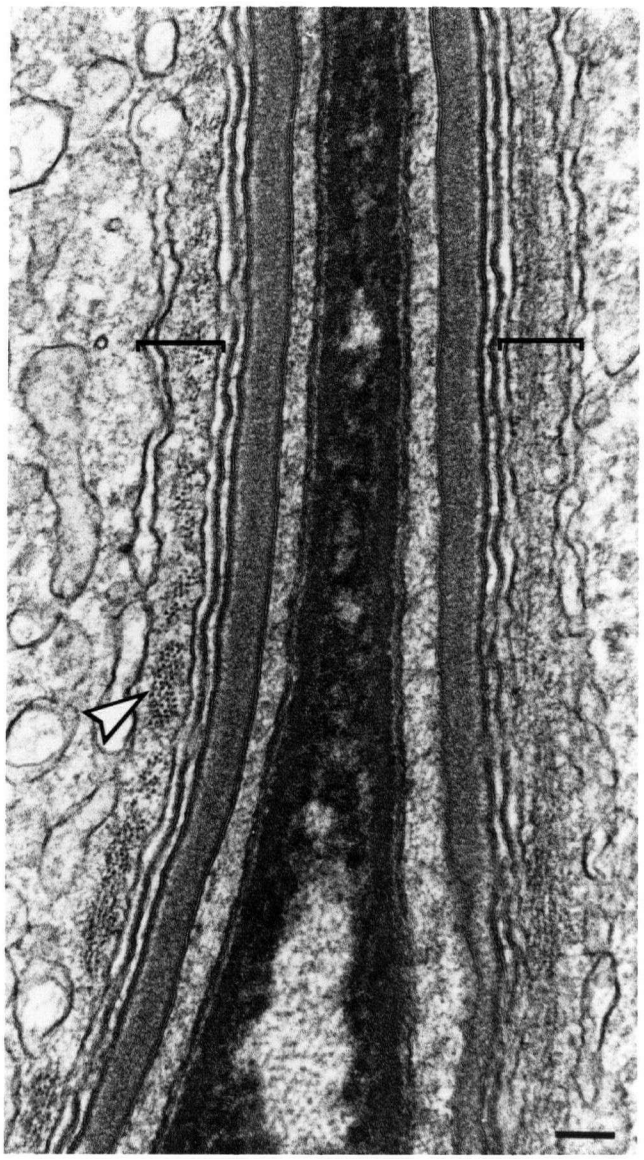
Other hypotheses of ES function have emphasized a more structural role at adhesion sites. Among these are proposals that ESs are involved in strengthening and supporting Sertoli cell adhesion sites (Russell, 1977a; Vogl and Soucy, 1985), or that ESs may serve as a scaffolding through which other cytoskeletal elements can act on adhesion sites to influence the positioning and movement of maturing germ cells through the epithelium (Russell, 1977a; Ross, 1976).

Common to the majority of hypotheses for ES function is the underlying theme of ES participation, either direct or indirect, in the process of intercellular attachment. Interestingly, in returning to the original descriptions of ESs (Brokelmann, 1963; Flickinger and Fawcett, 1967; Nicander, 1967), the suggestion was put forward that functionally ESs may not differ substantially from other filament associated junctions, where actin or intermediate filaments are involved in the intercellular adhesion process. These early descriptions provided little evidence for any interaction between ESs and the apical and basal intercellular attachment domains of the plasma membrane. Mounting evidence, however, now indicates that ESs are functionally associated with these membrane zones, and likely do play a direct role in intercellular adhesion. Observations supporting this conclusion include: (1) ESs occur only at sites of intercellular attachment, these being the basal Sertoli/Sertoli cell junctions and the apical Sertoli/spermatid adhesion sites; (2) When spermatids are mechanically separated from Sertoli cells, ESs remain attached to the spermatids (Russell, 1977a; Franke et al., 1978; Romrell and Ross, 1979; Vogl et al., 1985; Vogl and Soucy, 1985). For ESs to be removed, addition of trypsin is required, indicating that there is a proteinaceous linkage between ESs and the spermatids (Romrell and Ross, 1979). (3) Filaments of ESs are linked to the the plasma membrane through a proteinaceous layer of which vinculin may be a component (Grove and Vogl, 1989; Grove, Pfeiffer, Allen, Vogl, 1990). Vinculin is suspected to participate in the binding of actin filaments to the plasma membrane and is considered a protein marker for identifying actin associated adhesion junctions (see Actin Associated Adhesion Junctions). (4) ES dismantling during spermatogenesis, as germ cells move through the blood-testis barrier or prior to spermatid release from the epithelium, corresponds to a loss of intercellular adhesiveness. Experimental data further strengthens this point: disruption of ES actin filaments with the drug cytochalasin D results in a decrease or loss of adhesion between cells at apical sites and a change in permeability at basal Sertoli cell adhesion sites (Russell et al., 1988; Webber et al., 1988). (5) At the ultrastructural level, linkages spanning the intercellular space between apposed plasma membranes at ES sites have been reported (Russell et al., 1988). While one can not eliminate the possibility that these linkages simply represent artifacts of

tissue preparation, it is tempting to speculate that they may represent putative ES site adhesion molecules. Well developed intercellular linkages are consistently seen at adhesion junctions in other systems, e.g. zonula adherens junctions (Hirokawa and Heuser, 1981) and lens adherens junctions (Lo, 1988). At intercellular adhesion sites in general, actin filaments are thought to be linked to adhesion elements (CAMs) in the plasma membrane (see Actin Associated Adhesion Junctions).

In summary, the topographical association between ESs and Sertoli cell adhesion sites may involve more than a simple juxtapositioning of cytoskeletal elements with the plasma membrane. In fact, by analogy with other systems, there may be a physical linking between the actin filaments and as yet unidentified elements in the plasma membrane responsible for adhesion.

FIGURE 1.1 Shown here is a transmission electron micrograph of part of an apical Sertoli cell crypt containing an attached spermatid. Ectoplasmic specializations in the Sertoli cell cytoplasm immediately adjacent to the spermatid are indicated by the black brackets. Ectoplasmic specializations consist of a layer of actin filaments together with a cistern of endoplasmic reticulum on one side of the filament layer and regions of the plasma membrane involved with intercellular attachment on the other. Actin filaments within the layer are often grouped into distinct bundles, one of which is indicated here by the arrowhead. Bar = 0.1 um. X78,000. (Tissue shown in this figure was fixed and processed as described for Leydig cells in Chapter 5).



Leydig Cells

Unlike in Sertoli cells, cortical actin complexes involved with intercellular adhesion have not been identified in Leydig cells.

In mammals, Leydig cells occur as components of the tissue (interstitial tissue) between seminiferous tubules, and are the major endocrine components of the testis. Although Leydig cells exhibit some interspecies variation in number and internal structure (Christensen and Gillim, 1969; Fawcett, 1973), they generally have the morphology typical of steroid secreting cells (see de Kretser and Kerr, 1988). Leydig cells are responsible for virtually all of the androgen (testosterone) produced and secreted by the testis, and to a limited extent, they also synthesize and secrete estrogens (reviewed by Hall, 1988). The morphology of Leydig cells reflects this steroidogenic activity.

One morphological feature of these cells is the presence of lipid droplets in the cytoplasm. Cholesterol serves as the substrate for the synthesis of androgen by the Leydig cells. The source of this cholesterol appears to be both from plasma cholesterol, in the form of lipoproteins, and from the production of cholesterol by the Leydig cells themselves (see Hall, 1988). Lipid droplets, which serve as storage depots for steroidogenic cholesterol, are often conspicuous within Leydig cells. The size and number of these lipid droplets varies both with steroidogenic activity and with species (Christensen, 1975).

Mitochondria are the sites of conversion of cholesterol to pregnenolone in Leydig cells (see Hall, 1988). The structure of mitochondria differs between species (Christensen, 1975) Some, such as the ground squirrel, possess tubular cristae (Chapter 4, Fig. 4.5), whereas others, such as the rat, appear to have more lamellar (Christensen and Gillim, 1969) or "intermediate-looking" cristae.

The most prominent morphological feature of Leydig cells is the presence of an extensive network of interconnected tubules belonging to the endoplasmic reticulum (Christensen and Gillim, 1969; Christensen, 1975). Most of this reticulum is smooth, and is the site of

cholesterol synthesis and the conversion of pregnenolone to testosterone (see Hall, 1988).

In Leydig cells, unlike in Sertoli cells, there have been no reports of specialized complexes of actin filaments associated with intercellular attachment sites. Of the cytoplasmic filaments found in Leydig cells, actin filaments may represent only a small proportion of the total number, despite the fact that early descriptive studies reported abundant "microfilaments" or "fine filaments" within the cytoplasm (Belt and Cavazos, 1967; Chistensen and Gillim, 1969; Connell and Christensen, 1975). In reviewing the literature, it appears that the filaments described in many of these studies were most likely intermediate rather than actin, based on their appearance and location within the cell. Subsequent studies which clearly distinguished between actin and intermediate filaments revealed the latter type to be the most predominant (Russell and Burguet, 1977; David-Ferreira and David-Ferreira, 1980; Russell et al., 1987). Using the fluorescent probe NBD-phallacidin and fragments of the myosin molecule to localize actin filaments in rat Leydig cells, Russell et al. (1987) found actin filaments to be restricted to the cell perimeter in a limited network. Results of various actin disrupting experiments indicate that functionally this cortical network may be involved in the transport of cholesterol, and thus may assume a role in steroidogenesis (Hall et al., 1979; Bilinska, 1989). Russell et al. (1987) observed occasional actin filament associations with coated pits and noted that the peripheral location of actin filaments in Leydig cells is suited for a role in the uptake of cholesterol into the cells.

A peripheral location of actin is also consistent with a junctional related function, although, as indicated above, such an association with contact sites has not been reported.

The Thesis Problem

Sertoli cells:

Ectoplasmic specializations are complex actin filament containing structures found at specific regions of Sertoli cell attachment to neighboring cells. Based on cummulative observations of ES morphology and the interaction of these structures with attachment sites, the following hypothesis has been formulated: Ectoplasmic specializations are a form of actin associated intercellular adhesion junction. The filament bundles function to stabilize and reinforce membrane domains involved with intercellular adhesion (Vogl et al., 1986; Grove and Vogl, 1989; Grove, Pfeiffer, Allen and Vogl, 1990).

If the adhesion hypothesis is true, then components that characterize actin associated intercellular adhesion sites in general should be present in ectoplasmic specializations. In Chapter Two I provide evidence, using fluorescence microscopy, consistent with the prediction that vinculin is present in ectoplasmic specializations. In Chapter Three, I investigate whether or not cell adhesion molecules commonly found at actin associated adhesion sites are present at ectoplasmic specializations.

In Chapter Four, I describe the elaborate actin filament bundle changes that occur at apical ES sites in the ground squirrel during the course of spermatid maturation. I discuss these changes in the context of the adhesion hypothesis of ES function.

Leydig Cells:

In Chapter Five, I present evidence consistent with the hypothesis that actin associated adhesion junctions occur between interstitial cells of Leydig (a non-epithelial cell type) in the ground squirrel testis.

CHAPTER 2

Vinculin is Co-Distributed with Actin Bundles in Ectoplasmic Specializations of Ground Squirrel Sertoli Cells

Introduction

Ectoplasmic specializations are unique actin containing complexes found at two specific sites along the Sertoli cell plasma membrane - at sites of basal attachment between neighboring Sertoli cells and at sites of Sertoli cell attachment to spermatids (see reviews by Russell, 1980; Russell and Peterson, 1985; Vogl, 1989). As indicated in the General Introduction, ESs are speculated to be a form of actin associated intercellular adhesion junction (Grove and Vogl, 1989; Grove, Pfeiffer, Allen, and Vogl, 1990). If this hypothesis is true, one would predict that molecular components that characterize this form of junction in general should be present in ESs.

One of the marker proteins for actin associated adhesion sites is vinculin (Geiger, 1982; Geiger et al., 1983). Vinculin is a 130 kD (116 kD) peripheral membrane protein found at both cell/cell and cell/substratum adhesion junctions. This protein may aid in linking actin filaments to integral membrane proteins at adhesion sites (see reviews by Geiger et al., 1985a; Geiger, 1989).

Recent evidence indicates that vinculin may be a component of ESs. Immunological probes for vinculin react positively with a band of the appropriate molecular weight for vinculin on nitrocellulose blots of testicular fractions enriched for ESs (Grove and Vogl, 1989). In addition, the same probes label regions known to contain ESs in fixed frozen sections of rat seminiferous epithelium (Grove and Vogl, 1989; Grove, Pfeiffer, Allen and Vogl, 1990). Although these data are suggestive that vinculin is present in ESs, they are not conclusive. It could be argued that the reactive material on immunoblots may be due to vinculin from structures other than ESs that are contaminating the ES enriched fraction. In a similar way, it could be argued that the fluorescence emitted from antibody labelled sections is from structures other than ESs.

In ESs, actin filaments are cross-linked into bundles (Flickinger and Fawcett, 1967; Dym and Fawcett, 1970; Franke et al., 1978 Vogl and Soucy, 1985). In species such as the

ground squirrel, these bundles form distinct patterns around maturing spermatid heads (Vogl and Soucy, 1985). If vinculin is a component of ESs, and if the protein does in fact participate in relating actin filaments to the plasma membrane, one might predict that the patterns of vinculin distribution in ESs should co-distribute with those of actin filament bundles.

In this chapter I test the prediction that vinculin is co-localized with actin bundles in ESs. The approach I use is to react isolated ESs of the ground squirrel with fluorescently tagged probes for actin and vinculin. My data indicate that vinculin is co-localized with actin bundles in ESs and are consistent with the general hypothesis that ESs are a form of actin associated intercellular adhesion junction.

Materials and Methods

Animals:

Reproductively active golden-mantled ground squirrels (Citellus lateralis) were used in this study. Housing and maintenance of the animals generally followed that previously described (Vogl et al. 1983).

Fluorescence Microscopy:

(a) Tissue Preparation:

To facilitate visualization of ground squirrel apical ES sites, various stages of elongating spermatids together with their attached ESs were mechanically separated from the seminiferous epithelium in the following manner. Testes were excised from animals anesthetized with sodium pentabarbitone administered intraperitoneally. Each testis was perfused via the spermatic artery with 3% paraformaldehyde in PBS (150 mM NaCl, 5 mM KCl, 3.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, adjusted to pH 7.3 with 0.1 N NaOH) for 10 min at room temperature. Each testis was then decapsulated and minced, for 5 min, with two scalpels in fixative. This yielded a heterogeneous mixture of intact tubules, tubule walls, epithelial sheets and interstitial tissue which was transferred to glass centrifuge tubes and sedimented at low speed in a clinical centrifuge. Pellets were resuspended in PBS containing 0.1% triton-X 100 and incubated at room temperature for 5 min. Samples were then centrifuged and pellets resuspended in PBS and incubated for 10 min at room temperature. This step was repeated, after which pellets were resuspended in approximately twice their volume of PBS and gently aspirated 5-10 times through an 18G syringe needle. Drops of this tissue suspension were placed on polylysine-coated slides. Large aggregates of intact tubules and tubule walls were carefully removed with

micropipettes leaving only isolated clusters of spermatogenic cells and Sertoli cells, small groups of interstitial cells, and individual cells of various types (including elongate spermatids with attached ESs) on the slides. After 15 min, excess fluid was removed and the staining procedures for actin and vinculin were initiated.

(b) Single Label Experiments:

Localization of Actin:

The distribution of filamentous actin at ES sites surrounding isolated spermatids was revealed with the F-actin specific probe rhodamine-phalloidin. Slides with tissue samples prepared as described above were incubated at room temperature for 20 min in one of the following solutions: (1) PBS + 1.65 X 10^{-6} M rhodamime-phalloidin (test for filamentous actin); (2) PBS + 1.65 X 10^{-6} M rhodamine-phalloidin + 0.97 X 10^{-4} M phallacidin (competitive specificity control); (3) PBS + 1.04 X 10^{-4} M phallacidin (control for phallacidin in reagent in (2); (4) PBS (control for autofluorescence). Slides were then washed three times, for 10 min each, with PBS and mounted with 1:1 PBS:glycerol containing 0.02% sodium azide.

In some experiments, NBD-phallacidin (nitrobenzoxadiazole-phallacidin) was used instead of rhodamine-phalloidin to label filamentous actin. The controls done were similar to those used for experiments with rhodamine-phalloidin except that phalloidin (rather than phallacidin) was used in the competitive specificity sample (solution #2) and in solution #3.

Localization of Vinculin:

Indirect immunofluorescence was used to localize vinculin at ES sites. The probe used (previously prepared in this laboratory - Grove and Vogl, 1989) was an affinity purified

antibody raised against human platelet vinculin. Characterization of this antibody is described elsewhere (Grove and Vogl, 1989; Grove, Pfeiffer, Allen, and Vogl 1990).

Following the tissue preparation outlined above, slides were incubated for 30 min at room temperature with TPBS (0.05% Tween-20 in PBS) containing 0.1% bovine serum albumin (BSA) and 5% normal goat serum (NGS). Slides were then drained and incubated with the primary antibody at an approximate concentration of 50 ug/ml in TPBS containing 0.1% BSA and 1% NGS for 1 hr at 37^{o}C. After washing three times, for 10 min each, with TPBS containing 0.1% BSA, samples were incubated for 1 hr at 37^{o}C with secondary antibody (goat anti-rabbit IgG conjugated to fluorescein, Sigma) diluted 1:32 with TPBS containing 0.1% BSA. Samples were again washed three times with TPBS containing 0.1% BSA and then mounted with 1:1 PBS:glycerol containing 0.02% sodium azide.

Control slides included (1) non-adsorbed serum (1:50 dilution) substituted for the specific antibody; (2) preincubation of the specific antibody with antigen (1:10 mg/ml); (3) secondary antibody alone; (4) no antibodies. Smooth muscle cells of interstitial blood vessels served as a positive control for the specificity of the vinculin antibody.

(c) Double Label Experiments:

Slides to be double labelled for vinculin and actin were treated in a similar fashion to those used to label vinculin alone, except that rhodamine-phalloidin was included in the first of the three washes following incubation in the second antibody. Also, the second antibody was a goat anti-rabbit IgG conjugated to AMCA (7-amino-4-methylcoumarin-3-acetic acid) (Jackson Immunoresearch Laboratories, Inc). It was used at a dilution of 1:40.

All fluorescence microscopy in this study was performed on either a Zeiss Photomicroscope III or a Zeiss Axiophot Photomicroscope both fitted with filters for detecting fluorescein isothiocyanate, rhodamine, and AMCA.

Results

(a) Single Label Experiments:

Distribution of Actin:

By fluorescence, the distribution of ES actin filament bundles is easily visualized around isolated differentiating spermatids (Fig. 2.1e,e' - h,h'). Individual filament bundles appear as linear bands of fluorescence that course around spermatid heads. Collectively the filament bundles are organized into highly ordered patterns which undergo dramatic rearrangements during the course of spermatid differentiation. The overall staining patterns for ES actin filament bundles are similar to those which have been previously described in the ground squirrel (Vogl and Soucy, 1985). A more thorough description of actin filament bundle arrangement and patterning changes at ground squirrel apical ES sites is presented in Chapter 4.

No specific staining was observed in any of the control slides for rhodamine-phalloidin (Fig. 2.2) or NBD-phallacidin (data not shown).

Distribution of Vinculin:

In spermatids isolated with surrounding Sertoli cell ESs, specific staining occurs over the developing acrosome and nuclear regions with the affinity-purified probe for vinculin. As seen in Figure 2.1, the general patterns of vinculin labelling around representative stages of spermatid development (Fig. 2.1 a,a' - d,d') resemble those of ES actin labelling around similar stages (Fig. 2.1e,e' - h,h'). The vinculin staining appears somewhat more punctate than the

actin staining, but linear bands of fluorescence corresponding to known ES filament bundle arrangements are clearly evident.

A non-specific labelling of the acrosome with the vinculin antibody is seen in the early stages of spermatid elongation (Fig. 2.1a); however, this diminishes and is lost during the subsequent stages of spermiogenesis. When a 1:50 dilution of the non-adsorbed serum fraction is substituted for the primary antibody (Fig. 2.3b,b') different labelling patterns, unlike those which ES actin filaments exhibit (2.3a,a'), are seen. No specific fluorescence occurs when cells are incubated with primary antibody that has been preadsorbed with antigen (human platelet vinculin) (Fig. 2.3c,c'). Similarly, no specific fluorescence is seen when the primary antibody (not shown) and primary and secondary antibodies (Fig. 2.3d,d') are omitted from the staining procedure.

(b) Double Label Experiments:

In experiments in which isolated spermatids with attached ESs are labelled for both filamentous actin and vinculin, the staining patterns for the two proteins co-distribute around the spermatid head (Fig. 2.4a,b,c). Intercellular bridges, which contain filamentous actin (see Vogl, 1989), label strongly with rhodamine phalloidin (Fig. 2.4b), but not with the vinculin probe (Fig. 2.4c). This serves as an internal control for the specificity of vinculin labelling since intercellular bridges are not a form of adhesion junction.

Discussion

In the study presented here, I provide evidence that the protein vinculin is present in ESs of ground squirrel Sertoli cells.

Ectoplasmic specializations of mammalian Sertoli cells consist of a layer of hexagonally packed actin filaments together with a cistern of endoplasmic reticulum on one side of the filament layer and regions of the plasma membrane involved with intercellular contact on the other. The actin filaments are unipolar in arrangement, are cross-linked to each other and to adjacent membranes, are organized into bundles, and are not part of a contractile system. The structures occur basally at sites of attachment to adjacent Sertoli cells, and apically at sites of attachment to spermatids (see Chapter 1, and reviews by Russell, 1980; Russell and Peterson, 1985; Vogl, 1989).

At apical sites the filament bundles in ESs undergo pattern changes during spermatogenesis. In the ground squirrel, a species in which spermatogenic cells are large, the associated ESs are extensive and the actin bundles undergo particularly dramatic pattern changes (Vogl and Soucy, 1985). It is for this reason that I chose to use ESs of the ground squirrel for the co-localization study reported here. The well developed filament bundle patterns, in this species, readily permit comparison of junction-specific protein distribution with that of actin.

Little is known about the molecular composition of ectoplasmic specializations. There is some evidence for the presence of at least two members of the large class of actin binding proteins - alpha-actinin (Franke et al., 1978; Russell and Goh, 1988) and fimbrin (Grove and Vogl, 1989) . Both alpha-actinin and fimbrin are filament cross-linking proteins. In addition, alpha-actinin may participate in linking actin to the plasma membrane (Lazarides and Burridge, 1975; Burridge et al., 1988; Otey et al., 1990). Fimbrin cross-links actin filaments into bundles in which constituent filaments are unipolar (Glenney et al., 1981; Matsudaria et al., 1983; Mooseker, 1985). Besides actin, fimbrin and alpha-actinin, the only

other element that has been identified in ESs is plakoglobin (Cowin et al., 1986), a protein that is found both at actin and at intermediate filament associated adhesion junctions. Proteins that are present in actin networks in other systems and that appear to be absent from ESs include myosin II and villin (Vogl and Soucy, 1985; Horvat et al., 1990). Myosin II is an actin-based ATP-dependant mechanoenzyme (see Warrick and Spudich, 1987). Villin, a protein characteristically found in microvilli, is an actin bundling or actin severing protein depending on local calcium levels (Bretscher and Weber, 1980; Strossel et al., 1985).

Although only a few elements have been identified at ES sites, work on actin associated adhesion junctions in other systems indicates that there are likely several more. For instance, Tsukita and Tsukita (1989) have succeeded in isolating adherens junctions from rat liver cells and have tentatively identified at least 10 polypeptides that may be junction components. One of these, "radixin", (Tsukita et al., 1989) has been further characterized and appears to be localized only at adherens junctions. Another group of researchers (Ozawa et al., 1989) has identified three polypeptides, collectively called the "catenins", that co-isolate and co-distribute with uvomorulin (E-cadherin). Given that there are numerous proteins present at actin associated adhesion junctions in other cells, ESs are likely to contain more components than the few that have thus far been identified.

The function of ectoplasmic specializations is not entirely clear; however, there are a number of pieces of data (reviewed in Chapter 1) that are consistent with the view that these structures are a form of actin associated adhesion junction. If this is true, then proteins that typify actin related adhesion junctions in general should be found in ESs. In addition to actin and alpha-actinin, there is mounting evidence for the presence, in ESs, of vinculin - a protein that is currently considered the most definitive marker for actin associated adhesion sites.

Evidence for the presence of vinculin in ESs includes the following: (1) Affinity purified polyclonal antibodies raised in rabbits against human platelet vinculin react positively with a band of the appropriate molecular weight for vinculin on immunoblots of testicular fractions enriched for ESs (Grove and Vogl, 1989); (2) The same immunological probe labels regions in fixed frozen sections of rat testis that are known to contain ESs (Grove and Vogl, 1989; Grove,

Pfeiffer, Allen and Vogl, 1990); (3) As shown here, fluorescently labelled probes for actin and vinculin are co-distributed in isolated ESs of the ground squirrel. Since the completion of this study, further work has provided additional evidence that vinculin is present in ESs: At the ultrastructural level, immunological probes for vinculin label ESs of the rat (Vogl, unpublished data).

In this study I provide evidence that vinculin is present in ectoplasmic specializations of the ground squirrel. Moreover, this is the first demonstration that vinculin is co-distributed with individual filament bundles within an intercellular attachment plaque. The data are consistent with the general hypothesis that ESs are a form of actin associated intercellular adhesion junction. If this is true, one might further predict that the actin filaments in ESs should be related to adhesion molecules in the plasma membrane as is thought to occur at actin related adhesion junctions in other cell types.

FIGURE 2.1. In this figure are paired fluorescence and phase micrographs of ground squirrel spermatids together with attached ectoplasmic specializations of Sertoli cells that have been labelled with probes for vinculin (a,a' - d,d') and actin (e,e' - h,h'). The spermatids are at sequential stages of spermiogenesis. Notice that the staining pattern for vinculin occurs in linear arrays (arrowheads) and is similar to that for actin at each of the representative stages of spermatid development. In panel (a), nonspecific binding of the primary antibody to the acrosome of an early spermatid is seen. Notice that this binding is diminished in subsequent stages. The nucleus (N) and acrosome (A) are indicated in each of the phase panels. X1,184. Bar = 10 um.

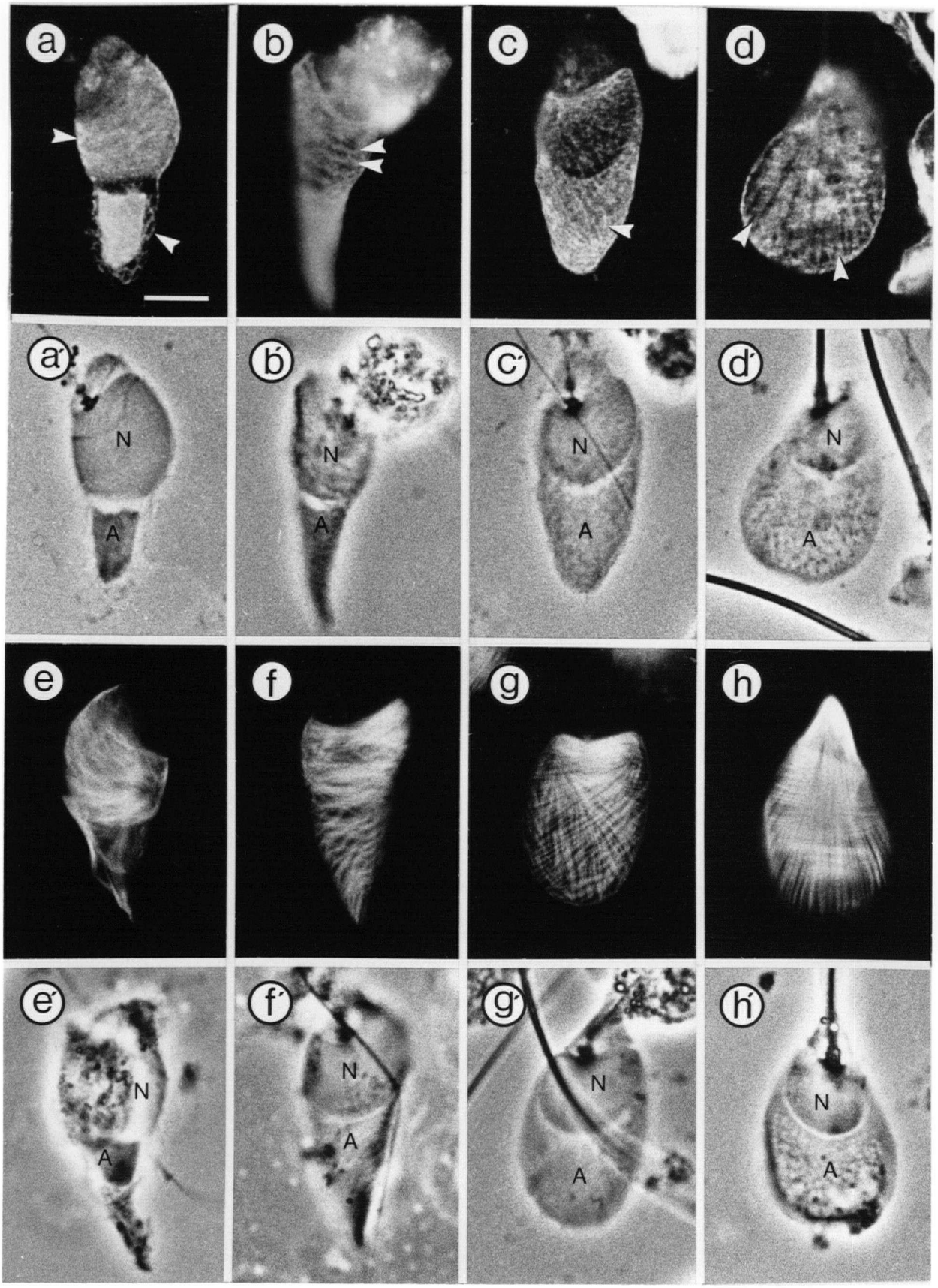

a
b
c
d
a´
b´
c´
d´
N
A
e
f
g
h
e´
f´
g´
h´

FIGURE 2.2. Shown here are controls for rhodamine-phalloidin staining for filamentous actin. The spermatid and attached ectoplasmic specialization in panel (a,a') has been stained with rhodamine-phalloidin. Note the elaborate pattern of actin filament bundles surrounding the spermatid head. In panel (b,b') similar material has been treated with rhodamine-phalloidin in the presence of phallacidin (competitive specificity control). Note the absence of staining. Likewise, notice the absence of staining in panels (c,c') and (d,d') that were treated with phallacidin alone and buffer alone respectively. X1,240. Bar = 10 um.

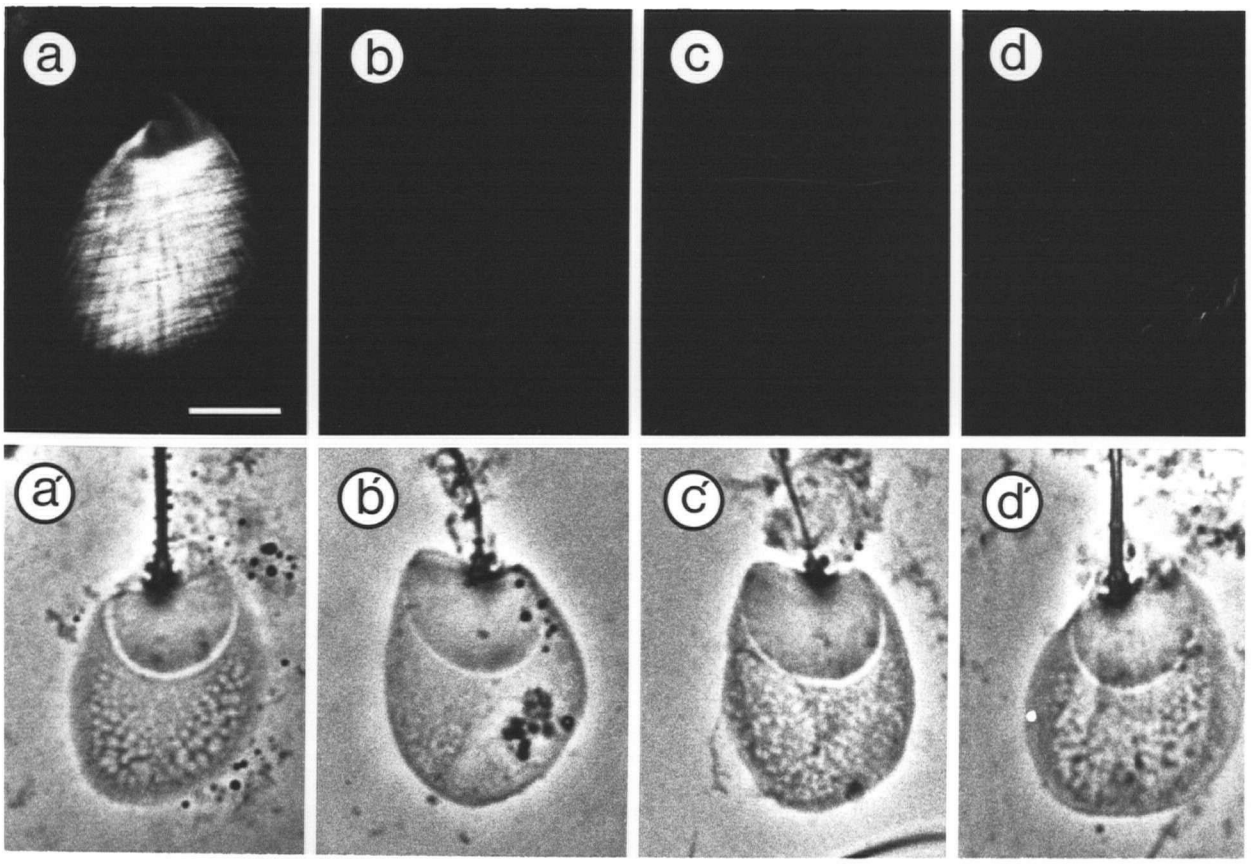

a
b
c
d
a'
b'
c'
d'

FIGURE 2.3. Shown here are controls for the localization of vinculin in ectoplasmic specializations of the ground squirrel. The spermatid and attached ectoplasmic specialization in panel (a,a') has been treated with an affinity purified polyclonal antibody raised in rabbits against human platelet vinculin. Notice that bands of fluorescence radiate across the spermatid head (arrowheads). In the protocol used to label the cell shown in panel (b,b'), the primary antibody has been replaced with the non-adsorbed serum fraction from the column used to purify the antibody used to treat the cell shown in panel (a,a'). Notice that, although labelling is present, the pattern is different from that when specific antibody is used. The cell shown in panel (c,c'), has been treated with primary antibody preincubated with and added to the cell in the presence of excess antigen. Notice that specific fluorescence is eliminated. No fluorescence occurs when cells have been treated with secondary antibody alone (data not shown) or with buffer alone (panel d,d'). X1,184. Bar = 10 um.

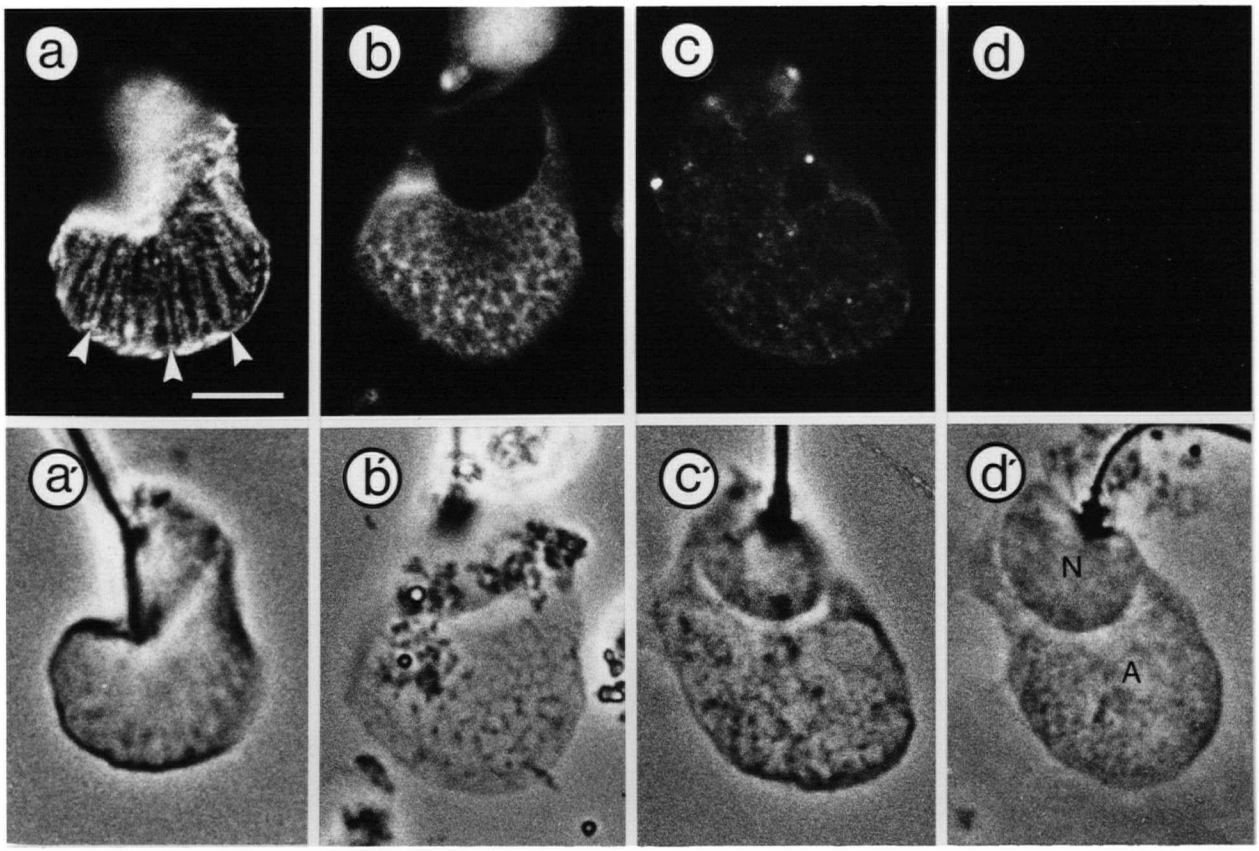
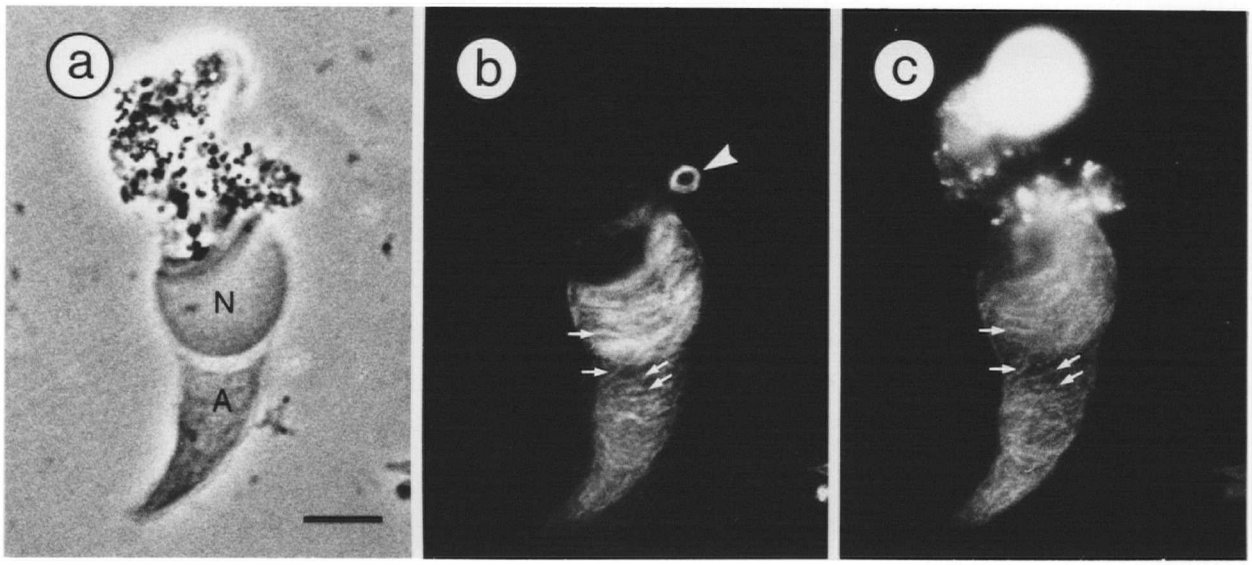

a
b
c
d
a'
b'
c'
d'
N
A

FIGURE 2.4. Shown here is a ground squirrel spermatid and attached ectoplasmic specialization that have been double labelled for actin and vinculin. The nucleus and acrosome of the spermatid are indicated by the N and A respectively in panel (a). Notice that the probe for actin heavily labels actin filament bundles (arrows) in the ectoplasmic specialization and also labels filaments of an intercellular bridge (arrowhead). The probe for vinculin (panel c,c') is co-distributed (arrows) with the actin probe in the ectoplasmic specialization, but is absent from the intercellular bridge. X1,095. Bar = 10 um.


a
N
A
b
c

CHAPTER 3

Adhesion Molecules in Ectoplasmic Specializations of Sertoli Cells

Introduction

At actin associated adhesion sites, intercellular attachment is mediated by specific molecules in the plasma membrane which are thought to be linked to the underlying cytoskeleton (see reviews by Takeichi, 1988; Geiger, 1989).

Surface adhesion molecules are of two general categories: (1) Cell adhesion molecules (CAMs) that are present at cell/cell attachment sites; (2) Substrate adhesion molecules (SAMs) that are present at cell/substratum adhesion sites (see Geiger,1989). Both categories of molecules are intimately involved with the processes of morphogenesis and differentiation, and in many terminally differentiated cells are concentrated at specific locations on the cell surface. In one current model of junctional complex formation, cell adhesion is a prerequisite to the positioning of cell junction molecules (CJMs - which include tight and gap junction elements) (Gumbiner et al., 1988).

Cell adhesion molecules are divided into two classes based on their requirement for calcium: Calcium-dependent and calcium-independent (Takeichi, 1977; Takeichi, 1988; Kemler et al., 1989). In the calcium-dependent group, calcium is required for adhesion. Additionally, CAMs of this group acquire protease resistance in the presence of calcium, but become extremely sensitive to proteases upon removal of this divalent cation (Takeichi, 1988). Takeichi and his colleagues proposed that a group of genetically related adhesion molecules exhibiting these properties be designated the "cadherins" (Yoshida-Noro et al., 1984; Hatta et al., 1985; Nose and Takeichi, 1986). Identified molecules in this group include E-cadherin, N-cadherin, P-cadherin, and others.

E-cadherin (124 kD) has been detected in a number of embryonic and adult cell types (Yoshida and Takeichi, 1982) where it is concentrated at adherens junctions (Hirano et al, 1987; Takeichi, 1988). It has been demonstrated, through amino acid sequencing, to be the same molecule as uvomorulin (Nagafuchi et al., 1987; Ringwald et al., 1987), which has been localized at the zonula adherens of intestinal epithelium (Boller et al., 1985). E-cadherin,

identified in mouse, is also thought to be homologous to chicken L-CAM (Gallin et al., 1987), human cell CAM 120/80 (Damsky et al., 1985), and canine Arc-1 (Behrens et al., 1985).

N-cadherin (127 kD), in addition to being found in a variety of embryonic tissues, has also been detected in adult neuronal tissue, fascia adherens of cardiac muscle and adherens junctions of lens epithelial cells (Hatta et al., 1985; Duband et al., 1987; Hatta et al., 1987). It appears to be very similar to and is possibly the same molecule as A-CAM (Volk and Geiger, 1984; Volk and Geiger, 1986a,b). N-cadherin also closely resembles and may be identical to N-Cal-CAM, an adhesion molecule identified by Lilien and co-workers in the chick neural retina (Bixby et al., 1987).

P-cadherin (118 kD) appears to be expressed predominantly in the placenta, both in embryonic and maternal regions (Nose and Takeichi, 1986).

Recently, new members of the cadherins group have been identified (R-cadherin and others) and there are likely additional members that have yet to be discovered. In addition, it appears that the glycoproteins (desmocollins) responsible for adhesion at desmosomes, are members of the cadherin family.

The calcium-independent CAMs are insensitive to calcium depletion and are members of the immunoglobulin superfamily. The best studied member of this group is N-CAM, a molecule found in a variety of tissues both early in development and in the adult (Edelman, 1983; Rutishauser, 1986). Other members of this group include Ng-CAM (L1) (Grumet and Edelman, 1984), Cell-CAM 105 (Obrink et al., 1988), and contactin (Ranscht and Dours, 1988).

Mounting evidence indicates that members of the cadherins group of adhesion molecules are concentrated at cell/cell actin associated adhesion junctions of several tissue types. Hirano and co-workers (1987) report that cadherins co-distribute precisely with underlying actin filament bundles, a relationship that is retained even after cytochalasin D treatment or detergent extraction, indicating a linkage between the filaments and the adhesion molecules. Of the calcium-independent CAMs, there is evidence that one form of N-CAM also associates with the cytoskeleton at sites of cell/cell contact (Pollerberg et al., 1987).

Based on analogy with other actin associated adhesion junctions in which actin filaments are co-distributed with and thought to be linked to CAMs in the membrane, one might predict that if ESs are a form of this junction type, a CAM should be present in ESs and should be co-distributed with the actin bundles.

That ES filament bundles are actin and that these filaments are bound to the plasma membrane through a layer of protein, of which vinculin appears to be a component, indicates that there is a similarity between the structural organization of Sertoli cell ESs and the adherens junctions of other cell types. This suggests that cell adhesion molecules of the cadherins class may be present at sites of Sertoli cell ESs. However, ES adhesion sites differ from adherens junctions in that their adhesion is not significantly altered by calcium depletion (Grove and Vogl, 1986). This may indicate that ES site CAMs differ from those known to occur at adherens junctions, ie: ES site adhesion molecules may be calcium-independent. The possibility that the calcium-independent molecule N-CAM (reviewed by Rutishauser, 1986) may be present at ES sites receives some support from the observations of Crossin et al. (1985) on N-CAM expression during chick embryogenesis. They reported N-CAM to be present in the germinal epithelium and the gonadal stroma of well differentiated chick embryos.

In this chapter, I test the prediction that a CAM is present in ESs and is co-distributed with the actin bundles. The approach I use is to immunologically probe ectoplasmic specializations for some of the known CAMs that are the most likely candidates for being present at these sites. These include E-cadherin, A-CAM (N-cadherin), and N-CAM. My results indicate that these three CAMs are not present in ectoplasmic specializations of the testis, which suggests that the ES site CAM, if present, may be a different adhesion molecule.

Materials and Methods

Immunological probes for each of the CAMs (E-cadherin, A-CAM and N-CAM) were tested for reactivity on fixed frozen sections of spermatogenically active testes from various animals depending on the probe used. In order to verify viability of the antibodies, each probe was also reacted with tissues or cultured cells known to express the CAM against which the antibodies were raised.

All animals used in these experiments were anesthetized either with sodium pentabarbitone administered intraperitoneally or with halothane administered via the respiratory system.

Tissue Preparation

(1) E-Cadherin:

Testis:

Testes were removed from anesthetized mice, rats (Sprague-Dawley strain), and ground squirrels (Citellus lateralis).

Testes from the mice were decapsulated and immersion fixed with 3% paraformaldehyde in HMF (Hepes-buffered magnesium-free saline supplemented with 2 mM $CaCl_2$) (Hirano et al., 1987). The organs were fixed for 30 min on ice, then washed three times with 50 mM TBS-Ca^{++} (50 mM Tris buffered saline containing 1 mM $CaCl_2$ - pH 7.4) at room temperature. The testes were then embedded in Tissue-Tek O.C.T. compound and frozen in liquid nitrogen.

Testes from rats and ground squirrels were perfused via the spermatic artery with 3% paraformaldehyde in PBS (room temperature) for 5 min then washed by perfusion for 30 min

with PBS. The organs were embedded in Tissue-Tek O.C.T. compound and frozen in liquid nitrogen.

Control Tissue and Cells:

Mouse small intestine was immersion fixed and processed in the same fashion as mouse testes. This tissue was chosen for staining because the zonula adherens is known to express uvomorulin (E-cadherin) (Boller et al., 1985).

In addition, human mammary tumor MCF-7 cells, also known to express E-cadherin (Hirano et al., 1987), were used as a control for E-cadherin staining. The cells were grown on glass cover-slips in F12/DME containing 10% FCS and 0.005 mg/ml insulin. The cover-slips were washed briefly (4-5 min) with HMF then fixed on ice for 30 min with 3% paraformaldehyde in HMF. They were washed thoroughly with TBS-Ca^{++} then further processed for immunofluorescence as described below for tissue sections.

The MCF-7 cells used in this set of experiments were kindly provided by Dr. Joanne Emerman's laboratory (U.B.C.).

(2) A-CAM:

Testes:

Testes from rats (Sprauge-Dawley strain) and roosters (Gallus domesticus) were used to test for the presence of A-CAM. Rat testes were perfusion fixed, for 5 min, with 3% paraformaldehyde in PBS then washed by perfusion for 30 min. The organs were embedded in Tissue TEK O.C.T compound and frozen in liquid nitrogen.

Testes were fixed by "whole body" perfusion (via the heart), for 10 min, with 3% paraformaldehyde in PBS and washed by perfusion for 30 min with PBS. The testes were

removed from the animals, cut into blocks, embedded in Tissue Tek O.C.T. compound and frozen in liquid nitrogen.

Control Tissue:

To control for A-CAM staining, blocks of heart from the perfusion fixed roosters described above were removed, embedded in Tissue Tek O.C.T compound and frozen in liquid nitrogen. Cardiac muscle cells are known to express A-CAM (N-cadherin) (Volk and Geiger, 1984) at fascia adherens of intercalated discs.

(3) N-CAM

Testes:

Testes from mature Sprauge-Dawley rats and a CD1 mouse were perfusion fixed, for 10 min, with 3% paraformaldehyde in PBS then washed by perfusion, for 30 min, with PBS. The organs were embedded in Tissue Tek O.C.T. compound and frozen in liquid nitrogen.

Control Tissue:

Twelve day old embryos were removed from the uterus of a halothane anesthetized CD1 mouse and immersion fixed, for 20 min, in 3% paraformaldehyde in PBS. They were washed for 30 min in PBS then embedded in Tissue Tek O.C.T compound and frozen in liquid nitrogen. Embryonic neural tissue is known to express N-CAM (Edelman, 1983; Edelman, 1985).

Immunofluorescence Protocol

Sections of 10 um thickness were cut from testes and control tissues on an AO HistoSTAT cryo-microtome. The sections were attached to polylysine-coated slides and immediately plunged into cold acetone (-20^{o}C) for 5 min. (Coverslips with attached MCF-7 cells, used to test for E-cadherin staining, were treated like tissue sections). Sections were air dried then rehydrated for 30 min with either PBS or HMF containing 0.1 % BSA (bovine serum albumin) and 5% normal serum from the type of animal in which the secondary antibody to be used in the staining protocol was raised. Following rehydration, slides or coverslips were drained and incubated with primary antibody for 1 hr at 37^{o}C. The material was washed then incubated with secondary antibody for 1 hr at 37^{o}C. Following a final series of washes, the samples were mounted with 1:1 glycerol:buffer containing 0.02% sodium azide.

Data was recorded using a Zeiss Photoscope III or a Zeiss Axiophot Photomicroscope both fitted with filter sets for detecting fluorescein.

Antibodies

Primary antibodies and the dilutions used were as follows. The immunological probe for E-cadherin was a rat monoclonal antibody raised against mouse E-cadherin and was a generous gift from Dr. Masatoshi Takeichi at Kyoto University, Japan. It was used at dilutions of 1:500 and 1:250 in HMF containing 0.1% BSA and 1% normal rabbit serum. The probe for A-CAM was a mouse monoclonal antibody raised against A-CAM isolated from chicken cardiac muscle and was obtained from Sigma. It was used at a dilution of 1:20 in TPBS (PBS containing 0.05% Tween-20) containing 0.1% BSA and 1% normal goat serum. The probe for N-CAM was polyclonal antiserum raised in rabbits against mouse N-CAM and was a generous gift from Dr. Gerald Edelman at The Rockefeller University, USA. It was used at dilutions of 1:100 and 1:50 in TPBS containing 0.1% BSA and 1% normal goat serum.

Secondary antibodies were all obtained from Sigma and included (1) rabbit anti-rat IgG conjugated to FITC (fluorescein isothiocyanate) and used at a dilution of 1:32, (2) goat anti-mouse IgG conjugated to FITC and used at a dilution of 1:64, and (3) goat anti-rabbit IgG conjugated to FITC and used at a dilution of 1:32.

Results

Testis

E-Cadherin:

No positive staining with the immunological probe for E-cadherin was detected within the epithelium of the mouse (Fig. 3.1a,a') rat (data not shown) or ground squirrel (data not shown).

A-CAM:

Both in the rat (data not shown) and in the rooster (Figl 3.1b,b') no immunofluorescence was observed in regions known to contain ESs; however, in the rooster, specific staining appeared to occur at the base of the epithelium. Basal ESs are known to be absent in the rooster. In more apical locations, some punctate fluorescence was observed. Similar basal staining and punctate staining was not seen in any of the control sections.

N-CAM:

As with E-cadherin and A-CAM, no specific labelling was present at sites known to contain ESs in either mouse (Fig. 3.1c,c') or rat (data not shown) seminiferous epithelium. In the mouse testis, a weak specific fluorescence was observed in peritubular regions.

Control Tissues

E-Cadherin:

In cultured MCF7 cells, specific fluorescence occurred at cell peripheries (Fig. 3.2.a,a'). The staining was intense and was restricted to regions of intercellular contact. This staining pattern was similar to that reported by others (Hirano et al., 1987) who have previously localized E-cadherin in this cell line. When the primary antibody was omitted from

the protocol (Fig. 3.2b,b'), or when both the primary and secondary antibody were replaced with buffer (Fig 3.2c,c'), no fluorescence was detected.

Specific staining was also observed in regions of apical junctional complexes in mouse intestinal epithelial cells (data not shown). As indicated in the Materials and Methods, these regions are known from previous work to contain uvomorulin (E-cadherin). No specific staining at junctional regions was observed in any of the controls (data not shown).

A-CAM:

In cardiac muscle cells of the rooster, intense and specific labelling (large arrowheads in Fig. 3.3a,a') occurred along the course of the linearly arranged myocytes. The pattern of this fluorescence resembled that described previously in similar tissue treated with immunological probes for A-CAM (Volk and Geiger, 1984) and interpreted as corresponding to the fascia adherens of intercalated discs. Also observed was a faint punctate staining along lateral margins of the myofibrils (small arrowheads in Fig. 3.3a,a'). This pattern has also been observed previously in cardiac cells treated with probes for A-CAM. No specific fluorescence was observed in any of the control sections. These included sections in which (1) the primary antibody was replaced with normal mouse IgG (Fig. 3.3b,b'), (2) primary antibody was replaced with buffer (Fig. 3.3c,c'), and (3) primary and secondary antibody were replaced with buffer (Fig. 3.3d,d').

N-CAM:

A positive signal was observed in regions of the developing nervous system of mouse embryos (day 12) (Fig. 3.4a,a'). These regions have been described by others (Edelman, 1983; Edelman, 1985) as expressing N-CAM during development. When normal rabbit serum was substituted for the primary antibody (Fig. 3.4b,b'), there was some background labelling of neural and some other tissues; however, the signal obtained was weak and homogeneous rather than patterned as in specific antibody treated material. No fluorescence was observed in sections treated with secondary antibody alone (Fig. 3.4c,c') or with buffer alone (Fig. 3.4d,d').

Discussion

Ectoplasmic specializations are complex actin containing structures found at sites of Sertoli cell intercellular attachment. They have been proposed to be a member of the large class of actin associated adhesion junctions that exist between cells and neighboring cells and between cells and their substratum (Grove and Vogl, 1989; Vogl, 1989). Strong evidence supporting this hypothesis is the finding that an immunological probe for vinculin co-distributes with actin bundles within ESs (Chapter 2.). If ES are adhesion junctions one might predict that adhesion may be mediated by adhesion molecules (CAMs) within the plasma membrane.

By analogy with other systems, one might predict that the most likely type of adhesion molecule present at ESs would belong to the cadherin family. Members of this family are concentrated at certain sites of intercellular adhesion and are "linked", both structurally and functionally (Nagafuchi and Takeichi, 1989), to cortical actin filaments. Of the known cadherins, E-cadherin and A-CAM would appear to be strong candidates for the ES CAM type. Both E-cadherin and A-CAM are found in embryonic as well as in adult tissues. In the embryonic chick, N-cadherin (A-CAM) is expressed both by primordial germ cell and by putative Sertoli cells (Hatta et al., 1987). It has not been determined whether or not the expression of N-cadherin by these cells is maintained through further development of the gonads or if the expression of this CAM is a transitory event during gonad morphogenesis. In the adult, E-cadherin is concentrated at the zonula adherens of various epithelial cells (Boller et al., 1985), and A-CAM is localized at the fascia adherens of cardiac muscle cells and the adhesion junctions between adjacent lens cells (Volk and Geiger, 1984; 1986a, b).

In this study, I present evidence that E-cadherin and A-CAM are likely not present at ESs. Immunological probes for each of these cadherins do not react with sites, in fixed frozen sections of seminiferous tubules, known to contain ESs. The observation that the same probes react positively with sites in other tissues or cultured cells known to express E-cadherin or A-CAM suggests that the probes are truly unreactive at ESs in the seminiferous epithelium.

However, at this point the possibility the Sertoli cells may express an isoform of E-cadherin or A-CAM that differs from other tissues, and is not recognized by the monoclonal antibodies I labelled with, can not be ruled out. Interestingly, although I did not detect A-CAM at ESs, I did observe labelling for this molecule in basal regions of the seminiferous epithelium, at least in the rooster. This basal A-CAM labelling appears localized to lateral cell margins, most likely of early spermatogenic cells (spermatogonia) and possibly of Sertoli cells. This raises the intriguing possibility that Sertoi cells may express two different types of adhesion molecules. One, A-CAM, that functions to attach Sertoli cells to early spermatogenic cells. Another, found concentrated at ESs, that functions to attach Sertoli cells to spermatids and, in mammals, also to neighboring Sertoli cells.

The absence both of E-cadherin and A-CAM from ES sites should perhaps not be surprising since the cadherins are dependent on calcium for intercellular attachment, and adhesion at ESs appears to be insensitive to removal of calcium (Grove and Vogl, 1986). With regard to the latter point, ESs remain firmly attached to spermatids even in the presence of 20 mM EDTA (Vogl and Soucy, 1985). It is possible that the type of adhesion molecule present at ESs may be a calcium-independent CAM. Although N-CAM, a widely distributed and well characterized calcium-independent CAM, is expressed in the germinal epithelium and gonadal stroma of chick embryos (Crossin et al.,1985), my results indicate that it is not expressed in cells of the adult seminiferous epithelium. This result is not totally unexpected since N-CAM tends to be largely restricted to neuronal tissue in adult animals.

The most popular function attributed to ectoplasmic specializations is that they facilitate intercellular attachment and are a form of actin associated adhesion junction. A current model of ES structure predicts that actin filaments are linked, in some way, to cell adhesion molecules in the plasma membrane (Grove, Pfeiffer, Allen, and Vogl 1989). In this Chapter, I present evidence that E-cadherin, A-CAM and N-CAM, three of the most likely candidates for the ES adhesion molecule, are probably not present at ESs. This suggests that if an adhesion molecule is present at ESs, it may be one of the other known CAMs or perhaps an as yet unidentified tissue specific CAM.

FIGURE 3.1. In this figure, paired fluorescence and phase micrographs in which the absence of E-cadherin, A-CAM and N-CAM labelling at sites known to contain ectoplasmic specializations is demonstrated. In all panels, the large arrowheads indicate sites known to contain apical ectoplasmic specializations around differentiating spermatids. The small arrowheads at the base of each phase panel indicate the tubule wall.

Panel (a,a') shows a cross-section of a mouse seminiferous tubule that has been treated with a rat monoclonal antibody raised against mouse E-cadherin. There is a complete absence of staining within the epithelium. X480. Bar = 25 um.

Panel (b,b') is a similar section of a rooster seminiferous tubule treated with a mouse monoclonal raised against chicken A-CAM. No staining occurs in ectoplasmic specializations; however, there is specific staining associated with cells at the base of the epithelium. X576. Bar = 25 um.

Panel (c,c') shows a section of a mouse seminiferous epithelium treated with a rabbit polyclonal antibody raised against mouse N-CAM. No specific staining occurs within the epithelium; however, there is a weak specific fluorescence apparently associated with the tubule wall. X416. Bar = 25 um.

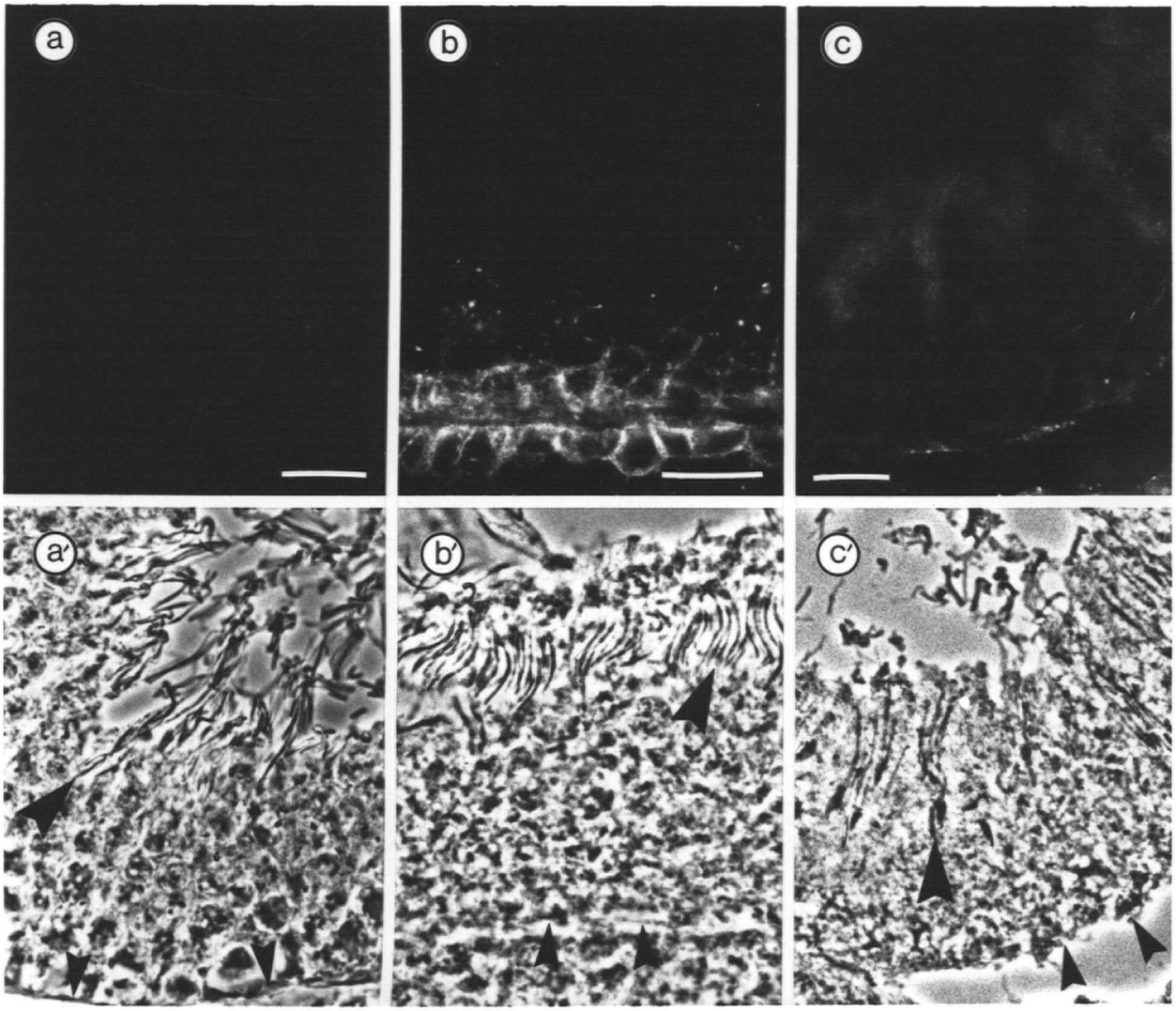

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FIGURE 3.2. Positive control for the immunological probe for E-cadherin. Shown here are paired fluorescence and phase micrographs of cultured human mammary tumor MCF-7 cells treated with the same probe for E-cadherin that was used to treat the section of mouse testis shown in figure 3.1a,a'. When treated with the probe, positive staining occurs in regions of intercellular contact (panel a,a') - sites known to contain E-cadherin. When similar cells are treated with secondary antibody alone (panel b,b') or with buffer alone (panel c,c'), no fluorescence is observed. X350. Bar = 50 um.

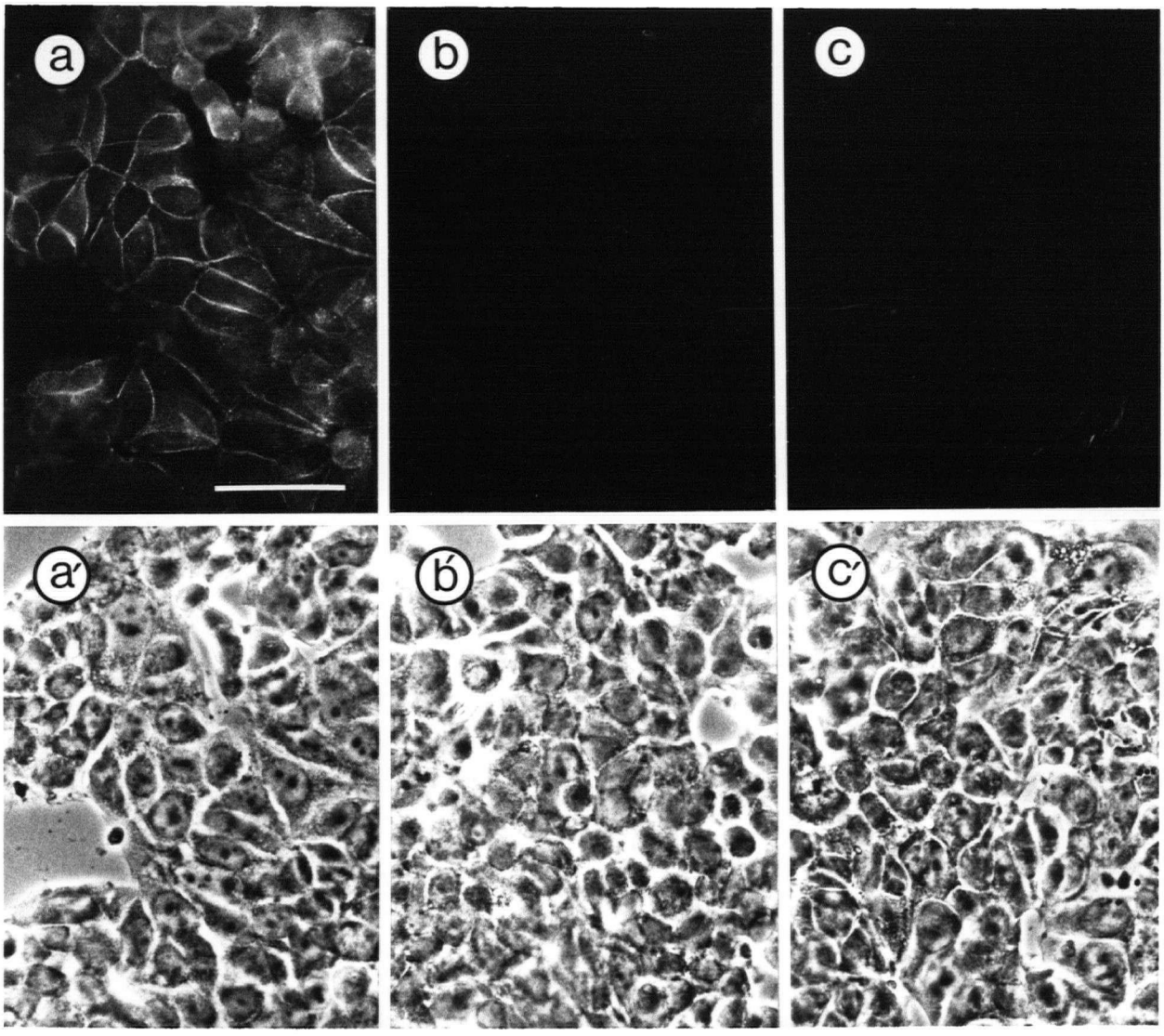
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FIGURE 3.3. Positive control for the immunological probe for A-CAM. Shown here are paired fluorescence and phase micrographs of fixed frozen sections of rooster cardiac muscle. When treated with the probe, positive staining occurs in bands (large arrowheads), which I interpret as intercalated discs, and faint puncta (small arrowheads), which are distributed along the lateral borders of the cells. When similar cells are treated with normal mouse IgG instead of the primary antibody (b,b'), with secondary antibody alone (panel c,c') or with buffer alone (panel d,d'), no fluorescence is observed. X1000. Bar = 10 um.

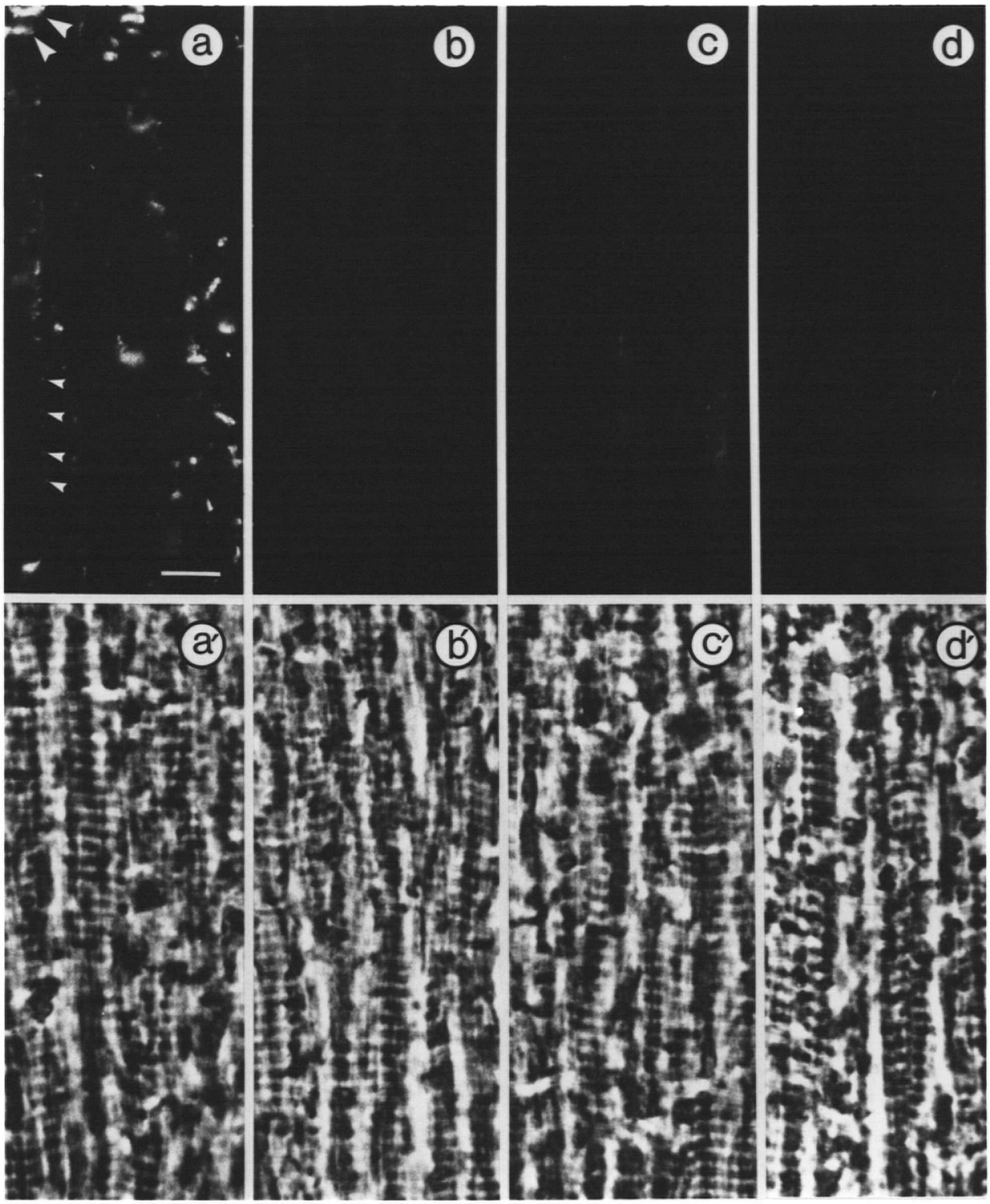
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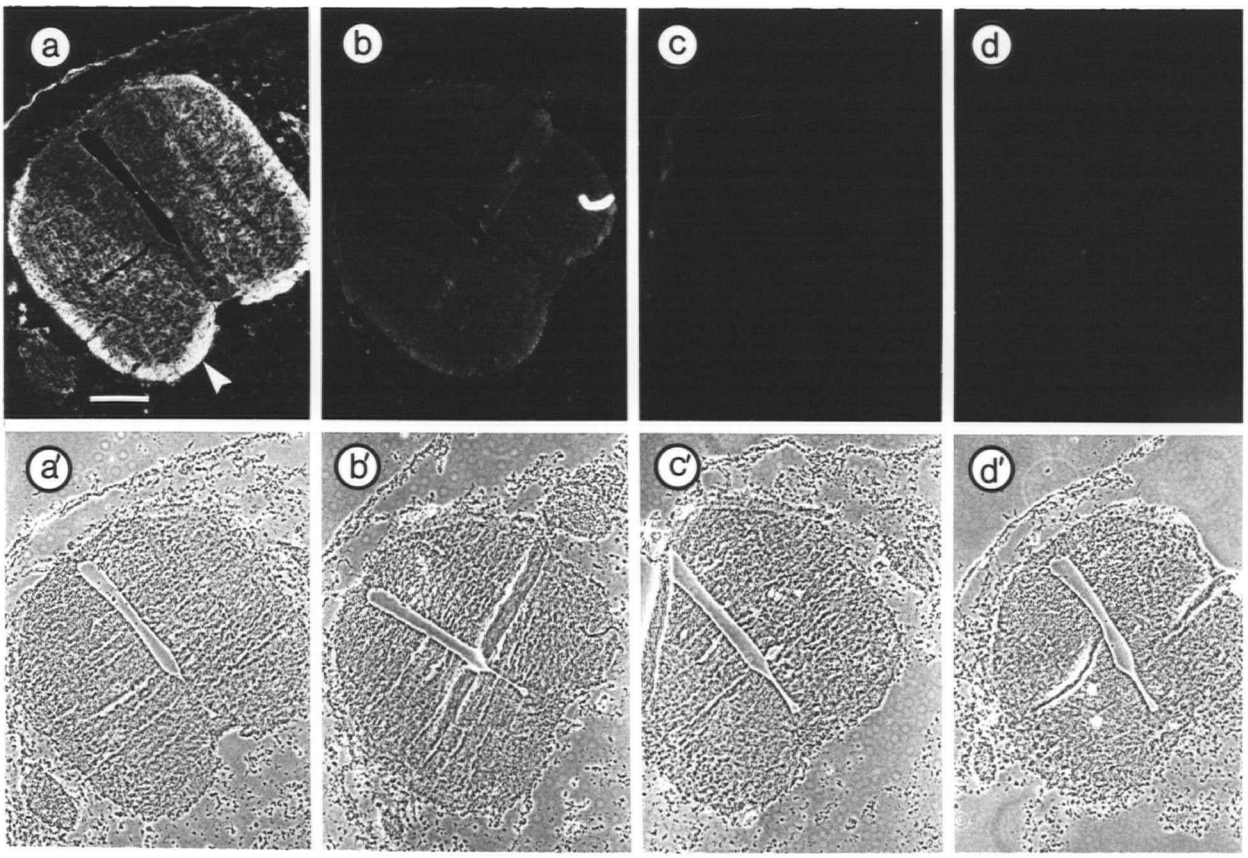
FIGURE 3.4. Positive control for the immunological probe for N-CAM. Shown here are paired fluorescence and phase micrographs of fixed frozen sections of the developing neural tube of a 12 day old mouse embryo. When treated with the probe, positive staining is most obvious in peripheral regions of the neural tube (arrowhead). When similar cells are treated with normal rabbit serum instead of the primary antibody (b,b'), with secondary antibody alone (panel c,c') or with buffer alone (panel d,d'), specific fluorescence is not observed. X40. Bar = 200 um.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CHAPTER 4

Filament Pattern Changes in Ectoplasmic Specializations of Ground Squirrel Sertoli Cells

Introduction

Mounting evidence, reviewed in the preceding chapters, indicates that Sertoli cell ESs function in intercellular attachment as a form of actin associated adhesion junction. At typical actin associated adhesion junctions a network of loosely arranged filaments is linked to the plasma membrane. A similar system of linkages also occurs at ES sites; however, a number of unique qualities separate ES actin filaments from those of actin associated adhesion junctions present between other cells. One such quality is that ES site actin filaments occur in tight bundles which form elaborate patterns. These patterns, in turn, undergo a series of organizational changes during the course of spermatid maturation.

In the ground squirrel, a species in which apical ES sites are very pronounced, the ES filament changes are particularly striking, and intricate patterns are formed by the filament bundles as they undergo a complex series of rearrangements at the adhesion site. For example, at certain stages of spermatid differentiation, ES filament bundles on one side of the flattening spermatid head become oriented in a different direction from those on the other. Although bundles on one side of the head may be continuous at their ends with those on those on the other side, it is possible that there may be a partitioning of filament bundles around the spermatid head into two groups. While the general changes of patterns are known (Vogl and Soucy, 1985), details of these changes at some stages are not yet sufficient to determine how the patterns change from one to another.

In this chapter, I define more concisely than has previously been done, the changes that occur in actin patterns in ectoplasmic specializations associated with spermatids of the ground squirrel. I also present an explanation for why these changes may occur.

Materials and Methods

Animals:

In this study, tissue from three reproductively active golden-mantled ground squirrels (Citellus lateralis) was used.

Fluorescence Microscopy:

(a) Tissue Preparation:

Spermatids with intact ESs were isolated from the seminiferous epithelium so that the arrangement of ES filament bundles around developing spermatid heads could be easily visualized. This was accomplished as follows.

Testes were excised from animals anesthetized with sodium pentabarbitone administered intraperitoneally. Each testis was perfused, for 10 min, via the spermatic artery with 3% paraformaldehyde in PBS. Each testis was then decapsulated and minced, with two scalpels, into small sections in fixative for 5 min. The resulting cells and epithelial fragments were collected then sedimented using low speed centrifugation. Pellets were resuspended in PBS containing 0.1% triton-X 100 and incubated at room temperature for 5 min. Samples were again centrifuged and the pellets washed in PBS. This step was repeated, after which pellets were resuspended in approximately twice their volume of PBS and gently aspirated 5-10 times through an 18G syringe needle. Drops of this tissue suspension were placed on polylysine-coated slides. Large aggregates of intact tubules and tubule walls were carefully removed with micropipettes leaving only isolated cells and cell clusters. Present within this tissue suspension was a cross section of the different spermatid developmental stages with ESs adherent and intact. After 15 min excess fluid was removed and the staining procedure for actin was initiated.

(b) Localization of Actin:

The distribution of filamentous actin at ES sites surrounding isolated spermatids was revealed with the F-actin specific probes rhodamine-phalloidin and NBD-phallacidin. When rhodamine-phalloidin was used, slides, with tissue samples prepared as described above, were incubated at room temperature for 20 min in one of the following solutions: (1) PBS + 1.65×10^{-6} M rhodamine-phalloidin (test for filamentous actin); (2) PBS + 1.65×10^{-6} M rhodamine-phalloidin + 0.97×10^{-4} M phallacidin (competitive specificity control); (3) PBS + 0.97×10^{-4} M phallacidin (control for phallacidin in reagent (2); (4) PBS (control for autofluorescence). When NBD-phallacidin was used to label F-actin, the controls done were similar to those described above except that phalloidin instead of phallacidin was used in the competitive specificity solution. After staining for actin, the slides were washed three times, for 10 min each, with PBS and mounted with 1:1 PBS:glycerol containing 0.02% sodium azide.

Fluorescence microscopy in this study was performed on either a Zeiss Photomicroscope III or a Zeiss Axiophot Photomicroscope both fitted with filters for detecting rhodamine and fluorescein.

Results

The mechanical separation process used in this study produces a mixture of tubule fragments, epithelial sheets and numerous individual cells. ES actin filaments separate away from Sertoli cells and remain adherent to dislodged spermatids. This provides an ideal system for studying the arrangement of ES actin filament bundles at apical adhesion sites. Unobscured by other cells, the actin filament bundles of these isolated ESs are easily visualized when stained with either NBD-phallacidin or rhodamine-phalloidin. Individual filament bundles appear as distinct linear bands or cables of fluorescence.

Collectively, ES actin filament bundles are arranged in a highly organized pattern which, as a whole, undergoes elaborate organizational changes during spermatid differentiation (Fig. 4.1). In round spermatids that are not polarized, no ESs are detected (Fig. 4.1a,a'). As the developing acrosome migrates to the plasma membrane, a very weak fluorescence becomes apparent over regions of the plasma membrane associated with the acrosome (Fig. 4.1b,b'). As spermiogenesis progresses and spermatids begin to elongate, fluorescence intensifies and linear bundles become obvious (Fig. 4.1c,c'). These filament bundles encircle the junction between the spermatid nucleus and extend over the spherical acrosome. With further elongation, the filament staining expands posteriorly over the nucleus. Filament bundles in this region course circumferentially around the spermatid, while over the acrosome filament bundles are oriented parallel to the long axis of the cell (Fig. 4.1d,d'-e,e'). In subsequent stages, the acrosome becomes less dense and assumes a cone-shaped form. Initially, filament bundles over the acrosome are oriented similar to those at earlier stages (Fig. 4.1f,f'); however, they eventually "shift" to an orientation similar to that over the nucleus (ie: perpendicular to the long axis of the cell (Fig. 4.1g,g'). At a slightly later stage (Fig. 4.1h,h'), and at all subsequent stages, staining over the acrosome can not be distinguished from that over the nucleus.

With further differentiation, the acrosome and nucleus flatten and the head changes from a conoid-shape to a more paddle-shape (Fig. 4.1i,i'). At these stages, filament bundles on one

side of the head can be visualized simultaneously with those on the other. The filaments on each side of the acrosome and nucleus course diagonally across the head; however, bundles on one side are oriented at right angles to those on the opposite. Viewed superimposed, the networks form a woven pattern (Fig. 4.1i,i'). As the cells continue to flatten and differentiate, the general filament orientation is further modified as the networks each undergo a slight rotational movement on their respective sides of the head (Fig. 4.1j,j'-L,L'). On one side, this rotation shifts filament bundles into an angle parallel to the cell's long axis, in which filaments splay out across the head in a fan-like arrangement (Fig. 4.1 L,L'). On the other side, the network rotates in the opposite direction bringing filament bundles into an orientation perpendicular to the axis of the cell (Fig. 4.1L,L'). As this occurs, there appears to be a gradual separation of filament bundles on one side of the head from those on the other. This separation occurs around the proximal end of the cell margin (Fig. 4.1L,L').

During maturation stages, the filament bundles of each network undergo a final rearrangement. On the side of the spermatid head where bundles course perpendicular to the cell's axis, there is a progressive loss of filaments beginning proximally and extending distally (Fig. 4.1L,L'-n,n'). On the opposite side of the head, filament bundles assume a more arched arrangement over the acrosome and nucleus (Fig. 4.1o,o'), then begin to disappear starting distally and extending proximally. Events around the spermatid head culminate with filament bundles remaining concentrated at two locations - over the nucleus on one side of the head and around the cell periphery on the other (Fig. 4.1p,p').

Discussion

In the ground squirrel, apical ES filament bundles undergo a remarkable series of rearrangements around developing spermatid heads (Vogl and Soucy, 1985). In this Chapter, I describe the details of these rearrangements and interpret them in the context of the adhesion hypothesis of ES function.

In early stages of spermatogenesis, filament bundles in ectoplasmic specializations are oriented in a circular fashion in Sertoli cell regions adjacent to the acrosome and nucleus of elongating spermatids. As spermatid heads flatten, bundles on one side of the head become oriented differently from those on the other. As this occurs, there is a loss in continuity at the periphery of the spermatid head between filaments on one side of the head with those on the other. This lack of continuity is maintained throughout the remaining stages of spermiogenesis during which time filament bundles on each side of the head appear to undergo independent orientational changes. These observations support the conclusion that there is a separation of filaments into two independent, or at least semi-independent, networks - one on each side of the head. This separation of filament networks would further indicate that, in the ground squirrel, the ES divides into two separate adhesion plaques in the latter stages of spermiogenesis.

Little is known about the functional significance of filament bundle positional changes at ES sites. Interestingly, the germ cell stages around which the ES filaments reorganize represent the series of cell stages over which the most substantial shape transformations occur during spermatogenesis. These transformations, which eventually yield the species specific shape of the mature spermatozoa, involve the processes of nuclear condensation and elongation, acrosome formation and shaping, and a considerable reduction of the germ cell cytoplasmic volume.

Various authors have suggested that the ES may play a role in shaping aspects of the spermatid head during these maturation steps (Ross, 1976; Fawcett, 1979). The fact that in the ground squirrel the organization of the ES filament bundles undergoes a predictable sequence of changes that correlates with the specific stages of spermatid shape transformation is

consistent with this hypothesis. However, several considerations lead me to conclude that apical ESs or their filament bundle rearrangements most likely are not involved in shaping spermatid heads.

Ross (1976) postulated that ESs may assume a role in shaping spermatid nuclei. If ESs do exert a molding influence on spermatid nuclei, one basic requisite for this would seem to be a close, if not direct, physical association between the ESs and the elongating nuclei. Numerous EM studies have shown this not to be the case. As nuclear elongation begins the ES is not associated with the nucleus but lies distant, in apposition with the developing acrosome. It is only after nuclear elongation is well underway that the ES acquires a more close relationship with the nucleus. Even then, and through to the conclusion of spermiogenesis, the ES remains separated from the nucleus by a layer of acrosome, or most distally, by a zone of spermatid cytoplasm. It is difficult to imagine how the ES could exert a molding force on the nucleus when spacially separated from it by these media. Perhaps a more plausible explanation for the underlying mechanism responsible for spermatid nuclear shaping was put forward by Fawcett et al. (1971). They proposed that the shape of the spermatid nucleus is regulated from within the nucleus itself by a species specific pattern of chromatin aggregation. Alternatively, others have suggested that microtubules of the spermatid manchette may be involved in nuclear shaping (Cole et al., 1988; Meistrich et al., 1990). Although the controversy between intrinsic and extrinsic factors has not been entirely settled, it appears unlikely, based on the observations discussed here, that the ESs are involved in nuclear shaping.

While some authors have suggested that ESs, together with other Sertoli cell elements, may be involved in shaping the acrosome (Fawcett, 1979), this hypothesis also appears unlikely based on the following observations:

1) ESs occur at apical and at basal sites, and are ultrastructurally similar at both locations. If these structures are primarily involved with acrosome (or nuclear) shaping, they would not be predicted to occur at basal locations, which they do.

2) A change in acrosome shape does not occur when ESs separate from spermatids during sperm release. If the ESs and/or other elements of the Sertoli cell are actively involved in molding the

contour of the acrosome, then at spermiation, when these molding forces are removed, a change in head shape to a previous or different form might be expected. Such changes are not seen. After release from the epithelium the acrosome shape in the ground squirrel and in most other species remains largely unaltered (subtle reductions in acrosome size do occur during epididymal transit in some species, but gross changes are not seen. See Bedford, 1975).

3) Considerable diversity exists in the shape of developing spermatid heads between species. If involved in shaping the acrosome (or the nucleus) then apical ESs would have to be "programmed" differently among species in order to produce the variations observed in morphology; in other words, Sertoli cells would be the major determiners of acrosome contour. Alternatively, species specific acrosome shape may be regulated solely by the germ cells themselves. Observations of spermatid morphogenesis in the guinea pig are consistent with the latter argument. In this species, unlike in most others, refinements in acrosome shape do take place after spermiation (Fawcett and Phillips, 1969). Although these changes ultimately produce an acrosome morphology that is unique to this species, some of the shape transformations that occur resemble those seen in ground squirrel acrosomes before spermiation. These include an overall elongation together with a tapering and flexing of the proximal tip. These observations are significant because they indicate that, in the guinea pig, the Sertoli cell is not absolutely essential for determining acrosome shape - a shape that is similar to that generated before spermiation in the ground squirrel.

4) Spermatids differentiate in "clones" of interconnected, developmentally identical cells. As a result each Sertoli cell is in contact, via ESs, with many morphologically identical spermatids at any given time during spermatogenesis. In addition, at least at one stage during spermatogenesis ESs are associated with two developmentally staggered clones of maturing spermatids. The concept that a single Sertoli cell is responsible for the precise and simultaneous sculpting of multiple spermatid contours seems complex and is difficult to envisage. An alternative and far simpler possibility is that proposed above; that is, the primary regulators of germ cell contour are the germ cells themselves not elements within the Sertoli cell.

If the elaborate filament bundle rearrangements that take place at apical ES sites are not actively involved in shaping spermatid heads, what then is their function? As discussed in preceding chapters, considerable evidence now indicates that both the apical and basal ES sites function as a form of adhesion junction. At the apical ES site the Sertoli cell plasma membrane lies adjacent to the extremely dynamic contour of the developing spermatid head. Rather than producing these shape changes, the apical ES filament rearrangements may more simply represent an adaptive response of the adhesion site to the continually changing cell shape to which it is apposed. Rearrangements of the filament bundles at the adhesion site may be necessary in order to maintain and reinforce adhesion between the Sertoli cell and spermatid throughout the spermatid's developmental sequence of dramatic shape changes.

This explanation may account for the fact that at the basal ES sites elaborate filament bundle changes do not appear to occur. Relative to the apical ES sites, the apposed plasma membranes at the basal ES sites are far more static in terms of their shape. Without substantial changes in the neighboring cell's contour, the need to reorganize filaments at the adhesion site is not present.

In broader terms, this explanation may account for why interspecies variations are seen in the general patterning and rearrangement of ES filaments. It is now becoming apparent that the overall complexity of ES filament arrangements varies significantly between species with differently shaped spermatid heads. In the rat, for example, a species in which the developing spermatid head becomes sickle-shaped, ES filaments are organized into a relatively simple pattern of longitudinal bundles around the head (Vogl et al., 1985). The arrangement and positional changes of filament bundles are unremarkable when compared with that seen in the ground squirrel. In the explanation proposed above ES filament bundle rearrangements represent adaptations in the adhesion site in response to the changing shape of the spermatid head. If true, then if the basic plan of spermatid head shaping is modified between species, likewise modifications in the surrounding adhesion site in the form of different filament patternings might be expected. In the ground squirrel the large spermatid head, with its progression of very different shapes, may demand more exaggerated filament reorganizations in

the surrounding adhesion site (the apical ES) than does the much smaller, less dynamic spermatid head of the rat. As seen in this study, part of these ground squirrel "exaggerations" may involve a partial division of ES filament bundles into two networks around the head as it begins to flatten.

An as yet unaddressed question regards the underlying factors that govern the patterning of filament bundles at apical ES sites. Are the filament bundle reorganizations directed from within the Sertoli cell or does the germ cell regulate the patterning of ES filament bundles? Moreover, what are the directing factors? At this point in time one is unfortunately limited to only speculation on these intriguing questions.

One factor which could potentially influence the patterning of ES filament bundles is the distribution of adhesion molecules on the surface of the spermatid head. It seems probable that if selective addition or deletion of adhesion molecules occurs, or movement of adhesion molecules within the germ cell plasma membrane takes place, the distribution of cognate adhesion molecules on the surface of the Sertoli cell plasma membrane would be correspondingly altered. Movement of Sertoli cell adhesion molecules might then influence the arrangement of the underlying ES filament bundles to which they may be linked.

Given the elaborate shape changes that occur in spermatid heads during spermiogenesis, it does seem possible that changes in the distribution of adhesion molecules in the plasma membranes of these cells may take place. If positional changes in these adhesion molecules do occur, they might influence filament bundles in adjacent ESs in a number of ways. Our model of ES structure (Grove, Pfeiffer, Allen, and Vogl, 1990) predicts that actin bundles in ESs are linked to adhesion molecules in the Sertoli cell plasma membrane, and that these molecules are, in turn, linked to similar molecules in the adjacent germ cell membrane. Changes in the arrangement of spermatid adhesion molecules might influence the patterning of filament bundles in adjacent ESs through a direct "pulling" or "dragging" action. On the other hand, this influence might be more indirect. The rearrangement of ES filament bundles, in response to changes in the distribution of spermatid adhesion molecules, could take place through a progression of actin filament depolymerization/repolymerization steps. In other words, as

spermatid adhesion molecules "move", corresponding changes occur in the positions of similar molecules in the Sertoli cell plasma membrane, and the underlying ES filament bundles are not "dragged" along, but "follow" the changing adhesion molecule patterns.

The proposal that it is changes in spermatid adhesion molecules that direct changes in attached ESs is purely speculative. One could equally argue that it is not the movement of adhesion molecules on the spermatid surface that govern the arrangement of ES filament bundles but, instead, it is the repositioning of filament bundles at the ES site by the Sertoli cell that ultimately determines the distribution of germ cell adhesion molecules.

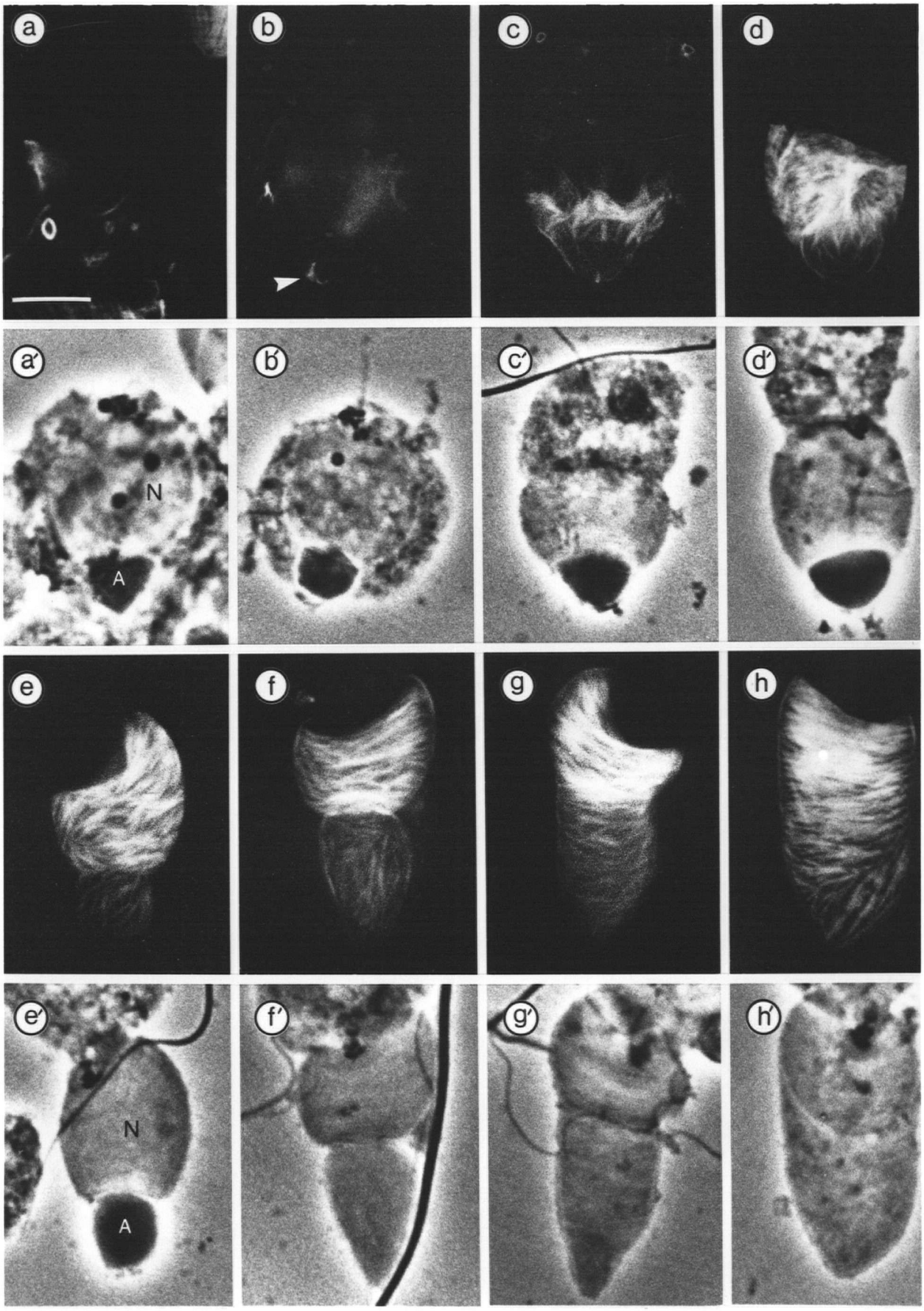
In summary, some of the fundamental questions that have been generated by the morphological data presented in this chapter include: (1) What is the function of the elaborate filament changes that take place at apical ES sites?; (2) Why do these patterns and the complexity of these patterns differ between species?; (3) What initiates changes in filament bundle patterns at ES sites?

To fully answer these questions and gain further insight into ES function, perhaps an in vitro approach utilizing cell culture techniques will prove fruitful. One intriguing and crucial experiment that might shed light on whether or not spermatogenic cells influence ES filament bundle arrangement would be to add undifferentiated spermatogenic cells (spermatogonia) of rat to the basal compartment of a morphologically differentiated Sertoli cell epithelium, derived from guinea pig, in culture and follow the differentiation process of the rat germ cells. If the rat germ cells differentiated as morphologically normal spermatids, and typical appearing rat ESs were formed around them by the guinea pig Sertoli cells, the following points could be made: (1) ES filament bundle arrangement is determined by the shape of the cell around which the adhesion site is formed and is not predetermined by the Sertoli cell; (2) Nuclear and acrosome shaping is genetically determined by the germ cells and not by the Sertoli cell. Together with the observation that spermatid heads of the guinea pig acquire their mature shape after spermiation, this data would support the argument that ESs are not involved with head shaping. While currently such an experiment may not be technically possible, given the rapid advances

being made in the area of the Sertoli cell culture system it seems quite likely that this and similar in vitro experiments may be feasible within the next 5-10 years.

In this chapter I provide a thorough description of the elaborate filament bundle rearrangements that occur at apical ES sites in the ground squirrel. I argue that the extensive pattern changes that occur represent a response of the Sertoli cell adhesion site to the dramatic contour changes that take place in adjacent spermatogenic cells, and that these pattern changes are not directly involved with influencing spermatid head shape.

FIGURE 4.1. Documented in this series of micrographs are changes in filament patterns that occur in apical ectoplasmic specializations of the ground squirrel. The spermatids, with attached ectoplasmic specializations of Sertoli cells, illustrated here have been mechanically fragmented from the seminiferous epithelium and stained with a probe for filamentous actin. The series begins with an unpolarized round spermatid (panel a,a') with which an ectoplasmic specialization is not yet detectable. Developing ectoplasmic specializations are first seen in Sertoli cell regions overlying the acrosome as the acrosome moves to the plasma membrane of the spermatid (arrowhead, panel b,b'). From this site, ectoplasmic specializations expand dramatically over regions adjacent to the acrosome and nucleus (panels c,c' - h,h'). As spermatid heads flatten, filament bundles one each side of the head course diagonally over the nucleus and acrosome. Seen superimposed, filaments on one side of the head appear oriented perpendicular to those on the other (panel i,i'). A filament bundle on each side of the spermatid head in panel (L,L') is indicated by an arrowhead. As spermiogenesis continues, filaments on one side of the head move to a position perpendicular to the axis of the associated spermatid (large arrowhead in panel L) while those on the other side "fan out" and move to a parallel position (small arrowheads in panel L). During this period, there appears to be a separation of filament bundles on one side from those on the other. This separation occurs around the perimeter of the head. Following this (panels m,m' - p,p'), the perpendicularly oriented filaments are gradually lost in an anterior to posterior direction and remain only in regions over the nucleus (large arrowhead in panel p). Simultaneously, filaments on the other side of the head assume an arched orientation and are gradually lost in a distal to proximal direction ultimately remaining as a band around the cell margin (small arrowheads in panel p) in regions overlying a raised fringe of the acrosome. These two filament networks are clearly distinct from one another. N = nucleus. A = acrosome. X1,450. Bar = 10 um.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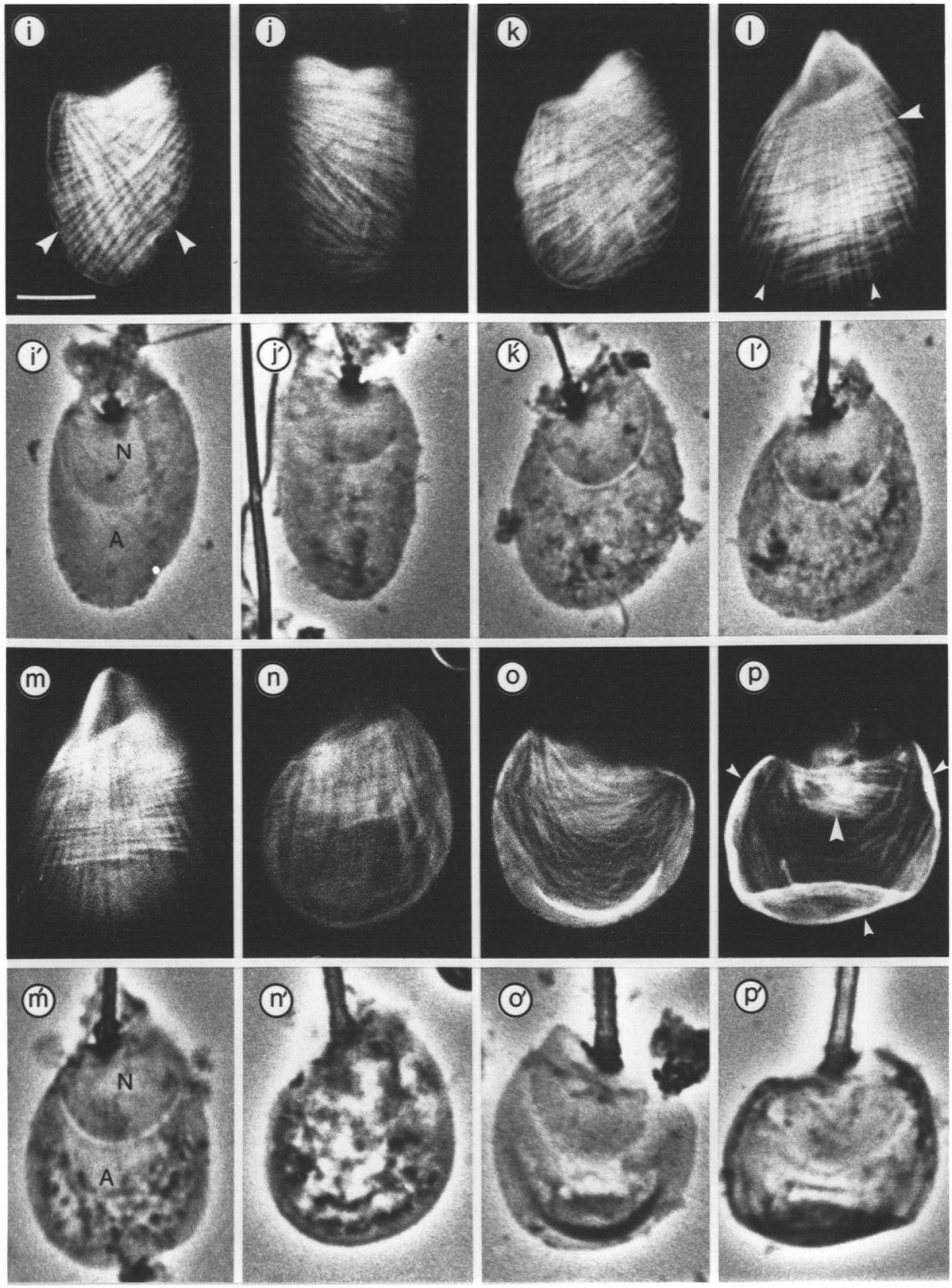
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CHAPTER 5

Evidence for the Presence of
Actin Associated Adhesion Junctions
Between Leydig Cells of the Ground Squirrel

Introduction

Situated between seminiferous tubules is a composite of connective tissue and steroid-producing cells - the interstitial tissue. Fawcett (1973) recognizes four different categories of interstitial tissue organization in mammals. Although there are considerable differences among the categories, there are also some constants in cell arrangements seen throughout. One such constant is the organization of Leydig cells into clusters. Leydig cells characteristically occur in clusters near or surrounding blood vessels. Often, when mechanically separated away from surrounding tissue, the integrity of the Leydig cell aggregations is maintained indicating that a mechanism for cell attachment is present. The morphological basis for this intercellular attachment is not entirely clear, although certain forms of intercellular junctions have been noted between Leydig cells (Belt and Cavazos, 1967; Christensen and Gillim, 1969; Connell and Christensen, 1975; Schulze, 1984; and others).

One class of junctions not previously reported between Leydig cells is the group of actin filament related adhesion junctions. In a set of experiments described earlier (Chapter 2), I fortuitously noticed that certain regions between ground squirrel Leydig cells label intensely for filamentous actin with the probe NBD-phallacidin. The nature of the staining pattern is indicative of a heavy concentration of actin filaments closely associated with the plasma membrane, such as occurs at adhesion sites in other cell types. This observation indicated to me that actin associated intercellular adhesion junctions may occur between Leydig cells of the ground squirrel and may play a role in maintaining cell attachment. Results reported in this Chapter support this conclusion.

Materials and Methods

Animals:

Reproductively active golden-mantled ground squirrels (Citellus lateralis) were used in this study. Housing and maintenance of the animals generally followed that previously described (Vogl et al. 1983).

Fluorescence Microscopy:

(a) Tissue Preparation:

To facilitate visualization of intercellular contact sites between Leydig cells, small clusters of interstitial cells were isolated from the seminiferous tubules in the following manner. Testes were excised from animals anesthetized with sodium pentabarbitone administered intraperitoneally. Each testis was perfused via the spermatic artery with 3% paraformaldehyde in PBS (150 mM NaCl, 5 mM KCl, 3.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, buffered to pH 7.3 with 0.1 N NaOH) for 10 min. Each testis was then decapsulated and minced with two scalpels into small sections in fixative for 5 min. This yielded a heterogeneous mixture of intact tubules, tubule walls, epithelial sheets, and interstitial tissue which was transferred to glass centrifuge tubes and sedimented at low speed in a clinical centrifuge. Pellets were resuspended in PBS containing 0.1% triton-X 100 and incubated at room temperature for 5 min. Samples were then centrifuged and the pellets resuspended in PBS and incubated for 10 min at room temperature. This step was repeated, after which the pellets were resuspended in approximately twice their volume of PBS and gently aspirated 5-10 times through an 18G syringe needle. Drops of this tissue suspension were placed on polylysine-coated slides. Large

aggregates of intact tubules and tubule walls were carefully removed with micropipettes leaving mainly isolated clusters of spermatogenic cells and Sertoli cells, individual cells of various types, and small groups of interstitial cells. After 15 min excess fluid was removed and the staining procedures for actin and vinculin were initiated.

(b) Single Label Experiments:

Localization of Actin

The presence and distribution of filamentous actin in regions of Leydig cell intercellular contact were revealed with the F-actin specific probe NBD-phallacidin (nitrobenzoxadiazole-phallacidin). Slides with tissue samples prepared as described above were incubated at room temperature for 20 min in one of the following solutions: (1) PBS + 1.65 X 10^{-6} M NBD-phallacidin (test for filamentous actin); (2) PBS + 1.65 X 10^{-6} M NBD-phallacidin + 1.04 X 10^{-4} M phalloidin (competitive specificity control); (3) PBS + 1.04 X 10^{-4} M phalloidin (control for phalloidin in solution #2); (4) PBS (control for autofluorescence). Slides were then washed three times, for 10 min each, with PBS and mounted with 1:1 PBS:glycerol containing 0.02% sodium azide.

In some experiments, rhodamine-phalloidin was used instead of NBD-phallacidin to label filamentous actin. The controls done were similar to those used for experiments with NBD-phallacidin except that phallacidin (rather than phalloidin) was used in the competitive specificity sample.

Localization of Vinculin

Indirect immunofluorescence was used to localize vinculin within Leydig cells. The probe used (previously prepared in this laboratory - Grove and Vogl, 1989) was an affinity purified

antibody raised against human platelet vinculin. Characterization of this antibody is described elsewhere (Grove and Vogl, 1989; Grove et al., 1990).

Following the tissue preparation outlined above, slides with samples were incubated for 30 min at room temperature with TPBS (0.05% Tween-20 in PBS) containing 0.1% bovine serum albumin (BSA) and 5% normal goat serum (NGS). Slides were then drained and incubated with the primary antibody at an approximate concentration of 50 ug/ml in TPBS containing 0.1% BSA and 1% NGS for 1 hr at 37°C. After washing three times, for 10 min each, with TPBS containing 0.1% BSA, samples were incubated for 1 hr at 37°C with secondary antibody (goat anti-rabbit IgG conjugated to fluorescein, Sigma) diluted 1:32 with TPBS containing 0.1% BSA. Following incubation, samples were washed three times with TPBS containing 0.1% BSA and then mounted with 1:1 PBS:glycerol containing 0.02% sodium azide.

Control slides included (1) non-adsorbed serum (1:50 dilution) substituted for the specific antibody; (2) preincubation of the specific antibody with antigen (1:10 mg/ml); (3) secondary antibody alone; (4) no antibodies. Smooth muscle cells of interstitial blood vessels served as a positive control for the specificity of vinculin staining.

(c) Double Label Experiments:

Slides to be double labelled for vinculin and actin were treated in a similar fashion to those used to label vinculin alone, except that rhodamine-phalloidin was included in the first of the three washes following incubation in the second antibody. Also, the second antibody was a goat anti-rabbit IgG conjugated to AMCA (7-amino-4-methylcoumarin-3-acetic acid) (Jackson Immunoresearch Laboratories, Inc).

All fluorescence microscopy in this study was performed on either a Zeiss Photomicroscope III or a Zeiss Axiophot Photomicroscope both fitted with filters for detecting fluorescein isothiocyanate, rhodamine, and AMCA.

Electron Microscopy:

Squirrels were anesthetized with sodium pentabarbitone injected intraperitoneally. Testes were excised and perfused through the spermatic artery with 0.1 M sodium cacodylate (pH 7.3) containing 1.5% paraformaldehyde and 1.5% glutaraldehyde. After 30 min perfusion was stopped and each testis was cut into small blocks (1 mm^3) which were then immersion fixed for 2 hr. Tissue blocks were washed with 0.1 M sodium cacodylate (pH 7.3) and then postfixed with buffered 1.0% OsO_4 on ice for 1 hr. Tissue blocks were next washed with distilled H_2O, stained *en block* with 1.0% uranyl acetate (aqueous) for 1 hr, and again washed with distilled H_2O. Following dehydration through a graded series of ethyl alcohols, blocks were embedded in Polybed 812.

Thin sections, stained with uranyl acetate and lead citrate, were examined on a Philips 300 electron microscope operated at 80 KV.

Results

Fluorescence Microscopy:

(a) Cell Identification:

In intact testes, Leydig cells characteristically encompass interstitial blood vessels. I used this fact to help differentiate Leydig cells from other cells types present in my tissue preparation. Isolated segments of interstitial blood vessels with adhering aggregates of Leydig cells were frequently seen. Other distinguishing Leydig cell features included their roughly spherical shape and, when viewed with phase contrast microscopy, the numerous lipid droplets found in their cytoplasm. In comparison with the other cell types present, Leydig cells resembled only the early spermatids in size. In this case, the absence of acrosomal granules from Leydig cells was used as the differentiating trait between these two cell types.

(b) Single Label Experiments:

Distribution of Actin:

Filamentous actin, as visualized with NBD-phallacidin, is concentrated at the periphery of Leydig cells (Fig. 5.1a,a'). The cortical cytoplasm of Leydig cell regions not in apposition with other cells displays a weak and patchy network of fluorescence immediately underlying the plasma membrane. Markedly different are those cell regions which lay adjacent to and in apparent contact with neighboring cells. At these sites, large accumulations of actin filaments are present. The strong fluorescence emitted from such areas appears as a solid linear band directly beneath and parallel to the plasma membrane (Fig. 5.1a). These prominent

fluorescence patterns correspond to and are restricted to those regions where, at the ultrastructural level, I observed sites of intercellular contact (described below).

No specific fluorescence was observed in any of the control slides for NBD-phallacidin labelling (Fig. 5.1b,b'-d,d').

Distribution of Vinculin:

Specific staining for vinculin is limited to only those regions of membrane/membrane apposition between adjacent Leydig cells (Fig. 5.2a,a'). The fluorescence appears as a line along the contour of the plasma membrane of these sites. Although somewhat less intense than that of the NBD-phallacidin staining, the specific fluorescence for vinculin corresponds very closely with that observed for actin at these sites. Unlike the actin staining, no specific labelling for vinculin is detected away from regions of apparent cell/cell contact.

Smooth muscle cells of interstitial blood vessels, used as positive controls for vinculin antibody specificity, reacted strongly with the antibody.

No specific staining was observed in any of the control slides (Fig. 5.2b,b'- e,e').

(c) Double Label Experiments:

In experiments where tissue is labelled with probes both for filamentous actin and for vinculin, the probes are co-distributed at specific sites of intercellular contact (Fig. 5.3 & 5.4). The intensity of fluorescence generated by the actin probe is greater than that emitted by the probe for vinculin. Also, some actin staining occurs generally around the periphery of the cells; however, vinculin staining is restricted to sites of intercellular attachment. This is particularly well seen in isolated groups of two or more Leydig cells that have separated from blood vessels but continue to maintain adhesion with one another (Fig. 5.3d- f).

Electron Microscopy:

Leydig cells, in thin sections of intact ground squirrel testis, are readily identified by their interstitial location, abundance of endoplasmic reticulum and mitochondria with tubular cristae. While the surface of each Leydig cell possesses microvilli and is free of contact with neighboring cells, small to medium expanses of membrane/membrane association between adjacent cells are common. Within these regions, gap junctions are observed as are areas of focal contact between adjacent membranes (Fig. 5.5a). Also, at certain locations within these regions of membrane/membrane association are sites of intercellular contact characterized by subsurface filament networks. These networks consist of a dense mat of indistinct filaments, which I interpret as actin. The filaments are not organized into bundles; rather, they appear to be arranged in a loose meshwork. Such filament concentrations are seen only in association with sites of intercellular contact, where they are restricted to the cytoplasm immediately below the plasma membrane on either side of the contact site (Fig. 5.5a,b). Each contact site is easily identified by a narrowing of the intercellular space relative to that in other areas of simple cell apposition. Intermediate filaments and microtubules are not found in the vicinity of these regions, nor are membranous organelles which appear to be excluded by the filament networks. Ultrastructurally, the Leydig cell filament associated contact sites appear reminiscent of actin related adherens junctions seen in many other cell types.

Discussion

In this study I present evidence that ground squirrel Leydig cells may possess actin associated adhesion junctions.

Testicular tissue breaks down into small groups of cells when subjected to mild mechanical separation procedures. In the face of this mechanical perturbation, Leydig cells maintain adherence to one another and frequently retain a clustered organization similar to that seen in intact tissue. Although the basis for this clustering is not entirely clear, results presented here indicate that it may be due to actin associated junctions.

The stains for filamentous actin NBD-phallacidin and rhodamine-phalloidin both label heavily certain sites of intercellular contact between these clustered Leydig cells. In addition, co-labelling at these contact sites for the protein vinculin occurs in double labelling experiments. As discussed in preceding chapters, vinculin is viewed as a marker protein for actin associated adhesion sites in general. It is thought to be involved in linking actin filaments to adhesion domains in the plasma membrane.

The ability of Leydig cells to maintain attachment to each other when exposed to mild tissue disrupting procedures, together with the co-distribution of vinculin and actin filaments at certain of these intercellular attachment regions indicate that actin associated adhesion sites are likely present between Leydig cells. Further data consistent with this argument are the ultrastructural observations of filament concentrations in the form of loose networks adjacent to certain sites of Leydig cell intercellular contact. In appearance these filament networks resemble those seen at actin associated adhesion sites in other cells.

In areas free of contact from neighboring cells a patchy and weak fluorescence pattern in labelling experiments for actin, but not vinculin, is present around much of the Leydig cell periphery. This indicates that a limited cortical network of actin is likely present in Leydig cells in the ground squirrel, similar to what has been described in the rat (Russell et al., 1987). Compared to the brilliant fluorescence emitted by labelled concentrations of actin

filaments in regions of cell/cell apposition, actin fluorescence over the rest of the cell is relatively pale.

At regions of cell apposition other forms of cell/cell contact frequently occur in the vicinity of the filament networks, some of which could potentially serve an accessory role in adhesion. Typical gap junctions are common. Several focal sites of apparent membrane fusion between adjacent Leydig cells are also present (fig. 5.5a). By appearance these contact sites resemble tight junctions; however, without an appropriate molecular probe for tight junctions it is difficult to determine if these sites are indeed tight junctions or if they represent a different form of contact, possibly focal gap junctions. Freeze-fracture studies have shown tight junctions to be absent from cells of the adrenal cortex (Friend and Gilula, 1972). Christensen (1975) points out that other steroid-secreting cells, including Leydig cells, may be similar in this respect.

In earlier studies of Leydig cells, one factor which intercellular adhesion has generally been attributed to is the presence of "desmosome-like" junctions. These junctions have been reported in various species, including the boar (Belt and Cavazos, 1967), squirrel monkey (Belt and Cavazos, 1971), dog (Connell and Christensen, 1975), and human (Schulze, 1984). That Leydig cells may possess rudimentary desmosomes is not surprising as various forms of desmosomes have been found in many tissue types. Interestingly though, in several micrographs presented in the previously cited studies of Leydig cells, the arrangement and appearance of filaments at these junctions appear more similar to that which typifies junctional related actin filaments than desmosomal intermediate filaments. The characteristic feature of desmosomes of an electron dense line (desmoglea proteins) between the apposed membranes of the contact site is also not present. In many cases the overall appearance of these desmosome-like junctions resembles that of the contact sites that I describe here, which I have interpreted as actin associated adhesion sites. This raises the possibility that in some instances the junctions previously described between Leydig cells as desmosome-like may, in fact, have been actin associated adhesion junctions. Corroborative evidence for this comes from the observations of Russell et al. (1987) of the distribution of intermediate filaments within rat Leydig cells. By

immunofluorescence techniques they demonstrated vimentin intermediate filaments to be present within the Leydig cell cytoplasm but did not find focal filament concentrations at the plasma membrane. This is contrary to what would be expected if intermediate filament associated junctions are present between Leydig cells. Their results, however, do not rule out the existence of desmosomes or desmosome-like junctions from Leydig cells. It is possible that the absence of vimentin labelling at the cell periphery is due simply to the fact that the Leydig cell intermediate filament associated junctions are too small to be detected by fluorescence microscopy. Nevertheless, their results indicate that, if present, intermediate filament associated junctions are limited in extent and that intercellular adhesion may be mediated, in part, by other factors. As shown in this study, one of these factors appears, in the ground squirrel at least, to be actin associated adhesion junctions.

FIGURE 5.1. Shown here are paired fluorescence and phase micrographs of ground squirrel Leydig cells. Filamentous actin, as visualized with NBD-phallacidin, is concentrated at certain sites of intercellular apposition (arrowheads in panel a). Tissue in panel (b), was treated with NBD-phallacidin in the presence of excess phalloidin. Tissue in panel (c) was treated with phalloidin alone, and that in panel (d) received only buffer. Note that no specific fluorescence is seen in any of the control panels (b-d). X540. Bar = 20um.

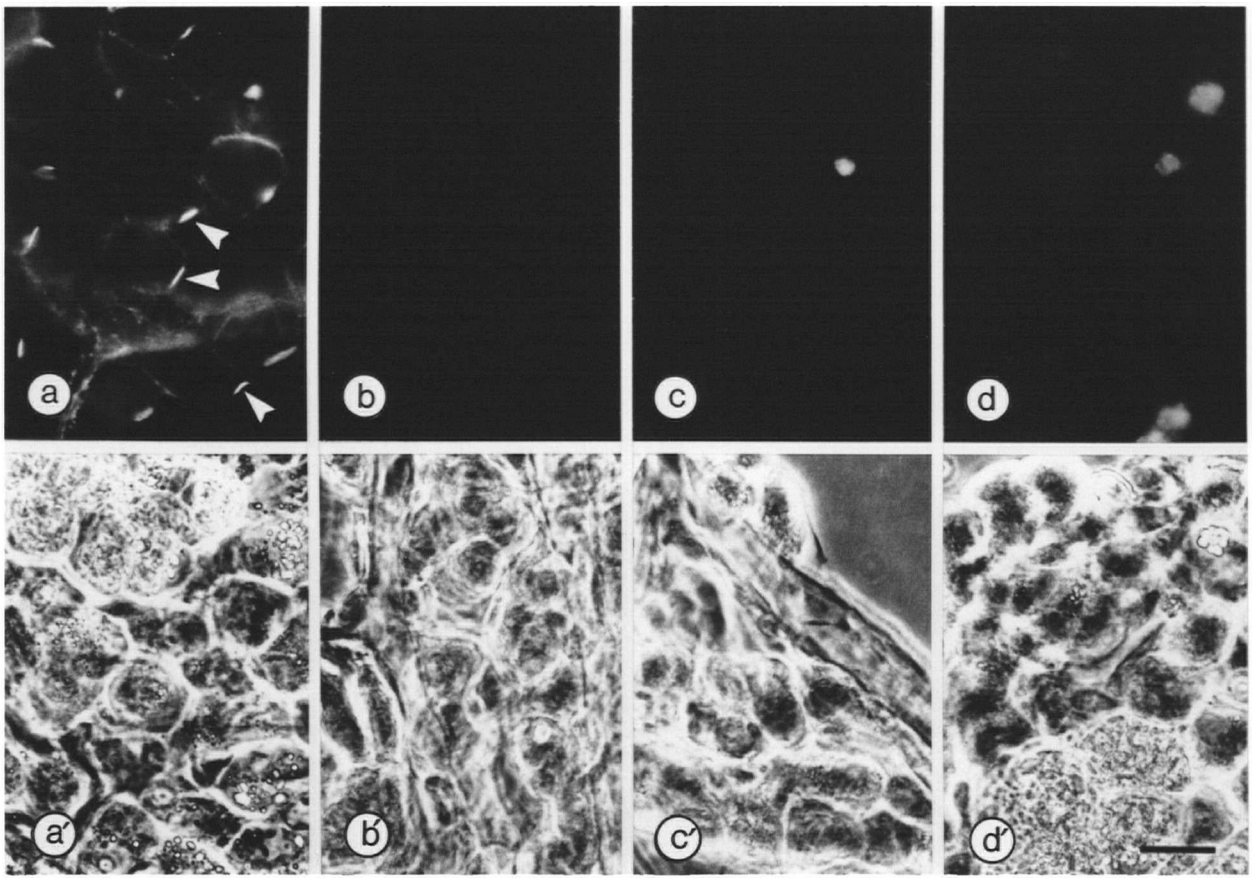
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FIGURE 5.2. Shown here is a series of paired fluorescence and phase micrographs, of ground squirrel Leydig cells, in which the immunological localization of vinculin is indicated. The tissue in panel a has been treated with an affinity purified primary antibody to human platelet vinculin and an FITC-conjugated secondary antibody. Note that specific labelling in Leydig cells occurs only in certain regions of intercellular contact (arrowheads). In panel (b), the non-adsorbed fraction collected from the column used to affinity purify the antibody used in panel (a) was substituted for the primary antibody. In panel (c) the primary antibody was pre-incubated with and added to cells in the presence of excess antigen. In panel (d), the primary antibody was omitted and in panel (e), both primary and secondary antibodies were omitted. X470.Bar=10um.

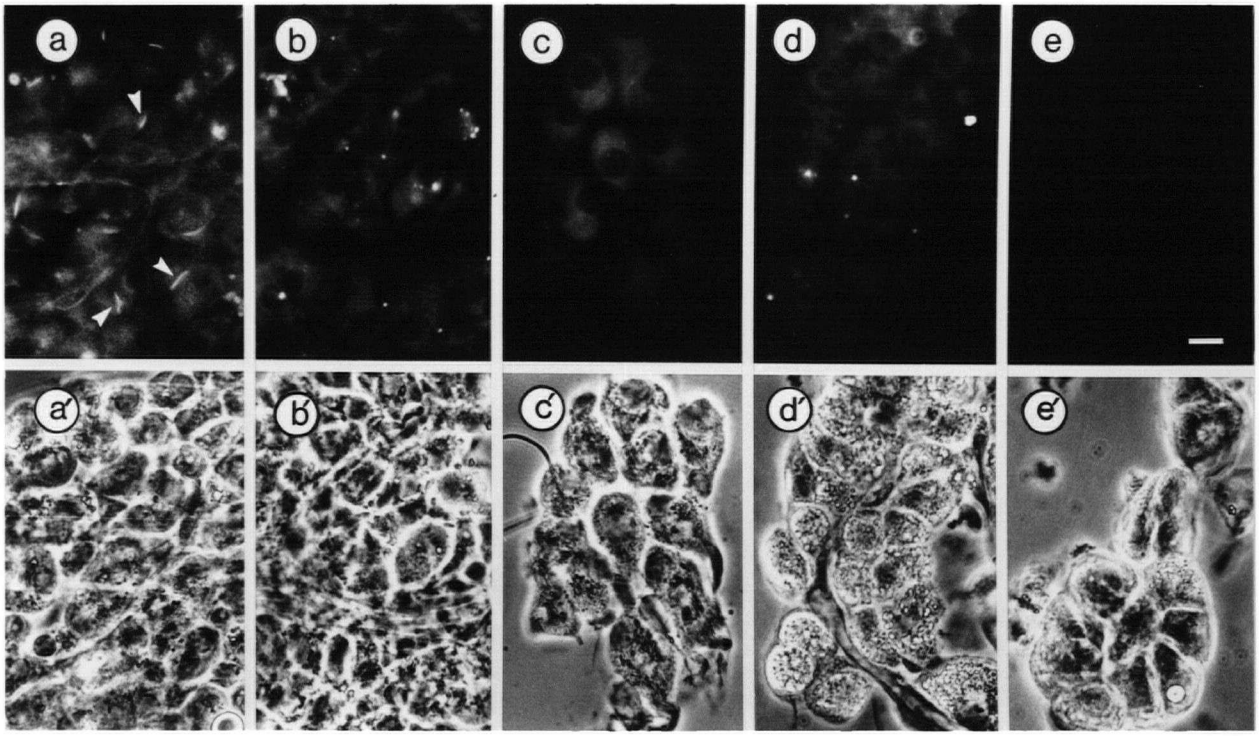
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FIGURE 5.3. Shown in this plate are clusters of ground squirrel Leydig cells (phase - panels a and d) that have been double labelled for actin (panels b and e) and vinculin (panels c and f). In panels (a-c), Leydig cells occur clustered around a small blood vessel (bv). Actin and vinculin are co-distributed at specific sites of intercellular contact (arrowheads). Notice that specific labelling, with each probe, also occurs in cells of the blood vessel. In panels (d-f), four isolated Leydig cells are shown. Again, actin and vinculin are co-distributed at sites of intercellular apposition (large arrows). Notice that a limited cortical network of actin is present in panel (e), but that vinculin (panel f) is restricted to the sites of intercellular attachment. Panels (a-c), Bar = 10um, X620. Panels (d-f), X815. Bar = 10um.

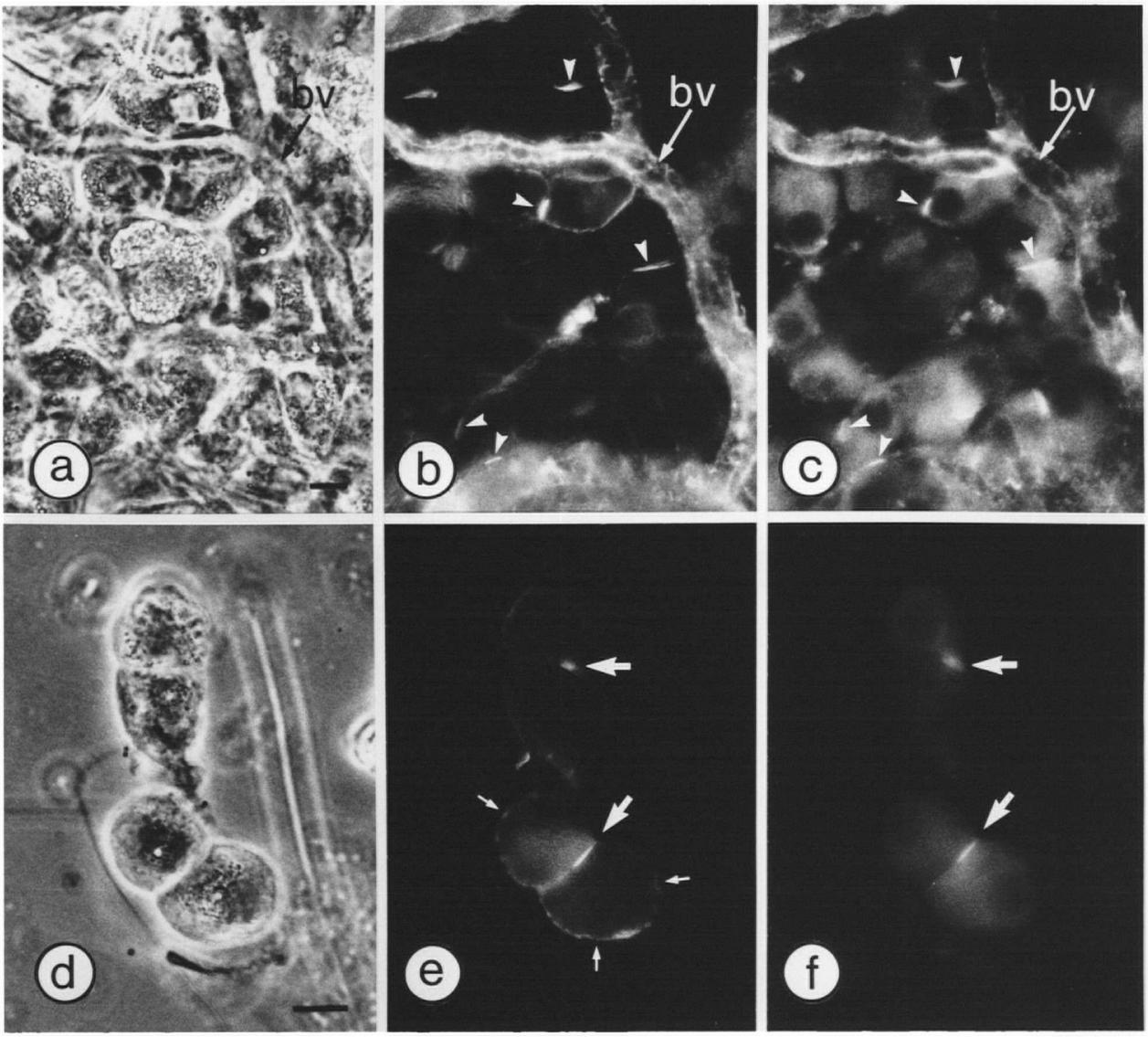
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FIGURE 5.4 Shown in these two micrographs is a cluster of Leydig cells around a blood vessel (bv). The distribution of actin (panel a) is co-distributed with vinculin (panel b) at sites of intercellular contact (arrowheads). The weak general background fluorescence in panel a corresponds to the limited overall cortical actin Leydig cells are known to possess (a slight overexposure of the print has exaggerated the intensity of the labelling). X870. Bar = 25u.

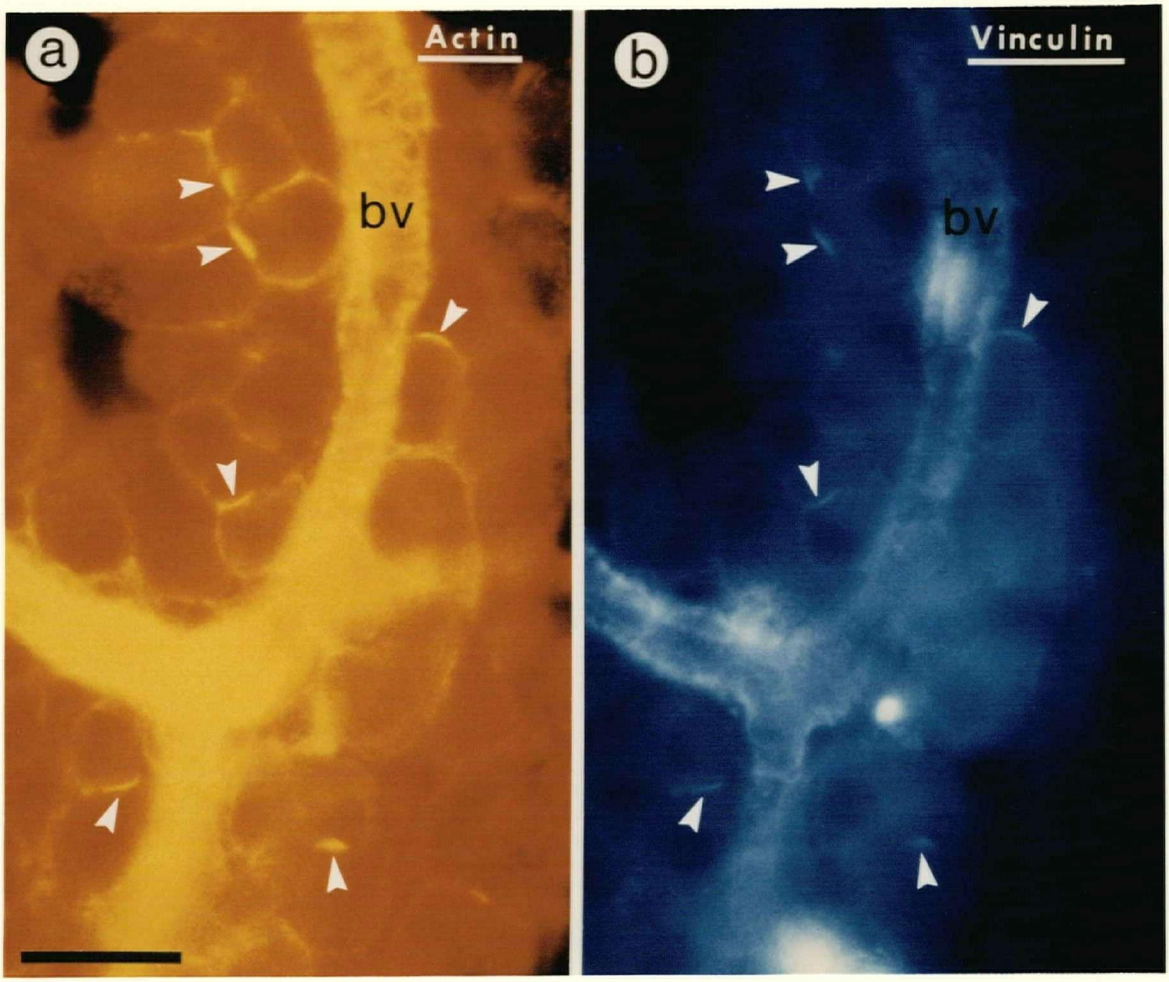
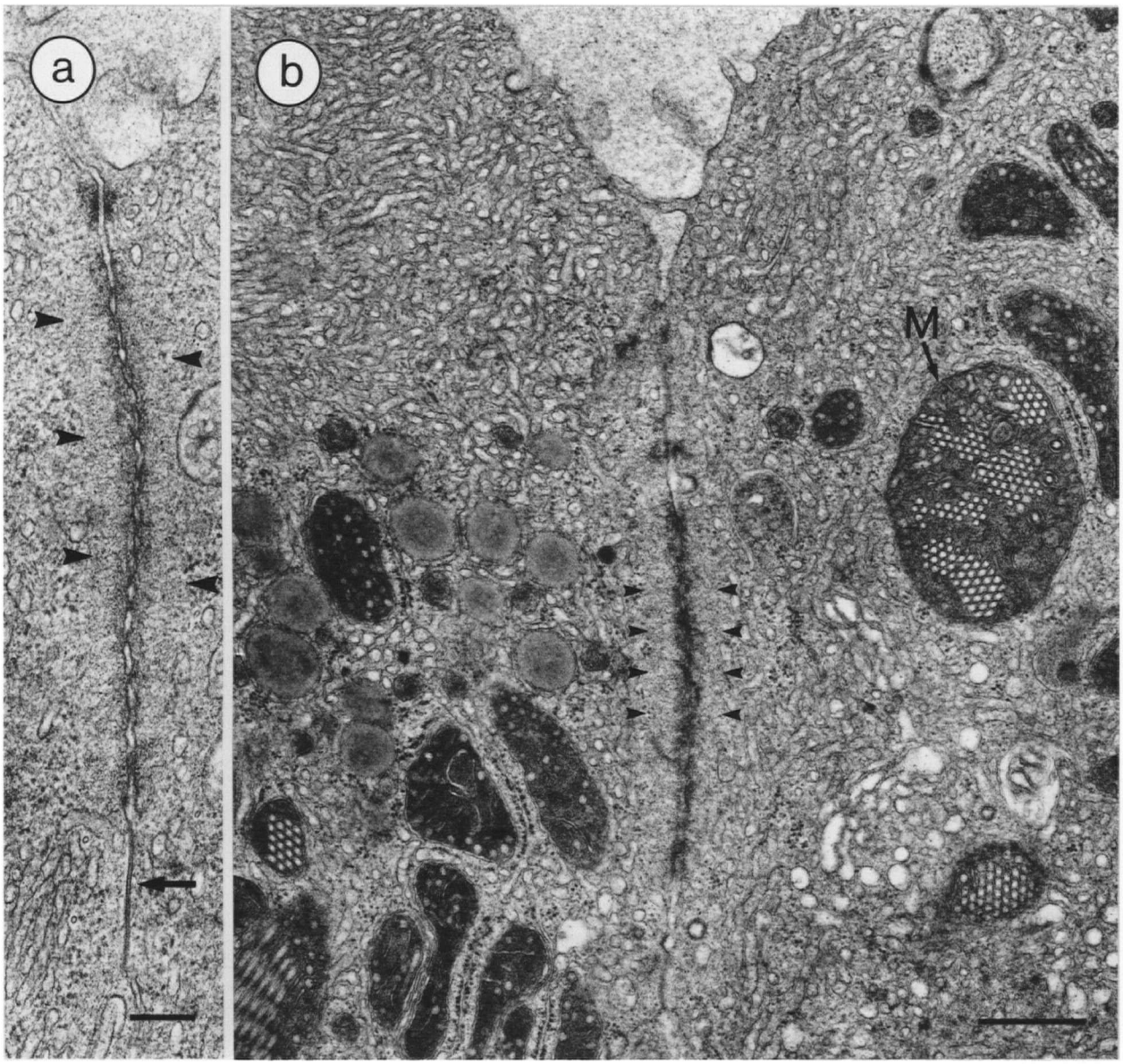

a
Actin
bv
b
Vinculin
bv

FIGURE 5.5. Electron micrographs of two examples of junctional sites between ground squirrel Leydig cells. In panels (a) and (b), a zone of filamentous material (arrowheads) is associated with certain regions of the junctional complex. As shown in panel a, the junctional complex consists of multiple junction types. An example of a gap junction is indicated by an arrow. Also visible in this micrograph are sites of apparent focal membrane contact. In panel (b), a good example of a mitochondrion (M) with tubular cristae is present. Panel (a), X41,667. Bar = 0.25um. Panel (b), X33,600. Bar = 0.5u.


a
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Summary and Conclusions

(1) Vinculin is co-distributed, at the light microscopic level, with actin in ectoplasmic specializations associated with spermatids of the ground squirrel. This observation is consistent with the prediction that vinculin is a component of ectoplasmic specializations and with the hypothesis that these structures are a form of actin associated intercellular adhesion junction.

(2) Immunological probes for some of the common cell adhesion molecules (E-cadherin, A-CAM and N-CAM) do not react with ectoplasmic specializations. This suggests that cell adhesion molecules commonly found at adhesion sites may not be present at ectoplasmic specializations. The adhesion molecule present at ectoplasmic specializations remains to be identified.

(3) Immunological probes for A-CAM appear to react positively at locations near the base of the seminiferous epithelium in the rooster. This suggests that (1) A-CAM is expressed by early spermatogenic cells and perhaps by Sertoli cells at sites other that ESs, and (2) that Sertoli cells, at least in the rooster, may express two different CAMs - one, A-CAM, at sites of attachment to early spermatogenic cells, and another, as yet unidentified, at ESs sites.

(4) In ground squirrel ESs, fluorescence data indicate that filament bundles on each side of the saucer-shaped spermatid head are discontinuous with each other and differ in their positional changes during spermatogenesis. These observations indicate that there may occur a partial separation of each ES into two adhesion plaques. I argue that the changes in filament patterns that occur during spermatogenesis are not associated with influencing spermatid head shape, as some authors have suggested, but rather are concerned with adjusting the contour of the adhesion junction to the changing cell shape of the attached spermatid.

(5) Probes for actin and vinculin react positively at specific sites of Leydig cell intercellular attachment. Ultrastructurally, concentrations of filaments that resemble actin networks found at adhesion sites between other cell types are present at certain sites of Leydig cell contact.

These data indicate that Leydig cells of the ground squirrel may possess actin associated adhesion junctions.

(6) Actin associated adhesion junctions appear to be present in epithelial (Sertoli) and non-epithelial (Leydig) cells of the testis. Morphologically these two sites differ considerably. Adhesion junctions in Leydig cells closely resemble those commonly seen in other cell types. However, those in Sertoli cells are unlike those seen between any other cell type in the body. Here, filaments are bundled and the bundles form elaborate patterns that change during spermatogenesis. Changes in the ES site may directly reflect dynamic changes that occur in the cell type (spermatid) around which the adhesion site is formed.

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