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The actin filament network associated to Sertoli cell ectoplasmic specializations

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ABSTRACT: Junctional devices in Sertoli cells conform the blood-testis barrier and play a key role in maturation and differentiation of germ cells. The spacial distribution of ectoplasmic specializations of Sertoli cells was studied by β-actin immunolabelling, using laser confocal and transmission electron microscopy. For confocal microscopy, β-actin immunolabelling of ectoplasmic specializations was studied over the background of either prosaposin or glutaredoxin immunolabelling of the Sertoli cytoplasm. Labelling was found near the basal lamina, surrounding early spermatocytes (presumably in leptotene-zygotene) or at one of two levels in the seminiferous epithelium: (1) around deep infoldings of the Sertoli cell cytoplasm, in tubular stages before spermiation, and (2) in the superficial part of the seminiferous epithelium, in tubular stages after or during spermiation. For transmission electron microscopy, β-actin immunolabelling of ectoplasmic specializations was also used. Ectoplasmic specializations were found at two different levels of the seminiferous epithelium. We also used freeze fracture to analyze the characteristics of tubulo-bulbar complexes, a known component of apical ectoplasmic specializations. Also, these different approaches allowed us to study the complex arrangement of the actin cytoskeleton of Sertoli cells branches, which surround germ cells in different stages of the spermatogenic cycle. Our results show a consistent labelling for β-actin before, during and after the release of spermatozoa in the tubular lumen (spermiation) suggesting a significant role of the actin network in spermatic cell differentiation. In conclusion, significant interrelations among the β-actin network, the junctional complexes of the blood-testis barrier and the ectoplasmic specializations were detected at different stages of the seminiferous cycle.

Introduction

The concept of a blood-testis barrier originated from the physiological studies of Setchell (1969) and Setchell and Waites (1975) in the mammalian testis. They showed a differential transport of ions and metabolites between the basal area of the seminiferous tubules, the *basal compartmen*t, in wide contact with bodily fluids, and the apical sector of the seminiferous epithelium, the *adluminal compartment*, close to the tubular lumen and isolated by the barrier. The morphological correlate of such a barrier was found to be located in specialized junctional devices found between adjacent Sertoli cells, integrated by occluding or tight junctions that seal each inter-Sertoli space near the base of the seminiferous epithelium (Dym and Fawcett, 1970; Dym, 1973). Related to the last mentioned topic is the long-time controversy on the existence of a third compartment in the seminiferous epithelium (see Yazama, 2008, for review).

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The early interpretations of Russell (1977a, b, 1978) on the rat blood-testis barrier were superseded by our observations on monkeys (Dym and Cavicchia, 1977, 1978) as we showed that the impermeable junctions assemble and disassemble simultaneously without forming an actual compartment. Later, in the rat, using intratubular administration of intercellular tracers we arrived to similar conclusions (Cavicchia and Sacerdote, 1988).

The second junctional device found in the seminiferous epithelium is the *zonula adherens*, mainly made by a dense circumferential belt of actin filaments near the Sertoli cell membranes and located (1) at the level of the blood-testis barrier, i.e., in the Sertoli cytoplasm close to tight junctions and surrounding a smooth cistern of the endoplasmic reticulum (Dym and Fawcett, 1970) and (2) at a higher level in the epithelium, in close apposition to germ cells in progressive steps of differentiation (round and elongated spermatids) (Gumbiner *et al*., 1988). These two junctional devices , as a whole, were termed ectoplasmic specializations of the Sertoli cell by Russell (1977b).

Another junctional device found in ectoplasmic specializations at both levels are the so-called tubulobulbar complexes (Russell, 1979; Russell and Griswold, 1993). At the higher place of Sertoli-germ cell contacts, they are elongate processes developed from the spermatid plasma membrane and surrounded by a deep invagination of the Sertoli cell membrane and a specialized layer of Sertoli cell cytoplasm. This layer contains abundant β-actin filaments bundles surrounding the whole invagination. These tubulo-bulbar complexes have been found to develop in the regions previously occupied by the ectoplasmic specializations and have been proposed to internalize intact junctions during spermiation (Guttman *et al.*, 2004). Thus, these complexes are likely significant participants in the mechanism of junctional turnover in the seminiferous epithelium (Young *et al*., 2009), which is related to the well-known endocytic activity of Sertoli cells, residual body formation, their phagocytic activity during spermiation and Sertoli lysosome types including multivesicular bodies ("multivesicular endosomes"; Gruenberg and Stenmark, 2004) and secondary lysosomes (reviewed by Morales and Clermont, 1993). Significant progress has been made in the understanding of the mechanisms that regulate multivesicular transport and the degradation pathway that leads to lysosome formation since the classical study that observed a maximum number of lysosomes marked by acid phosphatase in stages V-VIII of the seminiferous tubules, close to spermiation (Chemes, 1986). To review the stages of the seminiferous tubules see Russell *et al*. (1990).

Recently, experimental evidence has been accumulating on the regulation and functional significance of Sertoli ectoplasmic specializations (Wong *et al.,* 2004, 2008; reviewed by Cheng and Mruk, 2010). The simultaneous occurrence of transit of new germ cells through the blood-testis barrier and the process of spermiation has been put into a theoretical scheme in which these two cellular events are coordinated by the release of definite protein products, and the presence of an "apical ectoplasmic specializations- blood-testis barrier-hemidesmosome axis" in Sertoli cell has been put forward as a crucial element in the coordination of these events (reviewed in Cheng and Mruk, 2010). Although the actin network was previously described in Sertoli junctional devices (Morales and Clermont, 1993) its role in the subcellular organization of blood-testis barrier and ectoplasmic specializations at different stages of the seminiferous cycle have not been thoroughly investigated, and its significance in current schemes of spermatogenesis is not well understood.

Thus, the main objective of this paper is to report our findings about the cellular and fine structural organization of β-actin rich intercellular junctions, and its possible role in germ cell maturation, differentiation and migration towards the lumen of theseminiferous tubule.

Materials and Methods

Animals

All studies involving experimental animals were conducted in accordance with the the UFAW Handbook on the Care and Management of Laboratory Animals (Pole, 1999) and were approved by the Ethical Board of the School of Medicine of the National University of Cuyo, Mendoza, Argentina. Young male Wistar rats bred in our laboratory, being 90-100 days old and weighing 300 ± 50 g, were used throughout this protocol. They were housed under controlled light (lights-on from 07:00 to 19:00 h) and temperature (24 ± 2ºC) conditions, and water and food were provided *ad libitum*. Rats were anesthetized with ether under a bell and perfused through the spermatic artery according to the technique of Dym (1973) with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (for light microscopy studies) or with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.1% glutaraldehyde for electron microscopy studies. Testes were dissected and post-fixed in

the same solution for 2 additional hours. Forty μm sections were first obtained in an Oxford Vibratome and were then embedded in either paraffin or epoxy resin.

Immunochemistry

Paraffin sections

Sections (5-10 μm thick) were mounted on sylanized slices (Saraceno *et al.*, 2010) and were incubated overnight in β-actin antibody (1:500 dilution) (Sigma, USA) and prosaposin (1:500 dilution) (Morales, *et al.*, 1998) or glutaredoxin antibodies (1:500) (Lönn *et al.*, 2008; Godoy *et al.*, 2010) for colocalization studies. Sections were washed several times with phosphate buffer-saline and incubated with the proper secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA), followed by incubation with a biotin streptavidin complex (HRP Histo Mark, Caramillo, CA USA). After several washings in buffer, sections were developed with AEC substrate kit (Invitrogen Gauttersburg, MD, USA) until staining was optimal. Finally, samples were examined in a Spectral Confocal Olympus FV-1000. Collected pictures were analyzed and compiled using Adobe Photoshop 8.0 CS3.

Epoxy resin sections

Sections were incubated overnight with a monoclonal anti-β-actin antibody (Sigma, USA; diluted 1:500 in phosphate buffer-saline). Following several washings with buffer, sections were incubated with biotinylated anti-mouse secondary antibody for 1 h (Amersham) and followed by incubation with ABC complex solution (Vectastain ABC kits, Vector Laboratories Inc., Burlingame, CA, USA) for another 1 h. After washing in buffer, sections were developed with diaminobenzidine solution until staining was optimal as examined by light microscopy. Sections were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in distilled water, and stained with 1% aqueous uranyl acetate overnight. Tissue sections were dehydrated in increasing series of ethanol concentrations followed by dry acetone, and embedded in epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland). Thin 1μm sections were stained with toluidine blue-sodium borate for light microscopy. Ultrathin sections less than 0.1μm (silver-gold interference colour) were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 900 electron microscope (Carl Zeiss Inc., Berlin, Germany).

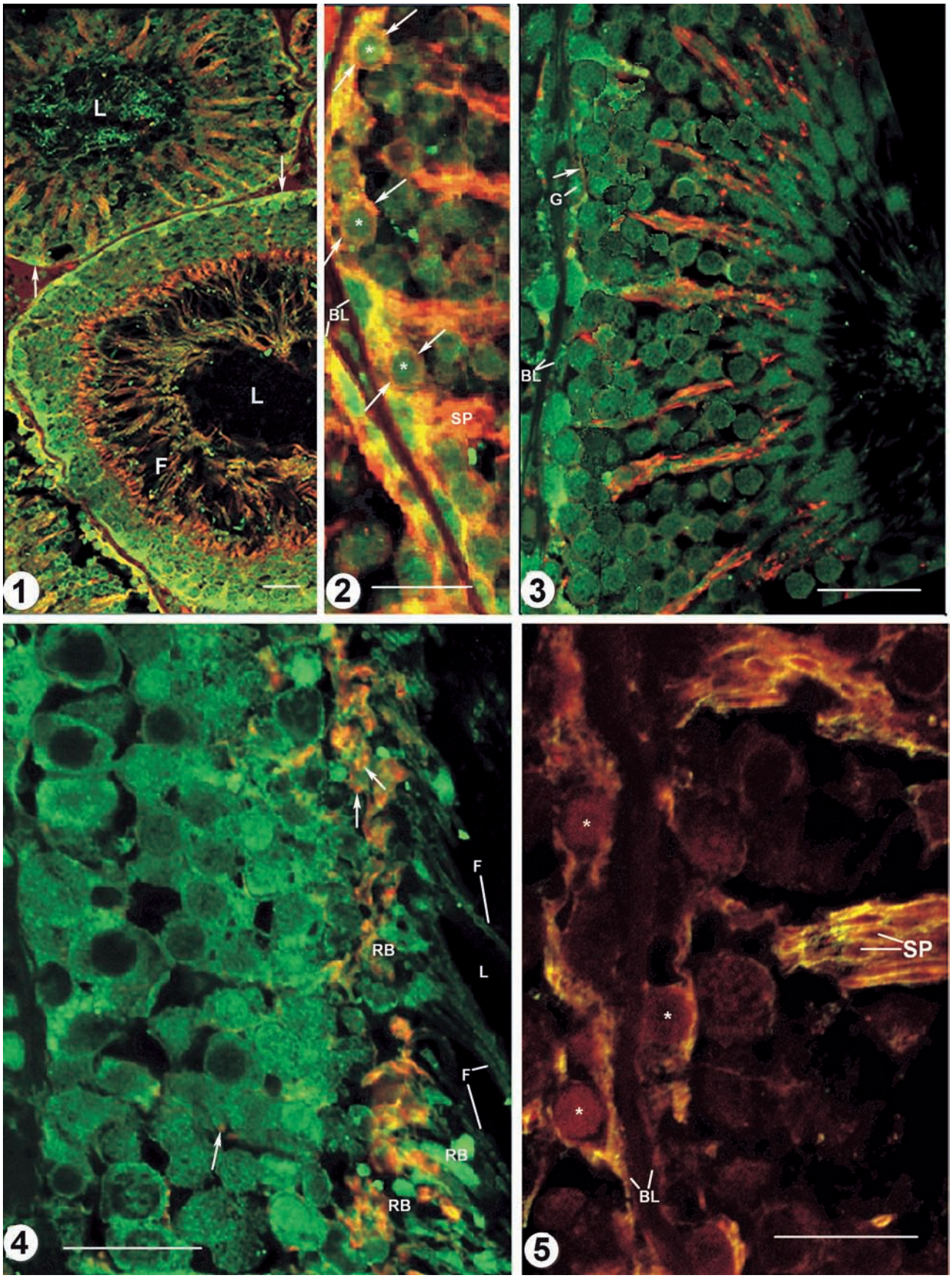
Freeze-Fracture

The method of Cavicchia *et al*. (1998) was used. In brief, rats were anesthetized and perfused as indicated above for immunohistochemistry, and the fixative used was 5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2-7.4. After perfusion, testes were removed, cut in small pieces and immersed in the same fixative for 2 h, placed for additional 2 h in 30% glycerol at room temperature (18°C), rapidly frozen in liquid Freon 22 and stored in liquid nitrogen. The material was fractured in a Balzers BAF 301 apparatus at -105°C and shadowed with platinum followed by carbon. Replicas were cleaned with sodium hypochlorite, mounted on copper grids and examined under the electron microscope.

Results

The combined immunocytochemistry for β-actin, prosaposin and glutaredoxin allowed us to observe significant details of the staining degree, the localization of the blood-testis barrier in relation to spermatocytes and to spermatids before, during and after spermiation (Figs. 1 to 5). Both prosaposin and glutaredoxin are proteins localized in the Sertoli cytoplasm (see Lönn *et al*., 2008; Godoy *et al*., 2010). In Figs. 2 and 5, and in the inset A of Fig. 6, β-actin condensation appeared surrounding early spermatocytes (probably leptotene-zygotene stages) at the level of the blood-testis barrier. When tubules at a later stage were observed (Fig.2) β-actin marked spermatids were observed above the blood-testis barrier, probably indicating β-actin marking of Sertoli junctional devices at this level. In an even later stage of development (Fig. 3) β-actin marking was associated to spermatid-Sertoli ectoplasmic specializations and lysosomes close to the tubular lumen.

Immunolabelling of β-actin observed under the transmission electron microscope showed spermatid heads located either in deep infoldings of the Sertoli cell membrane (pre-spermiation stages; Fig. 6, left) or located higher in the epithelium and with their tails protruding into the tubular lumen (post-spermiation stage; Fig. 6, right). Fig. 6 (inset A) shows at higher magnification a tubule in a pre-spermiation stage, in which β-actin condensations are also seen partially surrounding early spermatocytes, probably in leptotene-zygotene stages. Also, numerous immunolabelled β-actin bodies were found near the tubular lumen before spermiation (and probably corresponded to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multivesicular endosomes; Fig. 6, left), while much larger and less numerous electron dense bodies, were seen close to the basal lamina (they probably corresponded to secondary lysosomes, i.e., multivesicular endosomes in later stages of development, Fig. 6, right). In these bodies, β-actin immunolabelling appeared located in the intervesicular matrix of multivesicular endosomes (Fig. 6, inset B, right), while it was uniformly distributed within secondary lysosomes (Fig. 6, inset B, left).

When tubulo-bulbar complexes are favourably freeze fractured they exhibited their characteristic outline (Fig. 7) similar to that described by scanning electron microscopy (Russell and Griswold, 1993). When we compare freeze fracture of this structure with electron microscopy and the β-actin marking, it appears that the label is seen on the Sertoli cell cytoplasm surrounding the tubulo-bulbar processes, and is concentrated between it and the smooth reticulum cistern (Fig. 8).

Discussion

Mammalian spermatogenesis is probably the most exquisite known example of a spatially organized sequence of cellular associations, involving continuous synchronized cell proliferation, differentiation, meiosis, apoptosis, germ translocation, migration and morphogenesis. Spermatogenesis relies on a population of germ cells capable of self renewal, migration towards the lumen of the seminiferous tubules and differentiation into the male gamete (for review see Sharpe, 1994). To maintain close interaction with the surrounding Sertoli cells, both cell lineages possess a well-organized but still poorly understood system of intercellular communications, some of which were explored in the present paper.

The presence of actin in the ectoplasmic specializations in Sertoli cells originally raised the question about its involvement in cell movements related to spermiation, mostly the retraction of Sertoli cell apical cytoplasm. However, accumulated evidence has weakened such a hypothetical role for actin in Sertoli cells. Vogl and Soucy (1985) found no evidence of contraction in a glycerinated model of squirrel seminiferous epithelium. Then, Grove and Vogl (1989) postulated that actin could have a more structural than mechanical or contractile function in these locations since no myosin-based system was detected by immunohistochemistry.

In these two locations, actin consists of the β isoform, which forms filament bundles (Pollard and Earnshaw, 2008). Actin filaments in each bundle are

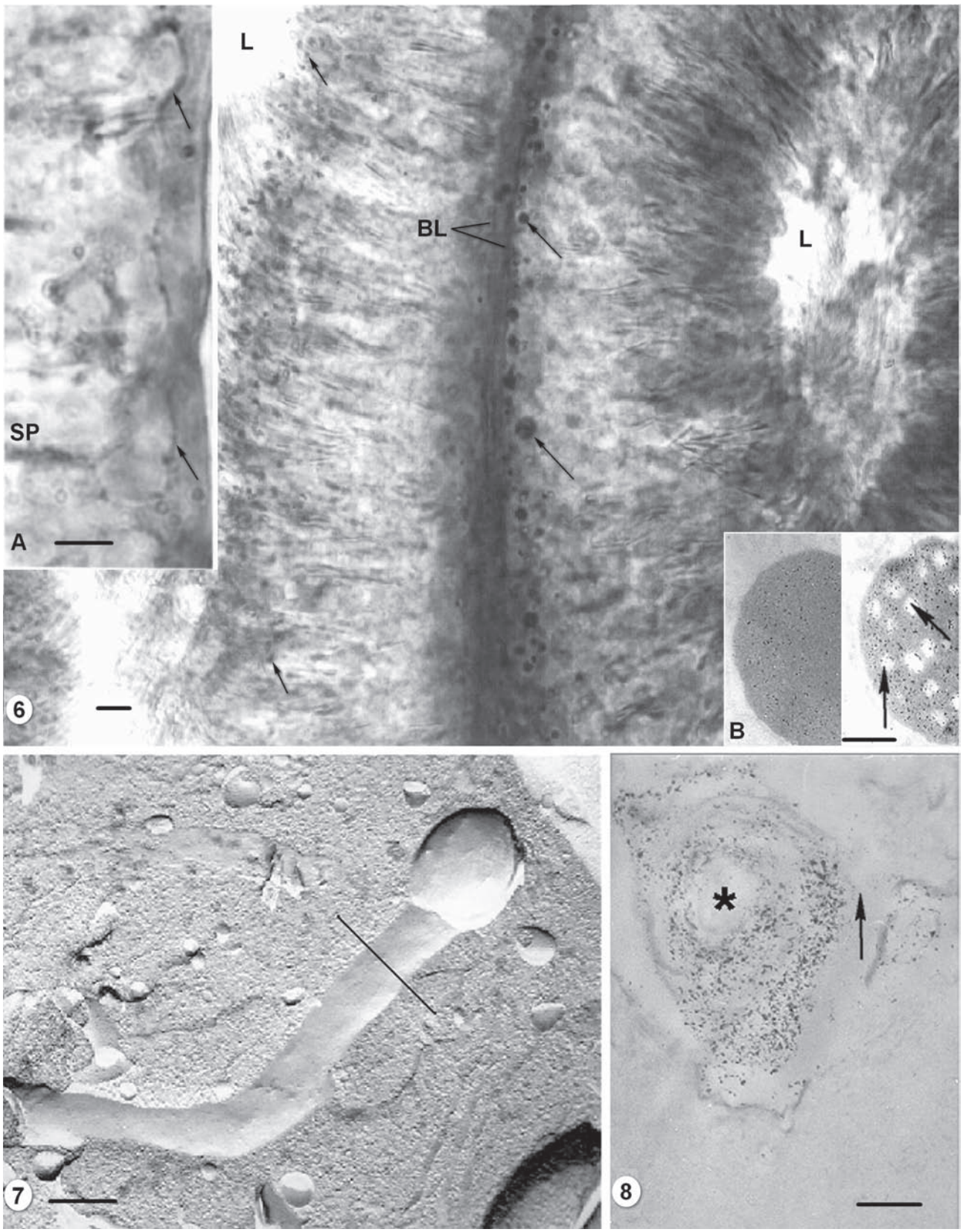
FIGURE 1. Immunostaining for both β-actin (orange-yellow) and prosaposin (green) showing cross sections of two seminiferous tubules in diametrically opposite stages. The upper tubule (pre-spermiation stage) displays elongated spermatids orange-marked for β-actin and which are located in deep infoldings of Sertoli cells, i.e., close to the basal lamina and thus to the blood-testis barrier. The lower tubule (post-spermiation stage) exhibits elongated spermatids with their flagella (F) protruding into the tubular lumen (L). Arrows indicate the basal lamina. Bar = 25 μm.

FIGURE 2. Spermatids (SP) located close to the blood-testis barrier, which is shown as a seemingly continuous band of β-actin condensation and by condensations around early spermatocytes (asterisks). Bar = 25 μm.

FIGURE 3. Immunostaining for both β-actin (orange-yellow) and prosaposin (green) showing a pre-spermiation stage in which spermatids are located higher than in the inset of Fig. 1. The thinly marked blood-testis barrier (arrow) is located above a spermatogonium (G). BL, basal lamina. Bar = 25 μm.

FIGURE 4. Immunostaining for both β-actin (orange-yellow) and prosaposin (green) showing a tubular section at a pre-spermiation stage (VII or VIII, i.e., near spermiation). Spermatids are located higher than in the lower tubule in Fig.1. Note the intense actin immunostaining surrounding the spermatid heads and numerous spermatid flagella (F) that are protruding into the lumen (L). Residual bodies (RB) are also shown. Numerous small round β-actin condensations probably correspond to multivesicular endosomes. Bar = 25 μm.

FIGURE 5. Immunostaining for both β-actin (orange-yellow) and glutaredoxin (dark red) showing adjacent basal portions of two seminiferous tubules which are close to each other and to the basal lamina (BL). The immune stain for glutaredoxin is observed in red, the immune stain for β-actin in yellow. In this particular stage, β-actin staining appears partly above and below early spermatocytes (asterisks), which are then probably in leptotene-zygotene stages. Also, intense β-actin staining (probably corresponding to ectoplasmic specializations) is surrounding elongated spermatid heads (SP) located deeply into the seminiferous epithelium (but above the blood-testis barrier). BL, basal lamina. Bar = 25 μm.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unipolar and hexagonally packed. The bundles are coupled to the adjacent Sertoli membrane and to each other.

A different function for the actin bundles at ectoplasmic specializations has emerged from works aimed to elucidate the dynamic state of intercellular junctions, while many other studies have been directed towards the identification of adhesion molecules in spermatogenesis and on the turnover of the elements of cellular junctions that have provided evidence about a support role of actin in these junctions (reviewed by Cheng and Mruk, 2010). The tubulo-bulbar complexes are known to be the structures following the ectoplasmic specializations both in the apical locations and in the basal location at the blood-testis barrier (Guttman *et al*., 2004). An immunohistochemical and ultrastructural study has shown that these tubule-bulbar complexes contain the integral membrane adhesion molecules nectin 2 (in Sertoli cells) and nectin 3 (in spermatids) as well as the actin-binding protein espin (Guttman *et al.*, 2004). Furthermore, these molecules (as well as other associated with ectoplasmic specializations) are enriched at the ends of the tubule-bulbar complexes and in the vesicles associated with these endings, which are also labelled with antibodies for lysosomal and endosomal markers (Guttman *et al.*, 2004). These observations have led to the proposal that tubulo-bulbar complexes are structures that internalize intercellular junction materials, both at the apical and at the basal ectoplasmic specializations (Young *et al.*, 2009).

Tubulo-bulbar devices have not been observed before using freeze fracture replicas, which permit a detailed observation of the cell membranes involved (Fig. 6). Moreover, using immune electron microscopy we detected β-actin surrounding the tubule-bulbar complex, extending previous observations by Morales and Clermont (1993) who reported the presence of actin but not of its specific β-isoform in this Sertoli specialization.

Our results show a significant correlation between the special organization of the β-actin filament network and the stages of the spermatogenic cycle of the seminiferous epithelium. Moreover, β-actin condensation appears modulated according to the degree of germ cell maturation and differentiation. We also observed a clear correlation between the localization of the β-actin network in the spermiation process and that of the germ cells that appear to ascend from the basal to the adluminal compartment.

Related to the last mentioned topic is the long standing controversy on the existence of a third compartment in the seminiferous epithelium. As we mentioned in the Introduction, Russell (1977a, 1978) was of the opinion that late leptotene spermatocytes in stages X-XI of the cycle of the seminiferous epithelium are the cells which cross the blood-testis barrier in the rat and penetrate into a third or intermediate compartment, which is limited, both below and above, by non-permeable inter-Sertoli junctions as seen by

FIGURE 6. Low magnification) of 40 μm epoxy resin sections showing two adjacent seminiferous tubules in different spermatogenic stages. The left one exhibits spermatid heads (SP) in deep infoldings of the Sertoli cell (as in Fig. 2). Numerous dense bodies marked for β-actin are located high, close to lumen (L) and some are indicated with arrows. They may correspond to the multivesicular endosomes.The seminiferous tubule to the right shows spermatid heads located high in the epithelium and with their flagella protruding into the lumen (L). In this particular seminiferous stage numerous electron dense bodies of β-actin reaction (arrows) are located close to the seminiferous basal lamina. They also seem to correspond to secondary endosomes. Bar = 25 μm. Inset A: It shows at higher magnification β-actin labelled spermatids (SP) located close to the blood-testis barrier. Note that β-actin labelling surrounds incompletely early spermatocytes, which appear to be in the leptotene-zygotene stage. Bar = 25 μm. Inset B: It depicts two electron dense bodies containing β-actin reaction (visible as small dots). The one to the right corresponds to a multivesicular endosomes. Notice that the reaction is mainly located in the matrix, while vesicles are almost devoid of the reaction. The image to the left shows the β-actin reaction homogeneously distributed inside the body. It may correspond to secondary lysosomes. Bar = 0.5 μm.

FIGURE 7. It depicts the freeze fracture image of a tubulo-bulbar process in longitudinal view. The line that crosses the tubular area approximates the region shown in the next figure under transmission electron microscopy. Bar = 0.5 μm.

FIGURE 8. Transmission electron microscopy of a thin sections immunolabelled for β-actin showing a cross section of a tubulo-bulbar process (asterisk), which is sandwiched between the tubulo-bulbar process and smooth cistern of the endoplasmic reticulum (arrow). Bar = 0.5 μm.

electron-opaque tracers. Simultaneously, in the monkey we observed a similar phenomenon but our interpretation was that these impermeable junctions are assembled and disassembled simultaneously without forming a real compartment. (Dym and Cavicchia, 1977, 1978). Later, using intratubular administration of intercellular tracers in the rat, we arrived to a similar conclusion (Cavicchia and Sacerdote, 1988). The current results also stand against the existence of the so-called third compartment, since we never observed leptotene-zygotene spermatocytes totally surrounded by β-actin. Thus, it appears that the dynamic changes of β-actin polymerization/depolymerization cycle contribute to a fast ascent of spermatocytes avoiding the formation of the postulated intermediate compartment.

Numerous multivesicular bodies were especially located in the upper regions of the seminiferous epithelium (for example, see Fig. 3 and Fig 5 inset B). Early electron microscopy studies (Trump *et al*., 1965) indicated the existence of multivesicular bodies, suggesting that they were related somehow to lysosomes and that contained characteristic accumulations of small vesicles in their lumen or matrix.

We also observed β-actin in the matrix of multivesicular bodies and in secondary lysosomes near the basal lamina of the seminiferous epithelium (Figs. 3 and 5, and Fig. 5, inset B). In MVB, it could be related to the fact that tubulo-bulbar complexes appear to internalize apical ectoplasmic specializations once that spermatids are released into the lumen of the seminiferous epithelium (Morales and Clermont, 1993; Young *et al.*, 2009). Secondary lysosomes (Fig. 5, right tubule) containing β-actin could have a similar function. In relation to our results, we remark that Young *et al*. (2009) postulate that tubulobulbar complexes are intercellular podosome-like structures that internalize intact intercellular junctions during epithelial remodelling events in the rat testis. Thus, our observations support the claim that tubulo bulbar complexes are actin-related, double-membrane projections that form at intercellular junctions in the seminiferous epithelium, and that intact junctions are internalized during sperm release and during spermatocyte translocation through basal junctional complexes between neighboring Sertoli cells.

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