Apocrine secretion in the mouse epididymis and rat and human vas deferens

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To my family…

Remember that "In life and in sport, whatever the play, always give it your best shot."

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ABSTRACT

Over the years, epithelial cells of the male reproductive tract have been suggested to be involved in apocrine secretion. However, this method of secretion has not been fully accepted, nor is it well understood. In the present study, apocrine secretion was examined in the mouse epididymis and rat and human vas deferens. Throughout these regions of the duct, the apex of the epithelial principal cells often shows protrusions extending towards the lumen of the duct, referred to as apical blebs (ABs). Of different shapes and sizes, ABs appear to form at the apex of principal cells, and morphological and immunocytochemical evidence suggests that ABs detach from the cell surface whereupon they eventually undergo fragmentation in the lumen. ABs contain selective organelles suggesting a segregation of their contents. Numerous polysomes suggest the synthesis of nonglycosylated proteins which upon the fragmentation of ABs in the lumen could act directly or indirectly on sperm. The presence of spherical vesicles of various sizes (20-200nm) within ABs as well as within the lumen of the duct supports AB fragmentation in the lumen and may play a role in modifying the sperm surface. Taken together the data provide strong evidence for apocrine secretion that could play important roles in relation to sperm maturation, viability and protection.

Resumé

Plusieurs études suggèrent que les cellules épithéliales des canaux reproductifs masculins secrètent leurs protéines par sécrétion apocrine. Ce processus de sécrétion est cependant controversé. Dans cette étude, la sécrétion apocrine est examinée dans l'épididyme de la souris et les canaux déférents du rat et de l'humain. Dans ces régions du canal, l'apex des cellules principales démontre des protrusions qui s'étendent dans le canal. Nous appelons ces protrusions des blebs apicaux (BA). De différentes formes et grosseurs, les BA se forment sur la partie apicale des cellules principales et certaines études morphologiques et immunohistochimiques suggèrent qu'une fois détacher de la surface les BA se fragmentent dans le lumen. Les BA contiennent des organelles sélectives qui suggèrent une ségrégation de leurs contenues. De nombreux polysomes suggèrent la synthèse de protéines nonglycolisées qui après la fragmentation des BA dans le lumen pourraient avoir des effets direct et indirect sur les spermatozoïdes. La présence de vésicules sphériques de différentes grosseurs (20-200nm) dans les BA ainsi que dans le lumen du canal support la fragmentation des BAs dans le lumen et pourrais avoir un rôle pour ce qui est de la modification de la surface des spermatozoïdes. En bref, les résultats suggèrent un mécanisme de sécrétion apocrine qui pourrait avoir un rôle important pour ce qui est de la maturation, la viabilité et la protection des spermatozoïdes.

Chapter 1:

Literature Review

Male Reproductive System

The male reproductive system consists of a number of individual organs acting together to produce functional spermatozoa, and to deliver these spermatozoa to the female reproductive tract. It consists of the testes, epididymis, vas deferens, and the accessory glands. The testes produce haploid germ cells in the seminiferous tubules resulting in immature spermatozoa which then undergo further maturation in the epididymis. The vas deferens carries the spermatozoa from the epididymis to the ampulla, where they mix with seminal vesicle and prostate gland secretions, as they pass through the ejaculatory duct. Transit continues through the prostatic urethra, membranous urethra, and finally the sperm exit via the penile urethra.

Testis

Structure

The testis in all mammals are paired encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissue. The testis develop abdominally and must migrate through the abdominal wall into the scrotum to ensure fertility (Gier and Marion, 1970).

The interstitial tissue fills up the spaces between the seminiferous tubules. It contains all the blood and lymph vessels and nerves of the testicular parenchyma (Fawcett et al., 1969, 1973). Also located in the interstitium are mast cells and macrophages, and the hormone-secreting Leydig cells (Hermo and Lalli, 1978; Nistal et al., 1984; Christensen et al., 1985). The seminferous tubules are lined by a seminferous epithelium. This is comprised of the germ cells and the supportive cells, the Sertoli cells.

The germ cells include spermatogonia, primary and secondary spermatocytes, round and elongating spermatids, and the spermatozoa. The Sertoli cells contact the basement membrane and extend to the lumen. Tight junctions are formed between the Sertoli cells to create the blood-testis barrier which creates two different compartments in the tubule: the basal and adlumenal compartments (Bardin et al., 1988; Morales and Clermont, 1993).

Function

The testis is the organ which generates the haploid germ cell, the spermatozoa. The process by which stem cells differentiate and develop into spermatids is called spermatogenesis. The round spermatids must then undergo spermiogenesis to give rise to mature elongated spermatids, and finally spermatozoa (de Kretser and Kerr, 1988). However, the spermatozoa produced by the testis are immature since they are non-motile and incapable of fertilizing an oocyte. Thus, further transit through the epididymis is necessary to produce mature viable sperm (Orgebin-Crist, 1987; Robaire and Hermo, 1988; Cooper, 1992; Hermo et al. 1994; Turner, 1995).

The testis are also the site of androgen production. Leydig cells have receptors for leuteinizing hormone and convert cholesterol to testosterone when stimulated. The testosterone produced by the Leydig cells acts both locally, on the seminiferous epithelium, and systemically (Dufau et al., 1983).

Efferent Ducts

Structure

The rete testicular fluid (RTF) and sperm are drained from the rete testis by eight to twenty convoluted, fat embedded tubules which pierce the tunica albuginea of the

testis. Closer to the epididymis, the tubules become highly tortuous before anastomosing to form a single duct which ultimately becomes the initial segment of the epididymis. The efferent ducts are surrounded by myoepithelial cells and are lined by a simple columnar epithelium composed of two cell types: ciliated and non-ciliated cells (Hermo and Morales, 1984; Ilio and Hess, 1994).

Function

The efferent ducts modify RTF and spermatozoa from the rete testis and transports them to the initial segment of the epididymis. Modification of luminal contents is achieved through water reabsorption, internalization of particulate matter and secretion of substances into the lumen. Approximately 90% of fluid secreted by the testis is resorbed by the active transport of salts followed by passive water diffusion (Hamilton, 1975; Ilio and Hess, 1994). The regulation of luminal contents in the efferent ducts is the first step to creating a specialized environment in the epididymal lumen in which the sperm will undergo maturation (Robaire and Hermo, 1988, Cooper, 1992; Hermo et al. 1994; Turner, 1995).

Epididymis

Introduction

After spermatogenesis in the seminiferous tubules, sperm enter the epididymis via the efferent ducts. When spermatozoa leave the testis, they are immature, immotile, and not capable of fertilizing an oocyte. The epididymis plays an important role in transforming sperm into fully mature cells that are motile and can recognize and fertilize eggs (Orgebin-Crist et al., 1975; Turner et al, 1995; Jones, 1999). In most species, this

status is only achieved by the time sperm enter the proximal cauda epididymis (Orgebin-Crist,1987; Robaire and Hermo, 1988; Cooper, 1992; Hermo et al. 1994; Turner, 1995).

Anatomy of the duct

The epididymis is a highly coiled duct that connects the efferent ducts to the vas deferens. The length of the epididymis varies according to species from approximately 6m in the rat and human to more than 50m in large breeding animals (Bedford, 1994).

The epididymis is divided into five regions depending on the species studied. The first region is the initial segment and is continuous with the efferent ducts. Sperm is then transported through the intermediate zone, caput, corpus, and finally the cauda where they are stored for a short period of time. These subdivisions are based on structural and functional differences. There are several different cell types that line the epididymis, and while some are present throughout the duct, there are others that are region specific (Hamilton, 1975; Robaire and Hermo, 1988; Hermo et al., 1994).

Histology of the Duct

The duct is circumscribed externally by two to three layers of myoepithelial cells and lined internally by a pseudostratified epithelium. The main cell type found throughout the epididymis of all mammals is the principal cell. It is present along the entire length of the duct, but shows structural differences in each region (Hamilton, 1975; Robaire and Hermo, 1988). Clear cells are found only in the caput, corpus, and cauda regions, while narrow cells appear only in the initial segment and intermediate zone

(Robaire and Hermo, 1988; Adamali et al., 1996). Basal and halo cells are found along the entire length of the duct (Hamilton, 1975; Robaire and Hermo, 1988).

Principal Cell

Depending on the region examined, the relative contribution of principal cells to the total epithelial cell population of the epididymis ranges from 65-80%, with the numbers declining from caput to cauda (Trasler et al., 1988). The height of the principal cells also decreases as one moves distally along the duct such that the luminal diameter of the caput epididymis is greatly increased (Robaire and Hermo, 1988).

The principal cell is an active secretory cell with an extensive endoplasmic reticulum and a well-developed Golgi apparatus. To date, essentially all proteins secreted into the epididymal lumen have been shown to be synthesized by principal cells (Flickinger 1979, 1981).

The principal cell also demonstrates endocytic activity with many coated pits, endosomes, multivesicular bodies, and lysosomes. Lysosomes differ structurally along the epididymis and often show region specific expression of different lysosomal enzymes such as B-hexosaminidase A, cathepsins A, B and D and sulfated glycoprotein-1 (SGP-1) (Hermo et al., 1992; Igdoura et al. 1995; Hermo et al., 1997; Luedtke et al., 1999).

Narrow Cells

Narrow cells are narrower than principal cells and reach the basement membrane via a thin process of cytoplasm. They are present only in the initial segment and intermediate zone of the rat and mouse epididymis (Abou-Haila et al., 1984; Adamali and Hermo, 1996). Many cup-shaped vesicles involved in endocytosis are found at the apex

of the cell. They function in secreting H+ ions into the lumen in order to create an acidic environment suggested to be important in inhibiting sperm motility (Hermo et al., 2000).

Apical Cells

Apical cells do not reach the basement membrane and can be characterized by an apically located spherical nucleus. They are found primarily in the initial segment and intermediate zone (Sun and Flickinger, 1980; Adamali and Hermo, 1996). It is known that they are involved in endocytosis and contain many proteolytic enzymes (Adamali et al., 1999), however many of their specific functions remain to be elucidated.

Clear Cells

Clear cells are present only in the caput, corpus, and cauda, with their number increasing from caput to cauda. They are found in many species including humans (Cooper, 1986; Robaire and Hermo, 1988). They are very active in resorption and display a prominent endocytic apparatus. Clear cells play a role in taking up the contents of cytoplasmic droplets released by spermatozoa as they traverse the duct (Hermo et al., 1988).

Basal Cells

Basal cells are found in all species studied to date including humans (Hamilton, 1975; Robaire and Hermo, 1988; Hermo et al., 1994; Yeung et al., 1994). They appear along the entire male excurrent duct system with the exception of the efferent ducts. These cells are hemispherical in shape and have a large area of contact with the basement membrane. They do not reach the lumen and are found between adjacent principal cells at the base of the epithelium (Robaire and Hermo, 1988). Basal cells are not stem cells as

they do not divide in adult animals (Clermont and Flannery, 1970). The presence of coated and uncoated pits along the cell surface together with multivesicular bodies and lysosomes suggests that these cells are capable of endocytosing factors from the blood or principal cells, although the functional significance of endocytosis has yet to be determined.

Halo Cells

Halo cells are small with a thin rim of cytoplasm and are found throughout the epididymis. They have been described as lymphocytes or monocytes. Through immunolabeling studies, it is now known that halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes, but not B lymphocytes (Flickinger et al., 1997; Serre and Robaire, 1999). They are thus believed to be, under normal conditions, the primary immune cell in the epididymis.

Functions of the Epithelium

The epididymis performs a variety of functions in order to produce viable mature sperm capable of fertilizing an oocyte. The epithelium plays a key role in creating a fluid microenvironment appropriate for sperm maturation. It plays a role in transporting the spermatozoa from the testis to a point from which they can be ejaculated, and provides protection from harmful agents along the way. The distal cauda plays a role in storing the mature viable sperm in a viable, but quiescent state for a variable period (Bedford, 1994).

Sperm Transport

Sperm transit time has been evaluated through the use of radioactively labeled spermatozoa (Monesi, 1962; Rowley et al., 1970). Although there is a large range in sperm production rate in different species, there is a remarkable consistency in the amount of time it takes for sperm to traverse the epididymis (Amann, 1981). Transit time is approximately 10 days in different mammals, although there is greater variability in humans (Rowley et al., 1970; Robb et al., 1978). Sperm movement through the epididymis requires an average of approximately 12 days, though some spermatozoa require a longer time, while others are much more rapid, taking from 1 to 4 days (Rowley et al., 1970).

The mechanisms of driving the sperm through the lumen of the epididymis include hydrostatic pressure, regular duct wall muscular contractions, and the action of cilia (Robaire and Hermo, 1988).

Sperm Storage

The cauda is the major site for storage of sperm in the mammalian excurrent duct system. Though transit time is approximately 10 days, sperm can be stored for longer than 30 days (Orgebin-Crist et al, 1975). A number of studies on mammalian species have shown that 50 to 80% of sperm present in the excurrent ducts are found in the cauda and approximately 50% of these sperm are available for ejaculation (Amann, 1981). Although many differences have been found between the luminal composition of the

cauda and other regions of the epididymis, the special conditions that allow for the storage of sperm in a quiescent state remain to be discovered, with the exception of immobilin in the rat which acts as a jelly-like substance to inhibit sperm movement (Hermo et al., 1992).

Sperm Protection

Epithelial cells of the epididymis also play a role in protecting sperm from damage by reactive oxygen species and other harmful agents (Veri et al., 1993; Hinton, 1996). Two major mechanisms of protection have been proposed. One involves the enzymes involved in regulating glutathione levels and its conjugation, thus controlling the levels of reactive oxygen species. The other mechanism involves the immune system.

Glutathione S-Transferases (GSTs) are a family of isozymes that catalyze the conjugation of glutathione with various electrophilic compounds. This protects DNA, RNA, and proteins from electrophilic attack through the formation of a less toxic product (Ketterer et al., 1990; Daniel, 1993). GSTs are soluble dimeric proteins that are grouped into 5 classes: alpha, mu, pi, theta, sigma (Buetler and Eaton, 1992). There is a high activity of GSTs along the length of the epidiymis, with the various forms being differentially distributed (Hales et al., 1989; Robaire and Hales, 1982). There is a need for many forms of GSTs as there is a wide variety of harmful agents that cell may encounter.

Another family of enzymes that may play an important role in protecting sperm from oxidative stress are the Gamma Glutamyl Transpetidases (GGTs) (Hinton et al., 1998). They play a major role in the regulation of glutathione degradation by cleaving

the gamma-glutamyl amide bonds. They are ubiquitous membrane-bound enzymes found on the cell surface (Chikhi et al., 1999; Hannigam, 1998).

In addition, tight junctions between principal cells (Suzuki and Nagano, 1978; Hoffer and Hinton, 1984) of the epithelium form a blood-epididymis barrier that allows the formation of a luminal environment distinct from the blood. Sperm are immunogenic and are recognized by the body as foreign, and therefore, the blood-epididymis barrier plays a role in protecting sperm from the immune system. Also, as mentioned, halo cells have been described as lymphocytes and monocytes (Flickinger et al., 1997; Serre and Robaire, 1999) and function in protecting the epididymal epithelium from pathogens.

Sperm Maturation

The key event for spermatozoal maturation is exposure to the luminal environment of the epididymis (Orgebin-Crist 1967, 1969; Bedford, 1967). The blood-epididymis barrier creates a unique luminal environment in the epididymis, with the concentration of organic and inorganic substances very different from that of the blood (Turner et al., 1984). Sperm from the proximal region of the human epididymis do not seem to be able to fertilize, and only develop this ability as they enter the proximal cauda region (Orgebin-Crist, 1987; Robaire and Hermo, 1988; Cooper, 1992; Hermo et al., 1994a; Turner, 1995). It is through the processes of secretion, endocytosis and fluid reabsorption that the distinct luminal environment is created.

Endocytosis

Endocytosis involves the receptor mediated uptake of specific substances and their subsequent degradation in lysosomes or recycling back to the cell surface (Robaire and Hermo, 1988; Hermo et al., 1994). Several substances have been shown to be endocytosed by principal cells such as androgen binding protein, α-2 macroglobulin, and transferrin (Djakiew et al., 1985, 1986; Gerard et al., 1988; Rankin et al., 1992; Hermo et al., 1998). Clear cells also demonstrate endocytosis of immobilin and other mouse epididymal proteins (Hermo et al., 1992; Vierula et al., 1995).

The endocytic receptor low density lipoprotein-related protein-2 (LRP-2) has been localized to the apical surface of principal cells of different epididymal regions (Zheng et al., 1994; Morales et al., 1996; Hermo et al., 1999). LRP-2 is found on many epithelial cells exposed to fluid filled spaces (Zheng et al., 1994). Studies have shown that clusterin binds with high affinity to LRP-2 and is endocytosed along the entire duct (Kounnas et al., 1995; Hermo, 1995; Hermo et al., 2000).

Endocytosed substances may be degraded in lysosomes by a variety of lysosomal enzymes such as cathepsins A, B and D, sulfated glycoprotein-1 (SGP-1), β-hexosaminidase A. These enzymes often show a cell-specific and region-specific distribution within the epididymal epithelium. This suggests that substances endocytosed from the lumen of the epididymis are degraded in lysosomes of different cell types in specific regions in order to provide a suitable environment for sperm maturation (Hermo et al., 1992; Igdoura et al., 1995; Hermo et al., 1997).

Fluid Reabsorption

Approximately 90% of fluid entering the efferent ducts from the rete testis is absorbed by the epithelium of the efferent ducts and proximal region of the epididymis (Levine and Marsh, 1971). Osmotic forces drive water from the lumen to the interstitial spaces and into vascular channels. The passive movement of sodium across the apical surface of the epithelium followed by its active transport across the basolateral membrane establishes the osmotic gradient (Wong, 1990).

However, the speed of this process is not fast enough to account for the rapid water movement required for an active regulation of water homeostasis. Recently, a novel class of transmembrane pore proteins, aquaporins, have been discovered and are thought to facilitate the osmotically driven transport of water. These water channels have a low activation energy and so far ten aquaporins have been cloned, with aquaporin-1 being the most ubiquitous (Agre et al., 1993; Nielsen et al., 1993; Wintour, 1997). Studies have shown that there is cellular, subcellular and region specific distribution of aquaporins along the epididymis (Badran et al., 2000).

Secretion

The epididymis is actively involved in the secretion of a wide range of ions, small organic molecules, proteins and glycoproteins. The principal cell is the main secretory cell of the epithelium. Many secreted proteins interact with the sperm surface and play a role in sperm maturation (Orgebin-Crist and Jahct, 1978) and sperm motility (Turner and Giles, 1982; Kirchoff, 1999).

Merocrine Secretion

The precise means by which proteins are secreted by principal cells into the lumen of the epididymis has been the subject of much controversy. The main focus of the scientific community has been to study proteins secreted in a merocrine fashion using routine morphology and radioactive tracers. In this model, proteins are formed in the rough endoplasmic reticulum (rER), glycosylated in the Golgi apparatus, and packaged into large secretory granules. These membrane bound granules then fuse with the apical plasma membrane and thereby release their contents into the lumen of the duct (Flickinger, 1981; Robaire and Hermo, 1988; Hermo et al., 1994). The large secretory granules are smoothed surfaced vesicles approximately 150-300nm in diameter (Hermo et al., 1994). There are many proteins that are differentially secreted by principal cells along the length of the duct (Orgebin-Crist, 1996).

Clusterin (SGP-2) is one example of a protein secreted in a merocrine fashion. It is secreted by epididymal principal cells and concentrations are found highest in the caput epididymis (Sylvester et al., 1984, 1989). Clusterin has been localized to ER, Golgi, secretory vesicles, the lumen, and at the sperm surface, suggesting a role in sperm maturation (Hermo et al., 1991).

Another protein secreted in a merocrine manner is immoblin (Hermo et al., 1992). It is secreted by the principal cells of the initial segment, intermediate zone and caput regions, but not by those of the corpus and cauda. In the lumen, immobilin is closely associated with sperm an appears to prevent sperm from movement (Usselman and Cone, 1983).

Apocrine Secretion

Over the years, apocrine secretion has received increased attention as an additional means to release proteins into the lumen, and has mainly been studied in the prostate, seminal vesicle, and coagulating gland (Wilhelm et al, 1998; Groos et al., 1998; Aumuller et al., 1999). Unlike merocrine secretion, apocrine secretion does not involve the Golgi apparatus and formation of membrane bound secretory granules. It involves an expansion and protrusion of a portion of the apical cytoplasm of epithelial cells into the lumen and the eventual detachment from the apical cell surface and liberation into the lumen. These protrusions are referred to as apical blebs. It has been suggested that proteins released into the lumen by this manner may also play a role in relation to sperm, but the precise mechanism is still unknown (see detailed section on apocrine secretion below).

Regulation of the epididymis

There are several hormonal factors that act to maintain the function of the epididymis. Vitamins A and D, estradiol, aldosterone, and androgens regulate the epithelial cells (Robaire and Hermo, 1988). Androgens play an important role in regulating the growth and development of the epididymis as well as the microenvironment produced in the lumen of the duct. The epididymis receives androgens both locally from testicular fluid and from the circulation (Orgebin-Crist, 1996).

The most important androgen is dihydrotestosterone. It is a reduced form of testosterone and is produced by 5-a-reductase, and enzyme found only in principal cells (Robaire and Hermo, 1988). Dihydrotestosterone is necessary for principal cells to

synthesize glycoproteins and enzymes, for ion transport. Studies with 5-α-reductase inhibitors have shown a decrease in the number of motile sperm and in the percentage of fertilized oocytes (Robaire and Viger, 1995).

Vas Deferens

Introduction

Despite the vast number of vasectomies performed each year, little is known about the structure and functions of the epithelial cells that line the vas deferens. It has been shown that humans do not have a bulbous cauda epididymis as in the rat, and that sperm storage occurs in the proximal vas deferens (Turner, 1991). There is evidence that the vas also plays a role in sperm maturation (Bergeson et al., 1994) and protection (Hinton et al., 1996). It is thus becoming increasingly apparent that the complexity of the different epithelial cells and regional differences in their morphology are important in maintaining the sperm in a viable and fertile state prior to ejaculation.

Anatomy of the Duct

The vas deferens begins where the coiled cauda epididymis straightens, turns towards the inguinal canal, and ends as the ejaculatory duct in the prostate. It is an epithelial lined muscular tube. In the human, it is approximately 35 cm in length, while in the rat it is approximately 6 cm. The vas deferens is divided into four regions based on the morphology and blood supply: the proximal, middle, distal and terminal regions (Flicklinger, 1973; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979, Andonian and Hermo, 1999c). Each region is histologically unique and is situated in a different part of the body.

Histology of the Duct

The epithelium is pseudostratified and is made up of principal, clear, narrow, and basal cells with differential distribution of each cell type along the length of the duct. Principal and basal cells are found in all regions, while clear and narrow cells are mainly found in the proximal regions. The epithelium rests on a thin basement membrane and is surrounded by layers of smooth muscle cells (Hamilton, 1975; Robaire and Hermo, 1988).

Principal cells have a uniform layer of tall microvilli apically, while dilated intercellular spaces were noted basally between adjacent principal cells and overlying basal cells (Andonian and Hermo, 1999a). Principal cells and their microvilli show a dramatic increase in size from the proximal to distal vas, and the nuclei shift from a basal to a mid region in the cell (Hamilton and Cooper, 1978; Kennedy and Heidger, 1979; Andonian and Hermo, 1999a). The lumen narrows and becomes convoluted, and there is a decrease in the content of spermatozoa. There is also a marked increase in the thickness of the smooth muscle layer surrounding the epithelium (Prins and Zaneveld, 1979).

Functions of the Epithelium

The vas deferens is often considered as simply a tube whereby sperm exit at the time of ejaculation. However, it is becoming increasingly apparent that it is actively involved in maintaining sperm in a mature state and that the epithelium plays a role in creating a unique luminal environment. Many of the functions are similar to those in the epididymis and will only be briefly reiterated here.

Endocytosis

The principal cells of the vas deferens show evidence of endocytosis. While specific proteins have yet to be characterized, immunocytochemical localization of several lysosomal enzymes such as SGP-1, cathepsins A, B, D have been reported in the vas (Hermo and de Melo, 1987; Andonian and Hermo, 1999c).

Water Transport

The vas deferens also plays a role in water transport. It has been reported recently that in the epithelium of the middle and distal vas deferens, large dilated intercellular spaces appear between adjacent principal cells and over basal cells which are suggestive of water transport (Andonian and Hermo, 1999a).

Aquaporins, the transmembrane pore proteins shown to be involved in water transport, have also been localized to the vas deferens. AQP-1 has been localized to the endothelium lining the large vascular channels of the lamina propria, AQP-6 and AQP-9 have also been found in the vas deferens (Andonian et al., 2001). Since the size of the lumen of the distal vas deferens decreases dramatically as compared to the proximal vas, the removal of water may function in concentrating sperm and provide more effective interactions with the secretory products of the accessory glands at the time of ejaculation (Andonian and Hermo, 1999a).

Sperm Protection

The cauda epididymis and proximal region of the vas deferens are major sites for sperm storage in many species, including humans (Turner, 1995). Therefore, the epithelium must play an important role in protecting sperm from harmful agents. These regions have a relatively low temperature which facilitates oxygen availability to the

sperm (Djakiew and Cardullo, 1986). However, this suggests the presence of reactive oxygen species which can potentially damage the sperm membranes through lipid peroxidation (Nonogaki et al., 1992).

Glutathione S-transferases (GSTs), a family of isoenzymes involved in cellular detoxification in the epididymis, are also expressed in principal, narrow and basal cells in a region specific manner in the vas deferens. This suggests that these cells encounter a changing environment to potentially damaging free radicals, and the absence of expression of a given GST by one cell type in any given region is usually compensated for by its expression in another cell type (Andonian and Hermo, 1999b).

Luminal Acidification

A distinct population of epithelial cells called the mitochondria-rich or narrow cells are found in the proximal vas deferens. They contain numerous small vesicles apically and lack a well-differentiated basal region. They have been noted to express high levels of a vacuolar proton-pumping ATPase (H+V-ATPase) on their luminal plasma membranes as well as in intracellular vesicles. These cells also contain cytoplasmic carbonic anhydrase II and they most likely play a role in acidifying the lumen. This low intraluminal pH helps to maintain the sperm immotile during their storage in the vas deferens (Brown and Breton, 2000).

Steroid Synthesis

The principal cells of the distal vas deferens have an abundance of smooth endoplasmic reticulum (sER) infranuclearly and basally (Niemi, 1965; Flickinger, 1973; Kennedy and Heidger, 1979; Hamilton, 1975). The ER cisternae consist of flattened parallel sheets that are layered on top of one another and at times form large concentric

whorls (Andonian and Hermo, 1999a). It is well recognized that the principal cells are involved in steroid metabolism and this occurs in the extensive whorls of sER present in these cells (Hamilton et al., 1969). It has been suggested that steroids are metabolized by the sperm and the vas would act to renew the steroid pool that would be depleted by sperm metabolism (Hamilton and Fawcett, 1970, Hamilton, 1971).

Merocrine Secretion

Principal cells in the vas deferens have also been recognized as active secretory cells. They have a well-developed rough endoplasmic reticulum (rER), Golgi apparatus, and secretory vesicles which is suggestive of the classical merocrine type secretion described in the epididymis. They are involved in the synthesis and secretion of various substances, including glycoproteins (Niemi, 1965; Friend and Farquhar, 1967; Flickinger, 1973; Hamilton, 1975; Robaire and Hermo, 1988).

To date, few studies have been done on the proteins secreted by the vas deferens. Recently, it was shown that the principal cells were immunoreactive for clusterin (SGP-2) and it was concluded that clusterin was being secreted along the entire vas deferens (Andonian and Hermo, 1999c). In the epididymis, clusterin has been suggested to play a role in sperm maturation and protection (Law and Griswold, 1994; Hermo et al., 1991), and may also perform the same functions in the vas deferens. Thus, while it is clear that the vas deferens is involved in merocrine secretion, little is known about the specific proteins secreted and their specific role in sperm maturation and protection.

Apocrine Secretion

Apocrine secretion has been demonstrated in the vas deferens of various species such as the rat, mouse, bull, monkey, and human (Niemi, 1965; Flickinger, 1973; Ramos,

1979; Riva et al., 1982 Agrawal and Vanha-Pettula, 1988a; Renneberg et al., 1995; Andonian and Hermo, 1999a; Andonian et al., 2000). As in the epididymis and other tissues of the male reproductive tract, protrusions of apical cytoplasm, called apical blebs, eventually detach and fragment in the lumen (Aumuller et al. 1997; 1999). This will be described in detail in the section below.

Apocrine Secretion

Apocrine secretion has been noted since the beginning of the past century as a method of secretion in the mammary gland and axillary sweat glands. The distinction between merocrine and apocrine secretion was inaugurated by a German histologist, Paul Schiefferdecker in 1917. The release of the apical pole of the cell during secretion was the essential feature of Shiefferdecker's description of apocrine secretion in human skin glands. Among these, the mammary glands and the axillary sweat glands were the most prominent (Aumuller et al, 1999).

Despite these early observations, apocrine secretion and apical blebs were neglected from analysis as they were initially considered to be fixation artifacts in the early years of electron microscopy. With the advent of vascular perfusion fixation of tissues through the heart in the 1970s, studies were slanted towards merocrine secretion and its role in sperm maturation, and apocrine secretion was largely ignored.

More recently apocrine secretion has been reported in a variety of male reproductive tissues of different species, but mainly by a few centralized groups of investigators (Aumuller et al.,1999). Apocrine secretion involves the formation of a bleb-like protrusion of cytoplasm at the apical pole of the cell referred to as an apical bleb. The latter, with time, appear to detach from the apical cell surface and be liberated

into the lumen of the duct, and in this way eventually release their contents. Over the years, apical blebs have been described in various species, namely the rabbit prostate gland (Nicander et al., 1974), turkey efferent ducts, epididymis, and vas deferens (Hess et al., 1976) rat prostate (Thompson et al., 1978), rat and seal submandibular gland (Messelt, 1980), human ampulla and ductus differentia (Riva et al., 1982), squirrel ductuli efferentes (Pudney and Fawcett, 1984), bull epididymis and vas deferens (Agrawal and Vanha-Pertulla, 1988a), cat epididymis (Morales and Cavicchia, 1991), serow infraorbital gland (Atoji et al., 1993), dog efferent ducts and epididymis (Ilio and Hess, 1994), bull seminal vesicles (Renneberg et al., 1995), mouse vas deferens (Manin et al., 1995), rat coagulating gland (Wilhelm at al., 1997), human epididymis (Aumuller et al., 1997, human vas deferens (Hermo and Andonian, 1999c), chimpanzee epididymis (Smithwick and Young, 1997), rat vas deferens (Andonian and Hermo, 1999b), and human prostate (Renneberg et al., 2001).

Studies of the cat epididymis have demonstrated apocrine secretion (Morales and Cavicchia, 1990). Ultrastructural studies reveal extrusion of the apical cytoplasm followed by pinching off and liberation of membrane-bound portions into the lumen. This process was present in all segments of the epididymis (caput, corpus, cauda) and there seems to be no regional ultrastructural differences. It was observed, however, that while some regions of the duct are engaged in secretion, others are resting. Another important observation is that the apical cytoplasm was clearly divided into two regions. The supranuclear region contained a tubular endoplasmic reticulum, mitochondria, lysosomes, and scanty ribosomes. However, the more distal region, which was destined to be extruded contained large vesicles and membranes, but no other organelles. A clear

separation between these two regions remained during the process of pinching-off (Morales and Cavicchia, 1990).

Apocrine secretion has also been studied in the seminal vesicle and ampulla of the vas deferens of the bull (Renneberg et al., 1995). With an antiserum developed against veisculosomes, small particles found in the bovine seminal vesicle fluid, immunocytochemical staining of these tissues revealed a strong reaction exclusively at the apical portion of secretory cells of the seminal vesicle and ampulla of the vas deferens, including apical blebs. The antiserum recognized both vesiculosomes in the lumen and plasma membrane proteins of the apical blebs of seminal vesicle epithelium. This supports the concept that the 'vesiculosomes' represent particles formed from plasma membranes surrounding apical blebs which are released in the lumen via apocrine secretion (Renneberg et al. 1995).

Further evidence for apocrine secretion has been shown in the mouse vas deferens (Manin et al., 1995). It has been demonstrated that a mouse vas deferens protein (MVDP) was detected in the cytoplasm of apical blebs of principal cells as well as in the lumen. MVDP is a major androgen-dependent protein of deferential fluid and belongs to the superfamily of aldo-keto reductases. It is specifically expressed in the epithelium of the mouse vas deferens. It is proposed that MVDP may be involved in the eventual production of fructose, the preferred substrate for glycolysis in sperm, in degradation of steroids, and may intervene as a detoxifying system, important in the survival of sperm in the vas deferens. MVDP lacks the ER signal sequence that normally triggers the translocation of secretory proteins across the ER membrane (Manin et al, 1995). Immunolocalization of MVDP revealed that it was distributed in the whole cytoplasm

and abundant in apical blebs and luminal fluid. It was never detected in the lumen of the ER, Golgi apparatus or secretory vesicles. It was also associated with the plasma membrane of spermatozoa (Manin et al, 1995).

The release of secretory material through apical protrusions has been convincingly shown in the rat coagulating gland (Wilhelm et al., 1997). It was shown that carbonic anhydrase (CAH) II was secreted in an apocrine fashion. Carbonic anhydrases are a family of enzymes that catalyze the reversible hydration of carbon dioxide and thereby regulate the electrolyte and acid-base balance in various organs. To date, six major isoforms of CAH have been described in mammals. Prior to studies on the rat coagulating gland, CAH IV, found in salivary glands, was described as the only secreted isoform. CAH II was believed to be a nonsecretory form found in the cytoplasm. Recent studies (Wilhelm et al., 1997) have isolated CAH II in large quantities from rat coagulating gland secretions suggesting that this protein represents a secretory protein rather than a cytoplasmic protein. Immunohistochemical studies showed the apical cell pole and apical protrusions of the epithelial cells to be intensely stained with an anti-CAH II antibody, whereas no staining was visible in the classical merocrine route. These ultrastructural data provide clear evidence that CAH II does not leave the coagulating gland by the classical pathway described for the salivary isoform CAH IV, but rather in an apocrine manner (Wilhelm et al., 1997).

It has also been suggested that apocrine secretion is necessary for the release of the protein transglutaminase from the rat coagulating gland (Groos et al., 1998). This enzyme is responsible for the formation of the intravaginal coagulation plug directly after copulation. Transglutaminase was compared with another secreted protein, named 115 K

protein (Wilhelm et al., 1997) in terms of rate of secretion and the route used. It was found that although they are simultaneously synthesized, they are transported intracellularly in an asynchronous manner and released by two different mechanisms. While 115 K protein followed a slow classical route of biosynthesis in the rough endoplasmic reticulum, intracellular transport through the Golgi, and merocrine release, transglutaminase was synthesized outside the endoplasmic reticulum and was released more rapidly by apocrine secretion. Immunohistochemical staining using an anti-transglutaminase antibody resulted in dense labeling of the cytoplasm of secretory cells and their apical blebs, whereas the cisternae of the rough endoplasmic reticulum, Golgi, and secretory granules were unlabelled (Groos et al., 1998).

There are some common features that are specific for the apocrine-synthesized proteins, transglutaminase and carbonic anhydrase II, studied in the rat coagulating gland (Wilhelm et al., 1997; Groos et al., 1998). Their biosynthesis and post-translational modification take place in the cytoplasm. The proteins lack an ER signal peptide, and thus transport proceeds without participation of the ER, Golgi apparatus, and secretory granules (Aumuller et al.,1999). Furthermore, blood serum derived transsudated albumin entering the secretory cells function as a carrier of the apocrine released proteins. During passage through the epithelial cells, it selectively binds to proteins destined for apocrine secretion by directing them into apical blebs, and simultaneously effectively excludes the resident cytoplasmic proteins (Wilhelm et al., 1999).

There are other important features of apical blebbing that have been discovered through studies on the rat coagulating gland. First, the participation of several cytoskeletal proteins in the release mechanism of apical blebs has been studied. The

blebs demonstrated a strong and homogenous immunofluorescence for myosin and gelsolin and a variable intensity for β-actin. The distribution of actin immunoreactivity is peculiar in that in smaller blebs, mostly closely located above the apical plasma membrane and sometimes still in contact with it through a nipple-like stalk, the strongest reactivity is seen. This pattern points to the significant role of terminal web-actin in the release process (Aumuller et al., 1999).

Furthermore, it has been noted that the biosynthesis of the apocrine synthesized proteins in the rat coagulating gland is hormone-dependent as was the number and form of these blebs (Zhao et al., 1993; Holterhus et al., 1993). As the coagulating gland is an androgen target organ, regular secretion of its glandular cells requires the presence of adequate levels of testosterone (Groos et al., 1998). Androgen deprivation is rapidly followed by an almost complete loss of transglutaminase immunoreactivity in secretory cells and all blebs vanish (Steinhoff et al., 1994). Contrary to this, estrogen treated animals still show some blebs, although their outline becomes irregular and the distribution of actin achieves a more generalized pattern (Aumuller et al., 1999).

Finally, apical blebs have been noted in the human reproductive tract and apocrine secreted proteins also play a role in the regulation of human sperm function (Aumuller et al., 1997). Through immunolocalization studies, three different proteins have been identified that are secreted in an apocrine manner from the epididymis, seminal vesicle, and prostate that also interact with sperm. Fibronectin, an extracellular matrix protein, is also secreted from the seminal vesicles and participates in the formation of the seminal clot. It is suggested that it may play a role in post-testicular regulatory function of sperm. 5'- nucleotidase, derived from the prostate, has been localized to the outer

leaflet of the plasma membrane covering the acrosomal region of the spermatozoa. When 5'-nucleotidase inhibitors are added, there is a clear decrease in sperm motility. Finally, a 100 kD membrane protein from the epididymis, seminal vesicle and prostate has been localized to the sperm head and principal piece of spermatozoa (Aumuller et al., 1997).

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Chapter 2:

Apocrine secretion in the mouse epididymis and rat and human vas deferens

ABSTRACT

Over the years, epithelial cells of the male reproductive tract have been suggested to be involved in apocrine secretion. However, this method of secretion has not been fully accepted, nor is it well understood. In the present study, apocrine secretion was examined in the mouse epididymis and rat and human vas deferens. Throughout these regions of the duct, the apex of the epithelial principal cells often shows protrusions extending towards the lumen of the duct, referred to as apical blebs (ABs). Of different shapes and sizes, ABs appear to form at the apex of principal cells, and morphological and immunocytochemical evidence suggests that ABs detach from the cell surface whereupon they eventually undergo fragmentation in the lumen. ABs contain selective organelles suggesting a segregation of their contents. Numerous polysomes suggest the synthesis of nonglycosylated proteins which upon the fragmentation of ABs in the lumen could act directly or indirectly on sperm. The presence of spherical vesicles of various sizes (20-200nm) within ABs as well as within the lumen of the duct supports AB fragmentation in the lumen and may play a role in modifying the sperm surface. Taken together the data provide strong evidence for apocrine secretion that could play important roles in relation to sperm maturation, viability and protection.

Introduction

It is well recognized that the epididymis and vas deferens play a role in the maturation, protection, transport and storage of sperm. A distinct luminal microenvironment is created through the various functions of the epithelium (Robaire and Hermo, 1988; Turner, 1995; Orgebin-Crist et al., 1996; Jones, 1999; Kirchoff, 1999). This allows the sperm to remain viable and develop the capacity to fertilize an oocyte. One of the major ways the unique fluid composition is attained is through the secretion of various proteins and other substances by the principal cells of the epididymis and vas deferens (Orgebin-Crist and Jahad, 1978; Turner and Giles, 1982; Kirchoff, 1999). However, the precise manner in which these proteins are secreted remains unresolved.

The main focus of the scientific community has been to study proteins secreted in a merocrine fashion. In this model, proteins are synthesized in the rough endoplasmic reticulum (rER), glycosylated and packaged in the Golgi apparatus, transported to the plasma membrane via large secretory vesicles, and released into the lumen after fusion of the latter with the plasma membrane (Flickinger, 1981; Robaire and Hermo, 1988; Hermo et al., 1994).

Over the years it has been noted that the principal cells of different male reproductive tract tissues are involved in another form of secretion referred to as apocrine secretion (Wilhelm et al, 1997; Groos et al., 1998; Aumuller et al., 1999). Unlike merocrine secretion, apocrine secretion does not involve the Golgi apparatus and formation of membrane bound secretory granules. It involves an expansion and protrusion of a portion of the apical cytoplasm of principal cells into the lumen, the

segregation of cytoplasmic organelles contained within, and the eventual detachment from the apical cell surface and liberation into the lumen. These protrusions are referred to as apical blebs.

Apocrine secretion was first observed in 1917 by a German histologist, Paul Schiefferdecker, in mammary and axillary sweat glands (Aumuller et al., 1997). Despite these early observations, apocrine secretion was largely ignored as apical blebs were believed to be fixation artifacts in the early years of electron microscopy (Robaire and Hermo, 1988). In the 1950s and '60s, tissues prepared for EM analysis were immersed in fixative, and consequently the morphology of cells and their organelles was disrupted. Vascular perfusion fixation techniques developed in the 1970s and '80s, have allowed tissues to be fixed optimally while maintaining the integrity of cells and their organelles. Despite these new techniques, studies were slanted towards merocrine secretion and its role in sperm maturation.

Good evidence for apocrine secretion has been demonstrated in the mouse vas deferens (Manin et al., 1995). Immunolocalization of a major mouse vas deferens protein (MVDP) revealed that it was distributed in the whole cytoplasm and abundant in apical blebs and luminal fluid. It was never detected in the lumen of the ER, Golgi apparatus or secretory vesicles. It was also associated with the plasma membrane of spermatozoa. MVDP lacks the ER signal sequence that normally triggers the translocation of secretory proteins across the ER membrane. Since MVDP was not localized over the ER or Golgi, although it was noted in the lumen, it was suggested that apocrine secretion was the mechanism whereby such proteins reach the lumen (Manin et al., 1995).

In recent studies, apical blebs have been observed in the male reproductive tract of a number of species and have been implicated in apocrine secretion. They have been documented mainly in the prostate, seminal vesicles, and coagulating glands of the rat (Wilhelm et al, 1997; Groos et al., 1998; Aumuller et al., 1999). The release of secretory material through apical protrusions has been convincingly shown in the rat coagulating gland. Two proteins, carbonic anhydrase (CAH) II (Wilhelm et al., 1998) and transglutaminase (Groos et al., 1998), have been demonstrated to be secreted in an apocrine manner. Immunolocalization studies have shown that while both of these proteins are found in the cytoplasm of epithelial cells and apical blebs, they are not localized to cisternae of the rough endoplasmic reticulum, Golgi, or secretory granules. Both proteins lack an ER signal peptide that normally allows translocation into the ER, and therefore they are synthesized on free ribosomes in the cytoplasm. Isolation studies have shown the presence of CAH II and transglutaminase in rat coagulating gland secretions, and therefore it has been suggested that they are secreted in an apocrine fashion (Wilhelm et al, 1997; Groos et al., 1998; Aumuller et al., 1999). Furthermore, it has been noted that the biosynthesis of the apocrine synthesized proteins in the rat coagulating gland is hormone-dependent as was the number and form of these blebs (Holterhus et al., 1993). Thus, the fact that they can be modulated hormonally is a further indication that they are not artifacts of fixation.

Apical blebs have also been noted in the human reproductive tract and apocrine secreted proteins also play a role in the regulation of human sperm function (Aumuller et al., 1997). Through immunolocalization studies, three different proteins have been identified that are secreted in an apocrine manner. The proteins were seminal vecicle-

derived fibronectin, prostate-derived 5'-nucleotidase, and a 100 K protein from the epididymis, seminal vesicle, and prostate. Since each one of these proteins was localized to apical blebs and also shown to interact with sperm, it was suggested that they are released by apocrine secretion (Aumuller et al., 1997).

While apocrine secretion has been studied in several male reproductive tissues, the presence and morphology of apical blebs in the epididymis and vas deferens of mice, rats, and humans have received little attention. In the course of our studies, we have noted that apocrine secretion appears to be a major activity of principal cells of the mouse epididymis and rat vas deferens, and the proteins released in this manner may be instrumental in sperm maturation and viability. Thus, the goal of the present study was to characterize apical blebs structurally and suggest possible functional roles for them. These tissues were examined by routine light microscope (LM) and electron microscope (EM) methods, and further immunocytochemistry studies were performed with the goal of determining proteins contained within apical blebs and secreted in an apocrine manner, and to ascribe a functional role for them in relation to sperm. Human vas deferens material was also examined for its role in apocrine secretion.

Materials and Methods

Routine fixation of mouse epididymis for LM and EM analysis

Six adult male Swiss mice (30-40g) obtained from Charles River Laboratories (St. Constant, Quebec) were anesthetized with sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON). The epididymis was fixed by perfusion through the heart with 5% glutaraldehyde buffered in sodium cacodylate (0.1 M) containing 0.05%

$CaCl_2$ at pH 7.4. After perfusing for 10 minutes, the tissue was removed and was divided into five regions, i.e. intermediate zone, initial segment, caput, corpus, and cauda. The tissue from each region was cut into small 1 mm^3 pieces and placed in the same fixative for an additional 2 hours at 4 °C. After an overnight wash in buffer, the tissue was post-fixed in potassium ferrocyanide-reduced osmium tetroxide for one hour to enhance the staining of membranes. The tissue was then rinsed several times in cacodylate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thick sections were cut with a microtome, stained with toluidine blue and examined under the light microscope. Thin sections of selected areas were cut with a diamond knife, placed on copper grids, counterstained with uranyl acetate and lead citrate and examined with a Philips 400 electron microscope.

Routine fixation of mouse epididymis and rat vas deferens for immunocytochemical analysis

Six adult male Swiss mice (30-40g) and six adult male Sprague Dawley rats (350-450g) obtained from Charles River Laboratories (St. Constant, Quebec) were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnitol, MTC Pharmaceuticals, Hamilton, ON), and the mouse epididymes and rat vas deferens fixed by perfusion through the abdominal aorta with Bouin's fixative for 10 minutes. After perfusion the epididymis and vas deferens were removed and immersed in Bouin's fixative for another 24hrs. Prior to immersion, the epididymis was divided into five regions, i.e. intermediate zone, initial segment, caput, corpus, and cauda. The vas

deferens was divided into proximal, middle, and distal regions so that the entire vas deferens would fit into one paraffin block. After fixation, the tissue was dehydrated and eventually embedded in paraffin.

Paraffin sections, 5-mm thick were deparaffinized in xylene and hydrated in a series of graded ethanol solutions. During hydration, residual picric acid was neutralized in 70% ethanol containing 1% lithium carbonate, and endogenous peroxidase activity was abolished in 70% ethanol containing 1% (v/v) H_2O_2. Once hydrated, tissue sections were incubated in a 300-mM glycine solution to block free aldehyde groups.

Mouse epididymis and rat vas deferens immunocytochemistry

Immunoperoxidase staining of tissue sections was carried out according to the procedure of Oko and Clermont (1989). Polyclonal antibodies reactive against ubiquitin (Sigma Chemical CO., St. Louis, Missouri) were kindly provided by Dr. Simon Wing and were used for staining of the mouse epididymis. Polyclonal antibodies reactive against aquaporin-6 (AQP-6) (Alpha Diagnostic International Inc, San Antonio, TX), were used on the rat vas deferens. Polyclonal anti-CFTR (Cystic Fibrosis Transmembrane Regulator) antibodies were also used on the rat vas deferens (Biogenesis, Poole, England). Finally, cytoplasmic droplets of spermatozoa in the rat vas deferens were stained using an antiserum to perforatorial proteins of the sperm (Sigma Chemical CO).

Before immunostaining, tissue sections were blocked for 15 minutes with Tris-HCl-buffered saline (TBS). This and subsequent treatments were accomplished by placing 100 µL of a solution onto a coverslip and overturning the tissue face of the slide onto the drop, thus ensuring that the entire tissue was treated with minimal fluid (Oko and Clermont, 1989). Coverslips were then removed by dipping the slides in TBS containing

1% Tween-200 (TWBS). Sections were then incubated in a 37 ^{0}C humidified incubation chamber for 1.5 hours with their respective polyclonal antibodies (all diluted at 1:100 in TBS). After four 5-minute washes in TWBS, sections were once again blocked with 10% goat serum in TBS. They were then incubated for 30 minutes (at 37 ^{0}C) with goat anti-rabbit IgG conjugated to peroxidase (Sigma) diluted 1:250 in TBS. This was followed by four 5-minute washes in TWBS.

The final reaction product was achieved by incubating the sections for 10 minutes in 500 mL of TBS containing 0.03% H_2O_2 , 0.1M imidazole, and 0.05% diaminobenzidine tetrahydrochloride (Sigma), pH 7.4. Slides were than washed in distilled H_2O and counterstained with 0.1% methylene blue for 1 minute. The tissue was dehydrated via a process that consisted of passing slides through a graded ethanol series, after which the sections were immersed in xylene and mounted with Permount. Tissue sections incubated without the primary antibody were used as controls.

Human Vas Deferens

Approximately 40 normal males aged 18-30 with proven fertility records were vasectomized by "no-scalpel' laser technique (by Dr. K. Jarvi in Toronto). Pieces of the vas deferens tissue were excised and immediately immersed in fixative of 5% glutaraldehyde with sodium cacodylate buffer (0.1 M) and pH 7.4. The 2-4 mm long samples were post-fixed in potassium ferrocyanide-reduced osmium and then dehydrated in alcohol. Finally they were embedded in Epon by conventional means. Thick sections were cut with a microtome, stained with toluidine blue and examined under the light microscope. Thin sections of selected areas were cut with a diamond knife, placed on

copper grids, counterstained with uranyl acetate and lead citrate and examined with a Philips 400 electron microscope.

Results

Structural features of Apical Blebs in the Mouse Epididymis

Light microscopy

The mouse epididymis consists of five anatomical regions: the initial segment (Figs. 1a and b), intermediate zone (Fig. 1 c), caput, corpus and cauda. Apical blebs (Figs. 1a-c) were noted in principal cells of all regions of the mouse epididymis, but especially in the initial segment, intermediate zone and corpus regions. Some blebs appeared to be protruding from the apical surface of the epithelium, while others seemed to be detached and lie deep in the lumen. The blebs had irregular shapes and sizes and were pale stained and homogeneous in appearance (Figs. 1a-c). As blebs have already been noted in the mouse vas deferens in previous studies (Manin et al., 1995), only the mouse epididymis was examined, although the apical blebs seen in the epididymis were noted to be of similar shape and size to those reported in the vas deferens.

Electron Microscopy

In the epididymis, areas of the apical cytoplasm of principal cells, referred to as apical blebs, were often noted to extend into the lumen of the duct, yet maintain their continuity with principal cells from which they protruded. These structures were regular features of principal cells and did not show many structural differences from one region of the duct to another. As such they appeared between the microvilli of principal cells

also extending from the apical cell surface into the lumen although they were markedly different from them. In all regions, apical blebs attached to principal cells took on different shapes and sizes, and while some were more or less spherical in appearance (Figs. 2, 3, 4, 8, 13, 14, 15, 16), others were irregularly shaped (Figs. 6b, 7, 9, 10, 11, 12a, 12b). The content of the apical blebs was also fairly consistent from bleb to bleb. Few organelles were present within apical blebs such as occasional ER elements, appearing as irregularly shaped tubular elements (Figs. 3, 4, 5, 6a, 9, 13, 14, 15), and glycogen granules (Figs. 5, 7). Notably conspicuous were numerous polysomes (Figs. 14, 15, 17a) and ribosomes (Figs. 8, 9 10, 13, 14, 15, 16) all of which were embedded in a homogeneous ground substance (Figs. 3, 4, 15, 16). Also found within apical blebs were occasional vesicular elements of various sizes. This included microvesicles with a diameter of about 20nm (Figs. 3, 4, 6b, 8, 10, 11, 17a, 17b, 17c) which were especially numerous in some blebs, and somewhat larger vesicles with a pale staining core and spherical shape (Figs. 4, 5, 6b, 10, 12a, 12b, 14, 15) which were also occasionally found dispersed in apical blebs. Other organelles such as mitochondria (Figs. 3, 4, 6a, 6b), large densely stained lysosomes (Fig. 9), endosomes (Fig 3) and the Golgi apparatus consisting of stacks of saccules (Fig.12a), notably present in the apical region of principal cells, were conspicuously absent from the cytoplasm of apical blebs. Coated pits and vesicles abundant in the apical region of principal cells (Figs. 3, 6b) were not present in apical blebs. Thus there appeared to be a distinct segregation of some organelles of principal cells between its apical region of cytoplasm and that of apical blebs.

While many apical blebs were connected to principal cells, various images suggested that blebs were in a continual state of formation as well as detachment from the

apical cell surface. Many blebs were seen protruding from the apical surface of principal cells, showing varying degrees of connections to the epithelium (Figs. 2, 3, 4, 5, 6-13). While some showed a broad base of attachment to the principal cell's apical surface (Figs. 3, 5, 6a, 7, 9, 13), others were attached by a narrow constricted area or thin stalk (Figs. 2, 6b, 8). Some images showed several blebs of varying sizes attached to a given principal cell suggesting that they were in a constant state of formation (Figs. 2, 4, 7). Other images suggested that apical blebs detached from the apical surface of principal cells (Figs. 10, 11). In such cases, fissures were noted in the region between the bleb and apical region of the principal cell. Within these fissures, microvilli were present indicative that the fissures represented areas of the lumen and that the blebs were being dismantled from the apical cell surface of principal cells.

In addition, numerous blebs were seen apparently detached from the apical surface and lying free in the lumen of the epididymis (Figs. 2, 3, 4, 12-17). While some were still close to the apical principal cell surface, others were far removed from it lying deep in the lumen of the duct. While the structural features of the detached blebs in the lumen were essentially similar to those attached to principal cell surface, there were some differences. Notable was the presence of large membranous profiles of various sizes at the periphery of some blebs, suggestive of a disrupting of their cell surface plasma membrane (Figs. 17b, 17c). Furthermore, the presence of vesicular elements of various sizes, as noted in the cytoplasm of apical blebs, as well as numerous microvesicles in the lumen of the epididymis (Fig. 17b) suggest that once liberated into the lumen, the apical blebs undergo fragmentation with subsequent release of their contents into the lumen of the duct.

LM immunocytochemical evidence for Apocrine Secretion in the Mouse Epididymis

When immunostained with an anti-ubiquitin antibody, an intense reaction was noted in the apical cytoplasm of principal cells of the corpus region (Figs. 18 a-c). A reaction was also present in the numerous apical blebs protruding from the apical cell surface of principal cells (Fig 18 a-c). The blebs were of different shapes and sizes and while many were still attached to or situated close to the principal cell surface, others were clearly detached, with many extending deep into the lumen.

LM immunocytochemical evidence for Apocrine Secretion in the Rat Vas Deferens

In the rat epididymis, principal cells did not exhibit much evidence of apical blebs. In fact, apocrine secretion does not appear to be a prominent feature of the rat epididymis (Robaire and Hermo, 1988). However, in the rat vas deferens many apical blebs were noted especially in its middle (Figs. 19 a-c) and distal regions (Fig 19d) as already described by Andonian and Hermo (1999a). In the present study, apical blebs were analyzed in the rat vas deferens using several different antibodies which stained fortuitously either the apical cytoplasm of principal cells or their apical plasma membrane. In appropriate sections of the vas deferens of material immunostained with an anti-CFTR antibody which stained the apical principal cell cytoplasm (Figs. 19 a-c), apical blebs, also reactive, were seen protruding from the principal cell surface and sometimes attached to them merely via a thin stalk. Numerous blebs were also seen free in the lumen at a considerable distance from the principal cell surface. When the distal vas deferens was immunostained with an anti-AQP-6 antibody (Fig. 19d), a rim of reaction was seen on the apical plasma membrane of principal cells. A reaction was also

noted outlining the membrane delimiting apical blebs seen at some distance from the principal cell surface.

EM analysis of Vesicular Elements and Membranous Profiles in the Lumen

Mouse Epididymis

In the lumen of the mouse epididymis, many vesicular elements were noted in close proximity to the apical blebs or located between the microvilli of principal cells (Figs. 3, 4, 7, 8, 9, 10, 11, 12a, 12b, 13, 14, 17b, 17c). The vesicular elements, surrounded by a membrane had a central pale-stained area and were of small, medium and large sizes. It was noted that some of the small sized vesicles were similar to those present in the cytoplasm of apical blebs (Figs. 4, 5, 6b, 10, 12a, 12b, 14, 15), but larger than the microvesicles also observed in blebs and the epididymal lumen (Figs. 3, 4, 6b, 8, 10, 11, 17a-c). Some of the larger sized vesicular elements were noted at the periphery of apical blebs detached from the principal cell surface and apparently undergoing breakdown (Figs. 17b, 17c).

Rat Vas Deferens

Vesicular elements of different sizes were also noted in the lumen of the vas deferens when examined by light microscopy and appropriate antibodies (Fig. 21 inset) and by electron microscopy (Figs. 20, 21). The vesicular elements had the same appearance and sizes as those found in the mouse epididymis and were located in the lumen and between microvilli.

When the vas deferens was immunostained with an antibody raised against rat sperm perforatorial proteins, the cytoplasmic droplets of sperm showed a strong reaction (Figs. 20 and 21 insets). Of interest was the fact that larger vesicular elements nearby did not show any reaction (Fig. 21 inset), suggesting that they were not related to the droplets. However, they appeared to be of the size of apical blebs stained with the anti-AQP-6 antibody (Fig. 19d). Thus these two different markers could serve to distinguish apical blebs from cytoplasmic droplets, with the former being considerably larger than the latter.

Structural Features of Apical Blebs in the Human Vas Deferens

Light Microscope

The epithelium of the human vas deferens was circular with folds extending into the lumen (Figs. 22 a-d). Numerous apical blebs were observed protruding from the principal cells into the lumen. They appeared as spherical or elongated structures of different shapes and sizes. Some of the blebs were still attached by thin stalk-like structures. Others appeared to be detached from the cell surface and were seen either close to the cell surface or far removed from it, deep in the lumen (Figs. 22 a-d). Only human vas deferens material was examined due to the relative ease of obtaining this tissue through vasectomy. However, the apical blebs that were observed in the vas deferens were of similar size and shape as apical blebs noted in previous studies on the human epididymis (Aumuller et al., 1997).

Electron Microscope

The principal cells of the human vas deferens contained a large nucleus including a well-defined nucleolus (Fig. 23). The cytoplasm contained many mitochondria,

lysosomes and lipofuscin granules as well as rough endoplasmic reticulum (Figs. 23, 24). Junctional complexes were seen at the apex of the epithelium between adjacent principal cells (Fig. 23). Microvilli were noted and occasionally interrupted by the existence of coated pits along the surface (Fig. 24).

Apical blebs appeared as protrusions of the apical cell surface of principal cells (Figs. 24-26). They varied in shape, size and number. Some blebs were attached to principal cells by varying degrees of stalk formation (Figs. 25, 26), while others were found free in the lumen far removed from the surface of the epithelium (Fig. 24).

The apical blebs presented a smooth plasma membrane devoid of coated pits and contained numerous polysomes, ribosomes and occasional ER elements and vesicular elements, all embedded in a fine ground substance (Figs. 24-26). However, lysosomes, Golgi stacks of saccules, and mitochondria were notably absent from the blebs and remained segregated to the cytoplasm of principal cells.

Discussion

It is well recognized that the principal cells of the epididymis (Orgebin-Crist, 1996; Kirchoff, 1999) and vas deferens (Flickinger, 1973; Hamilton, 1975; Hamilton and Cooper; 1978, Kennedy and Heidger; 1979) are active secretory cells. Proteins and other substances secreted into the lumen play a major role in sperm maturation, protection, concentration and viability (Robaire and Hermo, 1988; Cooper, 1992; Kirchoff, 1999). However, the precise means by which these substances are secreted remains unresolved.

Until recently, apocrine secretion was largely ignored by the scientific community (Hamilton, 1975; Robaire and Hermo, 1988). The immersion fixation techniques of early

electron microscopy often disrupted the morphological characteristics of cells and their organelles and apical blebs were thus considered to be fixation artifacts. However, over the years and with the advent of modern vascular fixation perfusion techniques, the focus of the scientific community has been on merocrine secretion and its role in sperm maturation. To this day, the existence of apocrine secretion remains controversial, and some still believe that apical blebs are merely fixation artifacts. In the present thesis, we will provide evidence that apical blebs are true structural feature of cells of the epididymis and vas deferens and that apocrine secreton may have several important functional roles in relation to sperm.

Structural Evidence for Apocrine Secretion

Apocrine secretion is characterized by the loss of an apical bulge of cytoplasm surrounded by the apical plasma membrane of a cell along with cellular material contained within. This phenomenon was first described in sweat glands by Schiefferdecker in 1917, although the most striking example of apocrine secretion occurs in the human mammary gland (Aumuller et al., 1999). However apocrine secretion has also been described in several tissues of the male reproductive tract. They have been documented mainly in the prostate, seminal vesicle, coagulating gland and vas deferens of different species: bull (Agrawal and Vanha-Pertulla, 1988; Renneberg et al., 1995), cat (Morales and Cavicchia, 1991), rat (Wilhelm at al., 1991; Andonian and Hermo, 1999a), mouse (Manin et al., 1995), human (Aumuller et al., 1997) and chimpanzee (Smithwick and Young, 1997).

In the male reproductive tract, apocrine secretion is an alternative to the classical form of secretion called merocrine secretion, whereby proteins formed in the

endoplasmic reticulum (ER) are then delivered to the Golgi apparatus where they are glycosylated and then packaged into secretory granules which ultimately fuse with the apical plasma membrane to release their content into the lumen of the duct (Hamilton, 1975; Flickinger, 1981; Robaire and Hermo, 1988; Hermo et al., 1994). The present study provides further evidence for apocrine secretion and documents the presence of apical blebs in the mouse epididymis and rat and human vas deferens. By studying the morphology and contents of the apical blebs, we hope to achieve a better understanding of their structure and functions.

Apical blebs: fact or fiction

In the present study, apical blebs were noted to be a consistent feature of principal cells of the mouse epididymis from animal to animal under the best methods of fixation to date. Furthermore, they were found to be especially prominent mainly in the initial segment, intermediate zone and cauda epididymidis. Thus, if they were simply fixation artifacts they should be randomly seen along the entire duct and not restricted to several of its regions. Also evident was the fact that apical blebs contained specific organelles and that there was a segregation occurring for their contents as has also been demonstrated for apical blebs of other tissues of the male tract (Morales and Cavicchia, 1990; Andonian and Hermo, 1999a). In fact, ribosomes, polysomes, occasional ER elements, microvesicles (20nm) and spherical vesicles of various sizes (50-200nm) were the main residents of apical blebs. Thus if apical blebs were merely fixation artifacts then they should also contain many of the organelles contained in the apical cell cytoplasm, such as mitochondria, Golgi stacks of saccules and microtubules, which in fact they never did. It was also apparent that the plasma membrane enveloping apical blebs was unique.

In fact it showed no coated pits, and endosomes, multivesicular bodies and large dense lysosomes, all organelles of the endocytic apparatus, were all consistently absent from apical blebs. This suggests that apical blebs are a specific entity of principal cells.

In addition, it was noted that apical blebs varied in size and shape from cell to cell and that blebs of increasing sizes were even noted along the surface of one cell suggesting that they are in a state of constant formation. This hypothesis also receives support from the data that the formation and number of apical blebs varies under hormonal regulation (Aumuller et al., 1999). Studies on the coagulating gland of the male rat have shown that androgen deprivation causes all blebs to vanish. Thus, the fact that apical blebs were hormone-dependent further indicates that they are not artifacts, but represent a dynamic phenomenon.

At times, apical blebs appeared to be attached to the apical surface of principal cells merely by a thin stalk-like process, while others showed fissures or clefts at the junction point between the bleb and apical cell cytoplasm. These images suggest the separation of one from the other and that apical blebs detach from the apical surface of principal cells. In addition, apical blebs as evidenced in our LM immunocytochemical studies using ubiquitin as a marker, which stains the apical cytoplasm of principal cells and blebs, revealed that blebs were noted not only near the apex of principal cells but deep in the lumen at a considerable distance from the principal cell surface. Various EM images also suggested that the plasma membrane of apical blebs released into the epididymal lumen undergoes fragmentation thus liberating the contents of blebs therein. Indeed, several of the structural features of the blebs such as the microvesicles and small sized vesicles (50-200nm) were also noted to be free in the epididymal lumen suggestive

of their release from the breakdown of apical blebs in the lumen. Thus it would appear that apical blebs represent a true structural feature of principal cells, and that apocrine secretion is an active and dynamic process of the mouse epididymis that may play important roles in relation to sperm.

In the present study we also provide further evidence for apocrine secretion in the rat vas deferens. Our previous work employing routine EM analysis demonstrated that apical blebs were consistently present in the middle and distal vas deferens (Andonian and Hermo, 1999a). Apical blebs revealed a segregation of specific organelles as compared to the apical cytoplasm of principal cells and morphological evidence for their detachment and eventual fragmentation in the lumen (Hermo et al., 2000). In the present study, we have utilized the fact that LM immunocytochemistry employing various antibodies, stained not only the apical plasma membrane or cytoplasm of principal cells but also the plasma membrane or cytoplasm of apical blebs. CFTR (Cysic Fibrosis Transmembrane Regulator) is a Cl- ion channel that is present in the apical membrane of chloride secretory epithelial cells, including the human vas deferens (Quinzii and Castenalli, 2000; Jezequel et al. 2001). However, because CFTR is an integral membrane protein, it will also be present, to some degree, in a variety of other membrane compartments including the endoplasmic reticulum, Golgi stacks, endosomes, and lysosomes (Bradbury, 1999). When the rat vas deferens was immunostained with an anti-CFTR antibody, a homogeneous reaction was seen throughout the cytoplasm of the principal cells as well as apical blebs. The presence of blebs attached to the principal cell surface as well as deep within the lumen strongly suggests that apical blebs form and detach from these cells in the rat vas deferens. In addition, LM immunocytochemical

studies employing an anti-aquaporin 6 (AQP-6) antibody also provide additional evidence for apocrine secretion by principal cells of the vas deferens. AQP-6 is a transmembrane pore protein involved in water transport (Agre et al., 1993; Nielsen et al., 1993; Wintour, 1997). When the vas deferens was immunostained with an anti-AQP-6 antibody, a reaction was noted on the apical plasma membrane of principal cells and also on the membrane of blebs seen adjacent to the principal cell surface or deep in the lumen. Taken together these data suggest that the principal cells of the vas deferens are involved in apocrine secretion.

Although the mouse and rat provide strong evidence for apocrine secretion, and allow the development of a model of the dynamic process of apical blebbing, it is recognized that human tissues must be examined to provide scientific importance. The present study also demonstrates that apical blebs are a consistent feature of the human vas deferens. In our LM studies, numerous blebs were seen protruding from the principal cells of the vas deferens. They were noted to be of different shapes and sizes. Some remained attached to the epithelium while others were free in the lumen. Further examination with the EM showed that the apical blebs appeared to form, detach, and liberate their contents into the lumen in a manner similar to that noted for the mouse epididymis. The contents of apical blebs of principal cells of the human vas deferens were remarkably consistent with those found in the mouse epididymis of the present study and rat vas deferens (Andonian and Hermo, 1999a; Hermo et al., 2000). While ER elements, polysomes, and ribosomes were consistently found in the blebs, organelles such as the Golgi stacks of saccules, endosomes, large dense lysosomes, microtubules and mitochondria were excluded from the blebs. Thus, although the human vas deferens was

excised from each individual and the material was immersed into fixative did not alter the contents of apical blebs. Thus apical blebs are not a consequence of immersion fixation but rather represent a true structural feature of cells of the vas deferens of the human. Reports on the presence of apical blebs have also been demonstrated in the human epididymis, prostate, seminal vesicle and coagulating gland (Aumuller et al., 1997; Renneberg et al., 2001) and see section below.

Functional Significance of Apocrine Secretion

Taking into account the fact that apocrine secretion is a dynamic event in the life of principal cells of the epididymis and vas deferens, why did cells evolve such a mechanism of secretion? To answer this we will examine the various components of apical blebs in light of their possible functional significance and why some organelles are retained in blebs and others excluded.

Polysomes and ribosomes:

Many proteins are secreted by principal cells into the lumen of the epididymis and vas deferens where they become closely associated with or an integral part of the plasma membrane of sperm. This interplay of proteins and the sperm surface appears to be an important factor whereby sperm attain their maturational characteristics enabling them to fertilize the ovum (Cooper, 1986; Robaire and Hermo, 1988; Kirchhoff, 1999). Glycoproteins synthesized within principal cells of the epididymis and vas deferens are packaged in secretory granules in the Golgi apparatus and released into the lumen via a process described above as merocrine secretion. However, many proteins are also synthesized on polysomes and ribosomes within the cytoplasm, but these proteins are usually used by the cell for general housekeeping requirements. Such proteins, including

glycoproteins, cannot cross the apical plasma membranes of cells to be released into the lumen. To overcome this, it would appear that several tissues of the male reproductive tract have evolved an alternative form of secretion, namely apocrine secretion.

In the present study, it was noted that in the mouse epididymis and rat and human vas deferens, the apical cell cytoplasm of principal cells protruded into the lumen to form an apical bleb. One of the most consistent features within the cytoplasm of apical blebs were the presence of numerous polysomes and ribosomes. This would suggest that proteins are being synthesized on these structures. These proteins would not be glycosylated and hence would not be incorporated into the secretory granules of the Golgi apparatus of the cell. How do such proteins therefore gain access into the lumen of the duct? Since proteins cannot cross the apical plasma membrane of apical blebs, it is suggested that the proteins formed on ribosomes would enter the lumen of the duct via the apical blebs and a process of apocrine secretion. Thus upon detachment from the apical cell surface of principal cells and eventual disruption, apical blebs would serve as the vehicle in which proteins formed on ribosomes could be delivered into the lumen. These non-glycosylated proteins could then interact either directly with the sperm surface and/or perform other specific functions.

What types of proteins are released via apocrine secretion? In the mouse vas deferens, evidence for apocrine secretion has been proposed by Manin et al. (1995) for a major mouse vas deferens protein (MVDP). MVDP lacks the ER signal sequence that normally triggers the translocation of secretory proteins across the ER membrane. Immunocytochemical localization revealed that MVDP was distributed throughout the entire cytoplasm of principal cells as well as in apical blebs and was also present in the

luminal fluid. It was never detected in the lumen of the ER, Golgi apparatus or secretory vesicles, but was shown to be associated with the plasma membrane of spermatozoa. Hence it was suggested that MVDP was secreted by principal cells of the vas deferens via apocrine secretion. MVDP belongs to the superfamily of aldo-keto reductases, and it was proposed that it may be involved either in the eventual production of fructose, the preferred substrate for glycolysis by sperm, in the degradation of steroids, or may intervene as a detoxifying system important in the survival of sperm in the vas deferens (Manin et al, 1995). Interestingly, the present study noted the presence of glycogen in the cytoplasm of apical blebs. This could serve as the substrate for MVDP in the production of fructose.

In addition, our previous studies on the vas deferens demonstrated that 3β-HSD was found in the cytoplasm of principal cells as well as in apical blebs (Andonian and Hermo, 1999a). 3β-HSD participates in a steroid converting pathway that efficiently transforms the precursor 5,16-androstadien-3 beta-ol into androstenol. (Dufour et al., 2001). It was suggested therefore that steroids made in apical blebs could then be released into the lumen of the duct where they could perform specific functions (Andonian and Hermo, 1999a).

It has also been proposed that principal cells of the vas deferens may be involved in apocrine secretion to release Glutathione S-Transferases (GSTs) into the lumen. GSTs are family of isozymes that catalyze the conjugation of glutathione with various electrophilic compounds. This protects DNA, RNA, and proteins from electrophilic attack through the formation of a less toxic product (Ketterer et al., 1990; Daniel, 1993). In the vas deferens of the rat, various isoforms of GSTs were expressed in the cytoplasm

of principal cells as well as within apical blebs (Andonian and Hermo, 1999b). It was thus postulated that apocrine secretion could serve as a means whereby GSTs are released into the lumen. In this way, GSTs would have direct access to reactive oxygen species and other electrophilic compounds present in the lumen of the vas deferens, where high oxygen levels are required to store sperm and where sperm are often seen to undergo degeneration (Djakiew and Cardullo, 1986; Nonogaki et al., 1992). In this way, GSTs would prevent the harmful effects of all of these substances on sperm viability.

In the present study, it is proposed that principal cells of the epididymis may also secrete ubiquitin in an apocrine manner. Ubiquitin is a normal component of most eukaryotic cells, and it is assumed that this protein plays an important role in intracellular proteolysis (Fried et al., 1987; Wing et al., 1996). In this pathway of proteolysis, ubiquitin becomes covalently linked to proteins for recognition and degradation by the large proteolytic complex, the 26S proteasome. Ubiquination is a multistep process and requires the presence of ubiquitin conjugating enzymes to allow ubiquitin binding to proteins targeted for degradation (Wing et al., 1996). While past studies have focused on the function of ubiquitin intracellularly in eukaryotic cells (Fried et al., 1987; Murti et al., 1988; Manetto et al., 1989), immunoreactive detection of ubiquitin in human seminal plasma suggests that this protein is secreted by epithelial cells of the excurrent duct system (Lippert et al., 1993). Further studies support this hypothesis through the immunolocalization of ubiquitin in the principal cells of the rat and human epididymis, and in the lumen of these structures interacting with sperm. (Martin et al., 1995; Fraile et al., 1996). However, the manner in which ubiquitin is secreted into the lumen of the epididymis has yet to be examined. In the present study, when the mouse epididymis was

immunostained with an anti-ubiquitin antibody, an intense reaction was seen in the cytoplasm of principal cells but also in their apical blebs. Since ubiquitin is normally a cytoplasmic protein, but was also detected in the lumen interacting with sperm (Martin et al., 1995; Fraile et al., 1996), it is suggested from the present data that ubiquitin is secreted in an apocrine fashion by principal cells in the epididymis. It is known that many proteins are found in the lumen of the epididymis such as those released from the contents of cytoplasmic droplets and degenerating sperm, and also those found in cellular debris of unknown origin (Robaire and Hermo, 1988). Once secreted, ubiquitin may be conjugated to these various luminal proteins and target them for degradation.

Finally, apical blebs have been noted in the human reproductive tract and apocrine secreted proteins may also play a role in the regulation of human sperm function (Aumuller et al., 1997). Through immunolocalization studies, three different proteins have been identified that are secreted in an apocrine manner from the epididymis, seminal vesicle, and prostate that also interact with sperm. Fibronectin, an extracellular matrix protein, is also secreted from the seminal vesicles and participates in the formation of the seminal clot. It is suggested that it may play a role in post-testicular regulatory function of sperm. 5'- nucleotidase, derived from the prostate, has been localized to the outer leaflet of the plasma membrane covering the acrosomal region of the spermatozoa. When 5'-nucleotidase inhibitors are added, there is a clear decrease in sperm motility. Finally, a 100 kD membrane protein from the epididymis, seminal vesicle and prostate has been localized to the sperm head and principal piece of spermatozoa (Aumuller et al., 1997). Thus aside from the proteins discussed above, one or all of these proteins may also be

secreted via apocrine secretion in the epididymis and vas deferens. This would need to be verified in future studies.

Microvesicles, spherical vesicles of different sizes and large irregular membranous profiles:

In the present study, numerous vesicles of different sizes were noted in the cytoplasm of apical blebs of the mouse epididymis. They consisted of microvesicles (20nm in diameter) as well as spherical empty looking vesicles ranging from 50-200nm in diameter. Vesicles of similar size as well as microvesicles were a consistent feature of the lumen of the epididymis. The origin of these structures has not as yet been demonstrated. It is therefore proposed from our morphological data that these structures arise from apical blebs as a result of their detachment and eventual disruption within the lumen of the duct. Microvesicles have been noted in the hamster epididymis and suggested to bind to the surface of sperm whereby they would modify their protein contents (Moore et al, 1986). A similar role for microvesicles may also occur in the mouse epididymis, but future studies would be required to evaluate their nature and functional significance.

The present data also suggest that apocrine secretion results in the release of vesicles (50-200nm) that may be involved in modifying the sperm's plasma membrane in several ways. Some of these vesicles may represent primary lysosomes derived from the Golgi apparatus that would be segregated within blebs at the time of their formation and then subsequently released via apocrine secretion into the lumen of the duct. Indeed, glycosidases have been noted biochemically within a heterogeneous population of membrane bound vesicles in the lumen of the rat epididymis (Tulsiani et al., 1995).

Glycosidases are also found in epididymal fluids as soluble enzymes. They have been associated with molecular changes of glycoproteins on the sperm surface and which could yield functional proteins and result in fertile and motile sperm (Tulsiani et al., 1998). Membranous vesicles in the lumen of the epididymis and their close association with sperm have also been implicated as a means of transferring lipid-anchored proteins to the sperm surface which may be crucial in promoting physiological changes to sperm and their maturational state (Cooper, 1998). From the present data it is suggested therefore that some of the vesicles containing glycosidases and those binding to the sperm surface arise from apical blebs after the latter breakdown in the lumen. Future studies employing specific lysosomal markers in conjunction with LM and EM immunocytochemistry would help resolve the nature and function of some of the vesicles seen in the lumen of the epididymis and apical blebs. Future studies directed at isolating apical blebs as well as their contents by raising antibodies to the proteins contained therein in conjunction with EM immunocytochemistry would aid us in understanding the nature of the other vesicles and their precise relationship with the sperm surface.

In the present study, large vesicular structures were noted in the lumen of the epididymis and vas deferens as seen in the LM. To determine whether or not such profiles may represent cytoplasmic droplets, the rat vas deferens was immunostained with antibodies that reacted specifically with droplets and not apical blebs. Cytoplasmic droplets are seen as punctate structures at the LM level and appear as small localized swellings of the cytoplasm along the tails of sperm (Oko et al., 1993). In tissue sections of the rat vas deferens immunostained with markers of the droplets, the latter were clearly reactive on the sperm tail, but larger membranous profiles were also noted in the lumen

of the duct that were consistently unreactive. Thus the larger membranous profiles do not appear to represent the droplets of sperm, but may represent apical blebs.

At the EM level, droplets contain exclusively flattened saccular elements that represent Golgi elements, but no polysomes and rarely vesicles. While droplets remain attached to the sperm tail as the latter traverse the epididymis, they do eventually detach and subsequently breakdown in the lumen. Thus some of the larger membranous profiles seen at the EM level in the lumen of the epididymis and vas deferens may correspond to the plasma membranes and contents of droplets once these structures breakdown in the lumen of the duct. However, some of the larger membranous profiles may also represent the plasma membranes of apical blebs that also breakdown in the lumen. Clearly, labeling the membranes of these structures with appropriate antibodies raised against them will help resolve their identity and provide further support for the existence of apocrine secretion.

Future directions:

In addition to GSTs, 3β-HSD and ubiquitin, it is hypothesized that other proteins may be synthesized on the polysomes of apical blebs of the epididymis and vas deferens. They would then be released into the lumen upon apical bleb dreakdown where they could interact with the sperm and play a role in their maturation and protection. Future studies would involve the isolation of blebs by differential centrifugation and their purification as well as isolation of their contents. Proteins of these structures could then be characterized by mass spectrometry techniques and Western blot analysis. Proteins would be screened and some would be identified in the gene data bank with a designated function. Novel proteins may also be present and one could raise antibodies to them,

which by LM and EM immunocytochemistry would resolve their localization within apical blebs and assess their interaction with the sperm surface. In addition, once a protein is characterized it may be possible to determine its function.

Through these and other studies, it may well be proven that apocrine secretion is an essential feature of principal cells that is necessary to ensure the maturation and survival of sperm in the epididymis and vas deferens. By gaining a better understanding of the role of apocrine secretion in relation to sperm, one may develop better methods of male contraception and aid in the understanding of the causes of male infertility. Further studies on apical blebs will also aid in understanding the precise means by which the principal cells restrict the entry of certain organelles into the apical blebs, the mechanism whereby apical blebs form on the apical cell surface, then detach from it and then breakdown in the lumen of the duct. Studies are also required to determine what factors regulate the formation and detachment from the cell principal cell surface.

Acknowledgements

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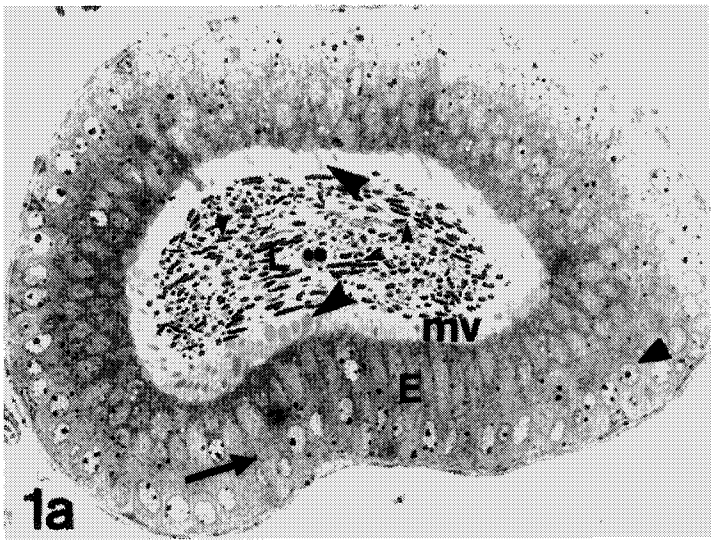
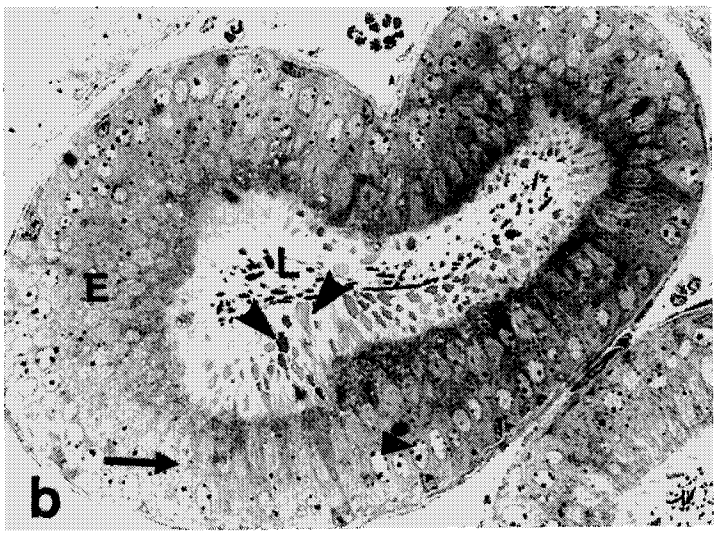
Figure Legend

Figure 1a: High Power light micrograph of a section through the region of the intermediate zone of the mouse showing tall columnar epithelium (E), consisting mainly of principal cells (arrow) where the nucleus (triangle) is located basally. Apical blebs (large arrowhead) are seen emanating from the surface of some principal cells. These apical protrusions appear embedded in the thick homogeneous layer of microvilli (mv) which is surrounding the sperm (small arrowheads) in the lumen (L). X 40

Figure 1b: High power light micrograph of a section of the mouse initial segment showing a taller epithelium (E) with a smaller lumen (L), principal cells (arrow), and their basally located nuclei (triangle). Note the numerous blebs (large arrowhead) extending from the epithelium and at times appear detached in the lumen where few sperm are found. X40

Figure 1c: High power light micrograph of the mouse corpus epididymis with tall columnar epithelium (E) consisting mainly of principal cells (arrow) with their basally located nuclei (triangle). Apical blebs (large arrowhead) are seen here as well, protruding into the lumen (L) of the corpus epididymis. X40


mv
E
1a


E
L
b

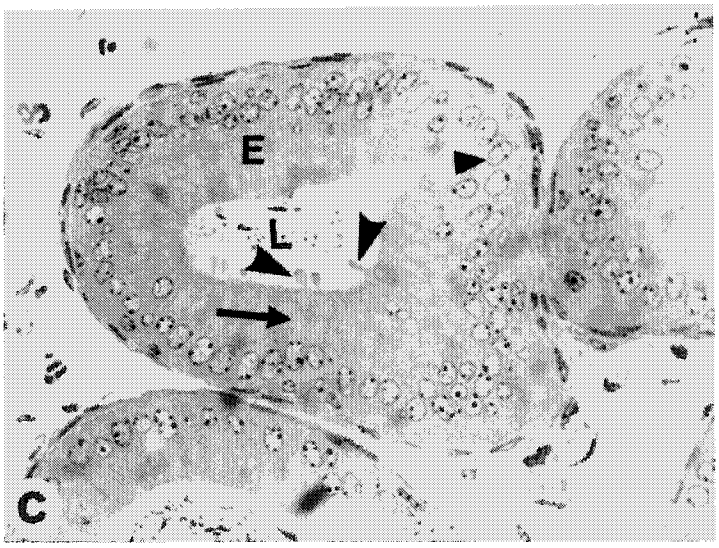

E
L
c

Figure 2: Low power electron micrograph of the mouse initial segment epididymis epithelium comprised mainly of principal cells (P). Apical blebs (AB) appear to be in the process of detaching and others seem to be already detached and free in the lumen (L). Blebs are surrounded by microvilli (Mv) which extend into the lumen as well. Also seen in the lumen is a cytoplasmic droplet (CD). X8250

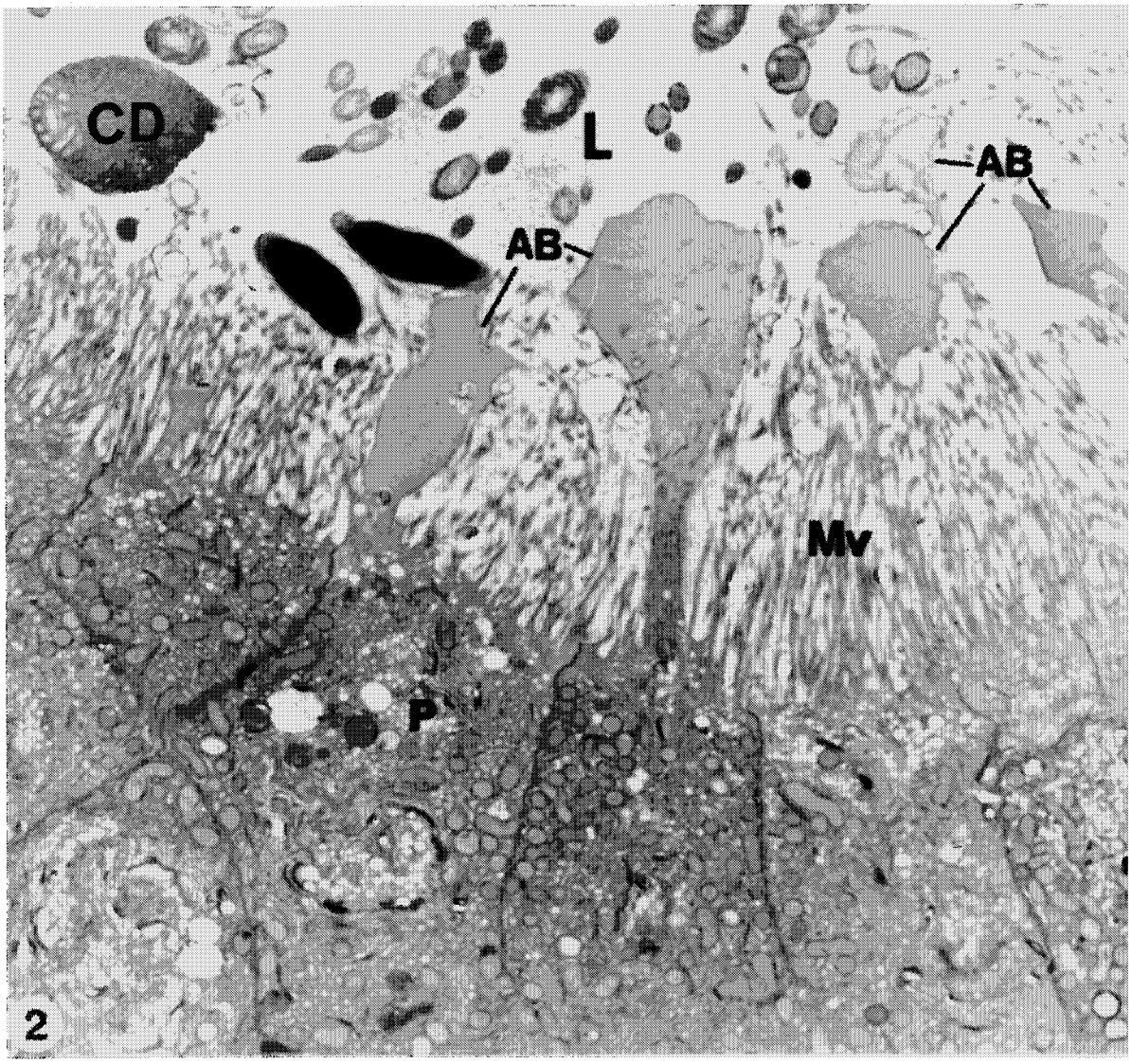

CD
L
AB
AB
Mv
P
2

Figure 3: High power electron micrograph of the mouse initial segment epididymis comprised mainly of principal cells (P). Some apical blebs (AB) are in the process of formation while others are seen free in the lumen (L) and surrounded by microvilli (Mv). Found within the blebs are ground substance (star), ER elements (arrow), and microvesicles (circle). Membranous vesicles of various sizes are found in the lumen (curved arrow) around the blebs. Other organelles such as mitochondria (m) and endosomes (En) are segregated from the bleb and remain in the principal cell. Also seen at the apex of the principal cell are coated pits (small curved arrow). X13,750

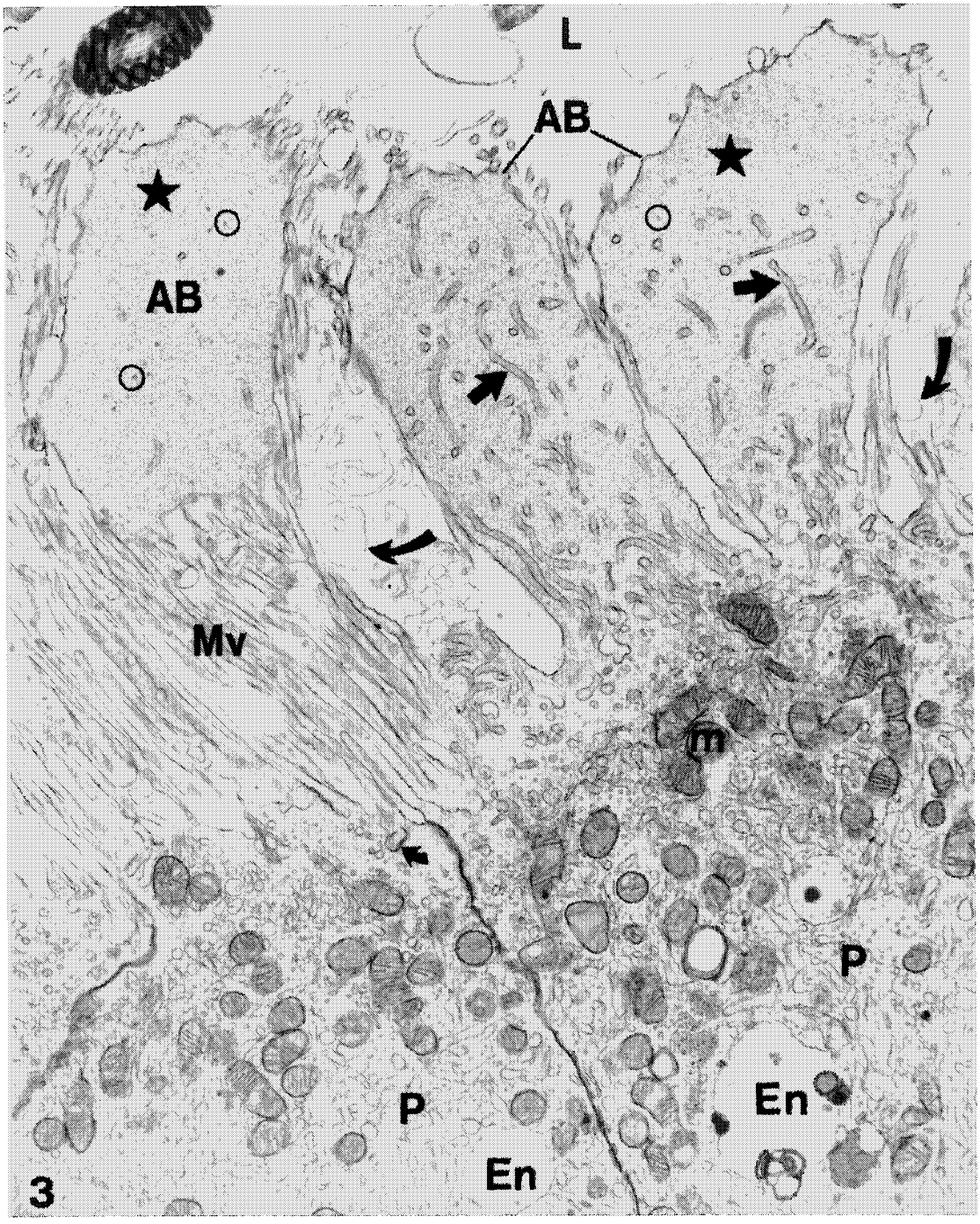

L
AB
AB
Mv
m
P
P
En
En
3

Figure 4: High power electron micrograph of the mouse initial segment epididymis comprised mainly of principal cells (P). Apical blebs (AB) are seen free in the lumen (L) while others are in the process of formation (encircled star) and surrounded by microvilli (Mv). Found within the apical blebs are ground sustance (star), ER elements (arrow), microvesicles (circle), and vesicles (large arrowhead). Membranous vesicles of various sizes are found in the lumen (curved arrow) around the blebs. Other organelles such as mitochondria (m) are segregated from the bleb and remain in the principal cell.

X13,750

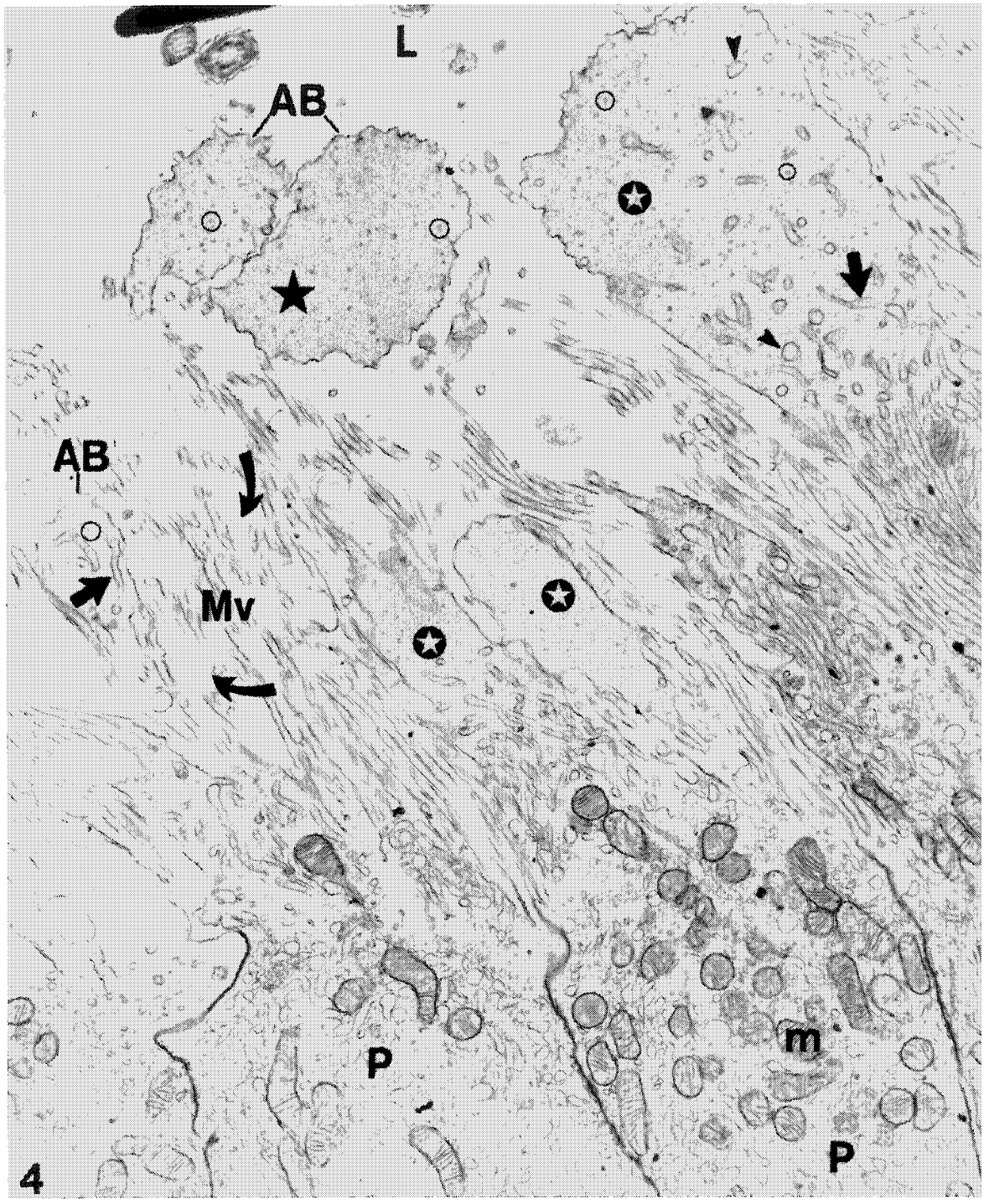

L
AB
AB
Mv
P
m
P
4

Figure 5: High power electron micrograph of an apical bleb (AB) protruding from a principal cell (P) of the mouse initial segment epididymis. Found within the bleb are ER elements (arrow), vesicles (large arrowhead), and glycogen (square). X45,600

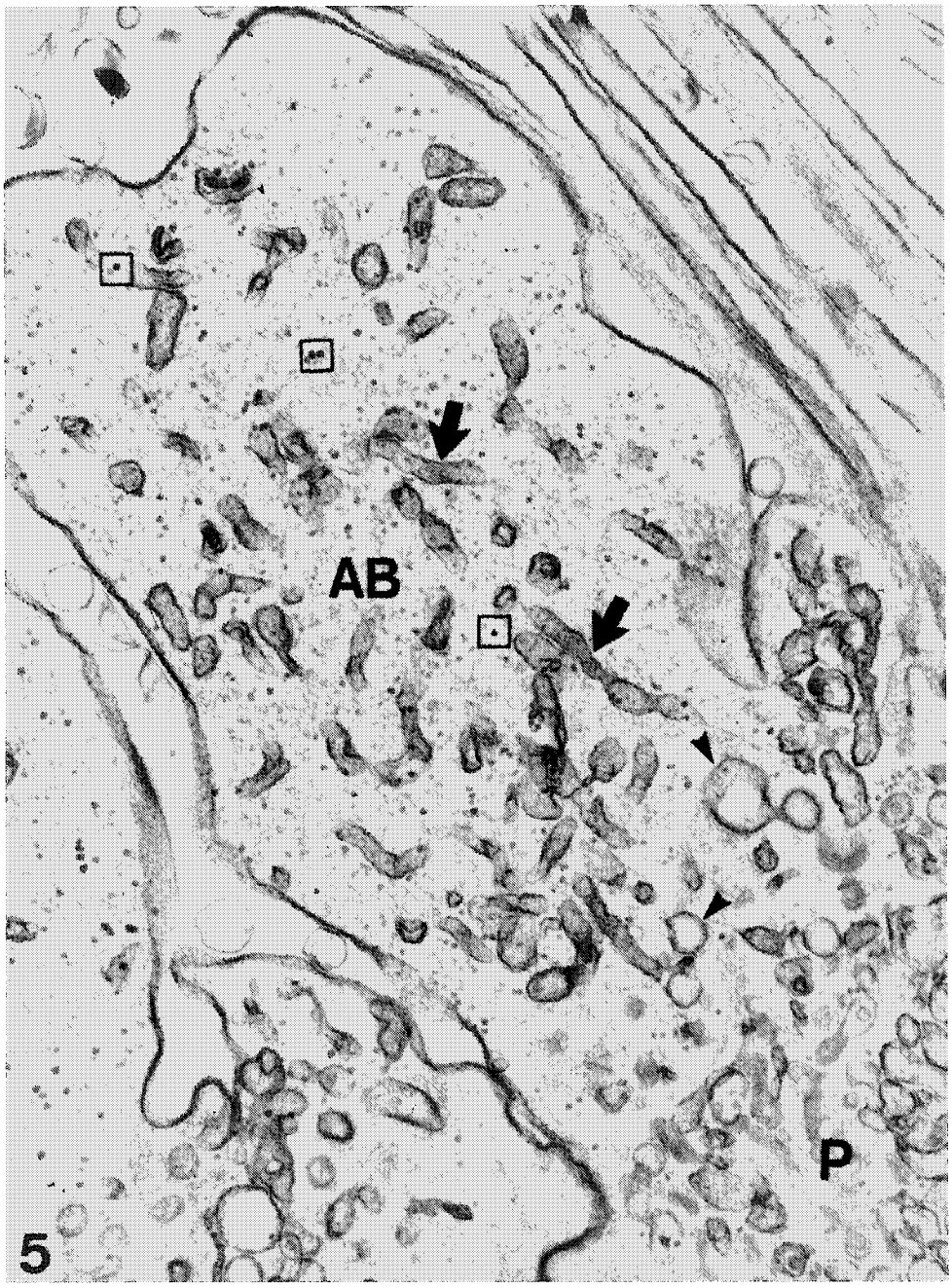
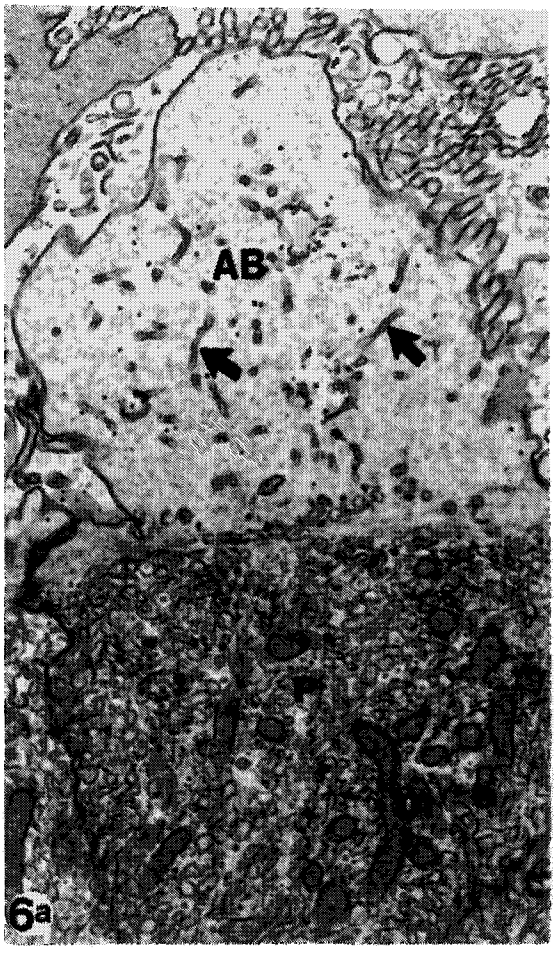

AB
P
5

Figure 6a: Low power electron micrograph of an apical bleb (AB) protruding from a principal cell (P) of the mouse initial segment epididymis. Note the segregation of organelles such that ER elements (arrow) are found within the bleb, while mitochondria (m) are not. X9900

Figure 6b: High power electron micrograph of an apical bleb (AB) protruding from a principal cell (P) of the mouse caput epididymis. Microvilli (Mv) are also seen emanating from the principal cell and surrounding the bleb. Found within the bleb are vesicles (large arrowhead) and microvesicles (circle), while mitochondria (m) remain segregated in the principal cell. Note the presence of coated pits (small arrowhead) in the apical membrane of principal cells. X29,250


AB
P
6a

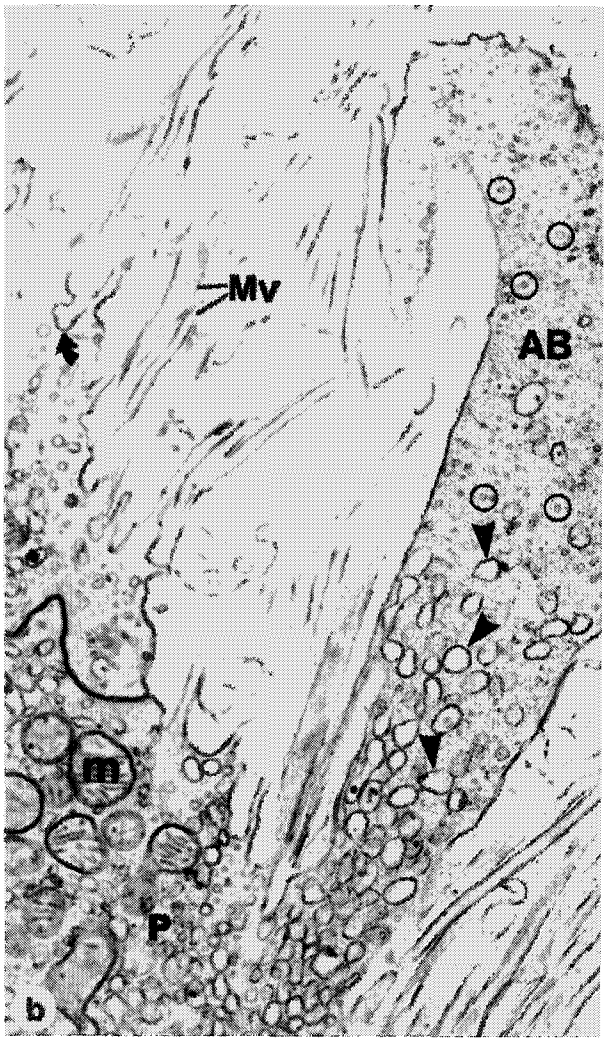

Mv
AB
m
P
b

Figure 7: High power electron micrograph of numerous apical blebs (AB) in different stages of formation seen protruding from principal cells (P) of the mouse initial segment epididymis. Microvilli (Mv) are seen around the blebs as well as vesicles of various sizes (curved arrow). Glycogen (square) is found within the apical blebs. X41,000

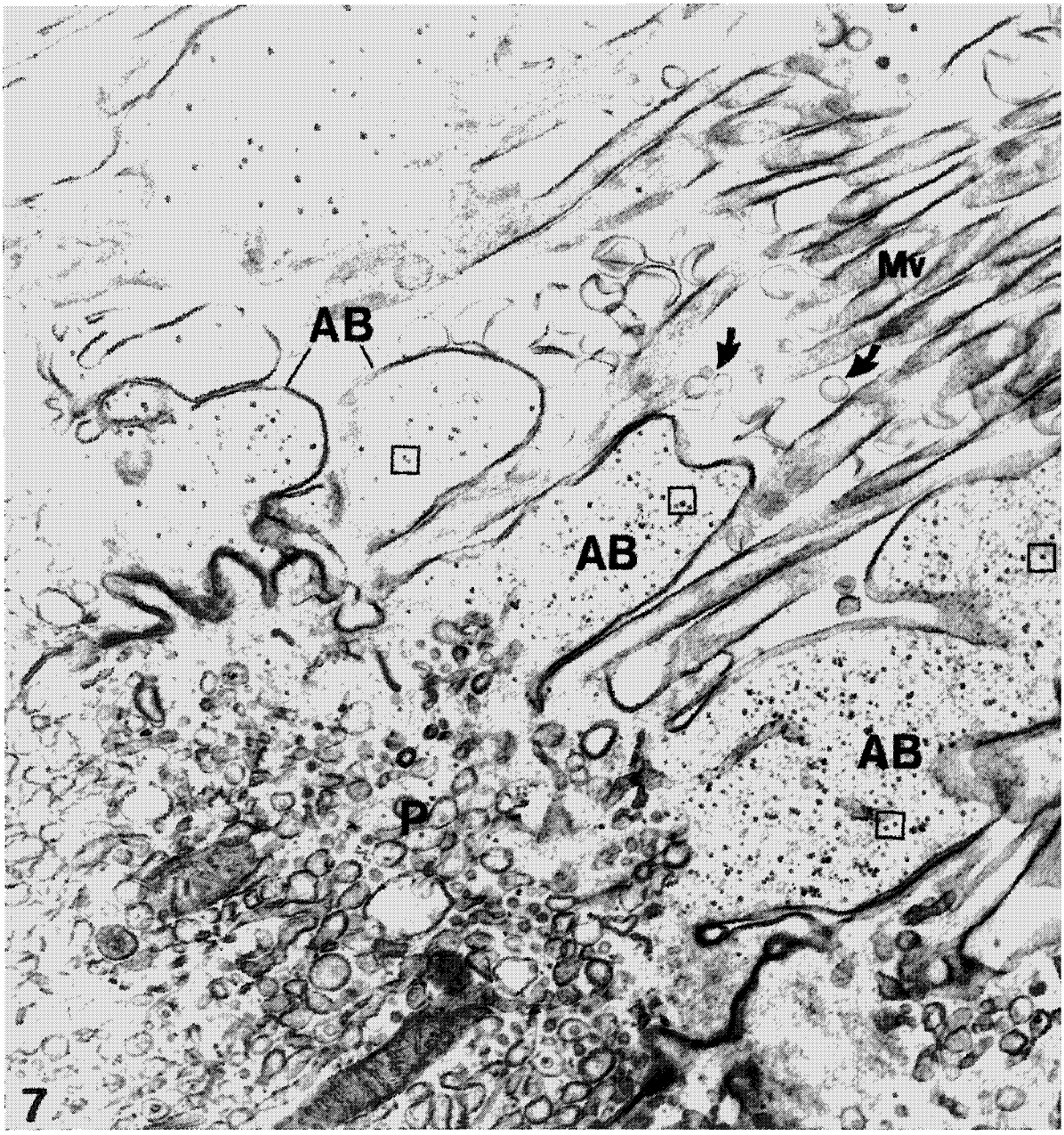

AB
Mv
AB
AB
P
7

Figure 8: High power electron micrograph of an apical bleb (AB) protruding from a principal cell (P) of the mouse initial segment epithelium via a thin stalk. Surrounding the bleb are microvilli (Mv) and vesicles (curved arrow) of various sizes. Found within the bleb are microvesicles (circle) and ribosomes (small arrowhead). X13,750

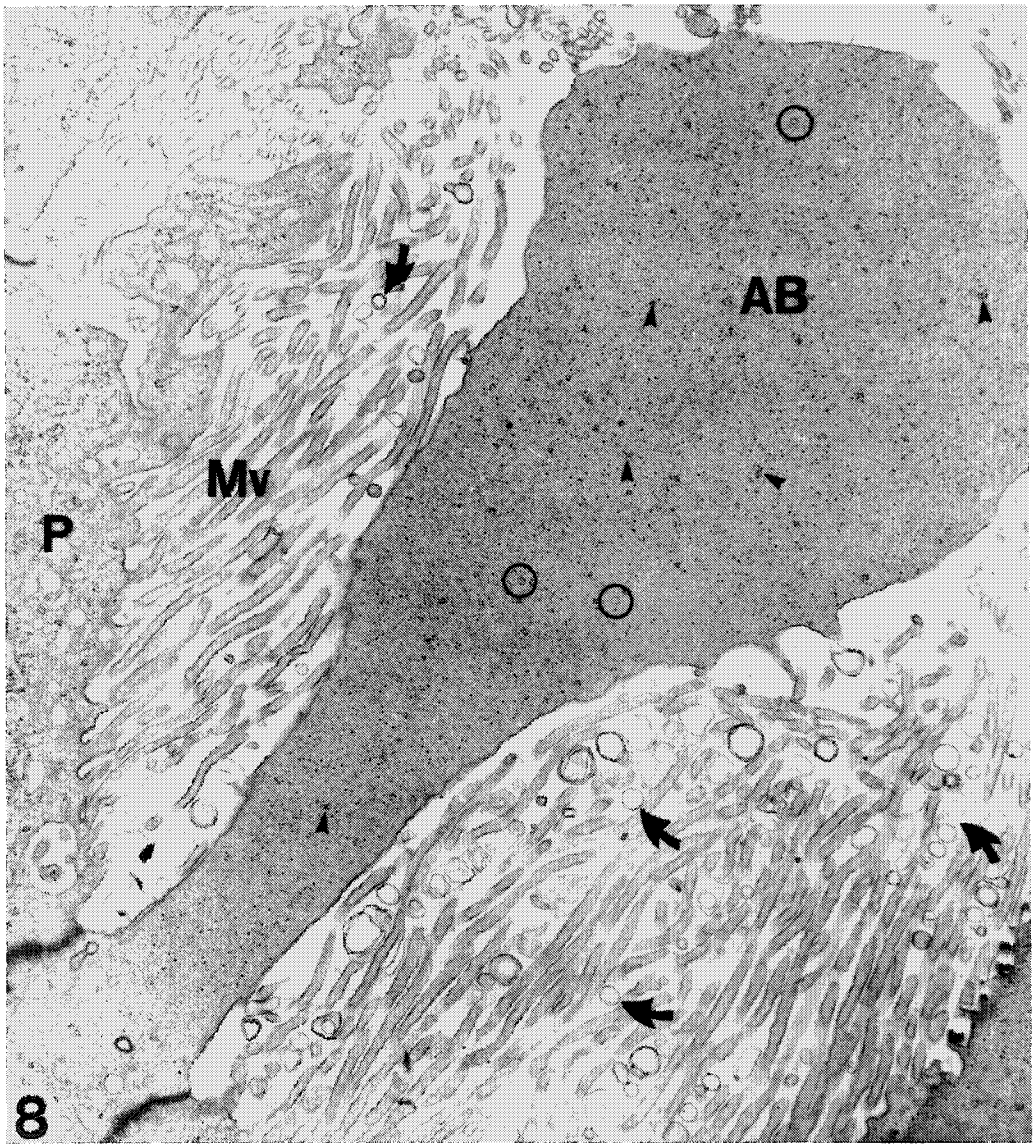

AB
Mv
P
8

Figure 9: High power electron micrograph of an apical bleb protruding from a principal cell (P) of the mouse caput epithelium. Note the vesicles of various sizes (curved arrow) surrounding the bleb. Found within the bleb are ER elements (arrow) and ribosomes (small arrowhead), while lysosomes (Ly) remain within the principal cell. X29,250

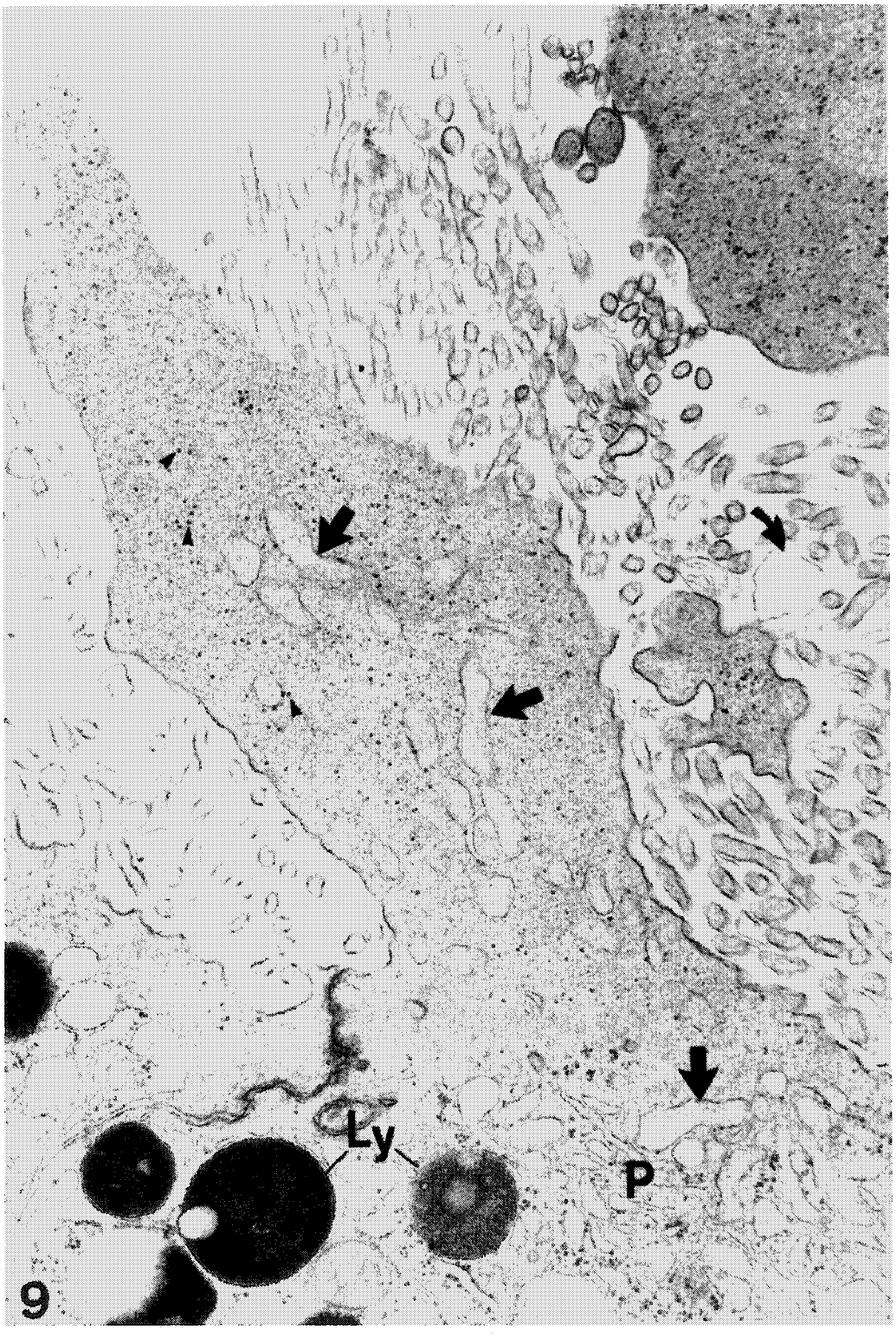

Ly
P
9

Figure 10: High power electron micrograph of an apical bleb detaching from a principal cell (P) of the mouse caput epididymis. Note the region of detachment (bent arrows) with microvilli (small arrow) found within. Vesicles (curved arrow) of various sizes are seen around the bleb. Ribosomes (small arrowhead), microvesicles (circle) and vesicles (large arrowhead) are found within the bleb, while ER elements (arrow) are seen at the base of the bleb. X29,250

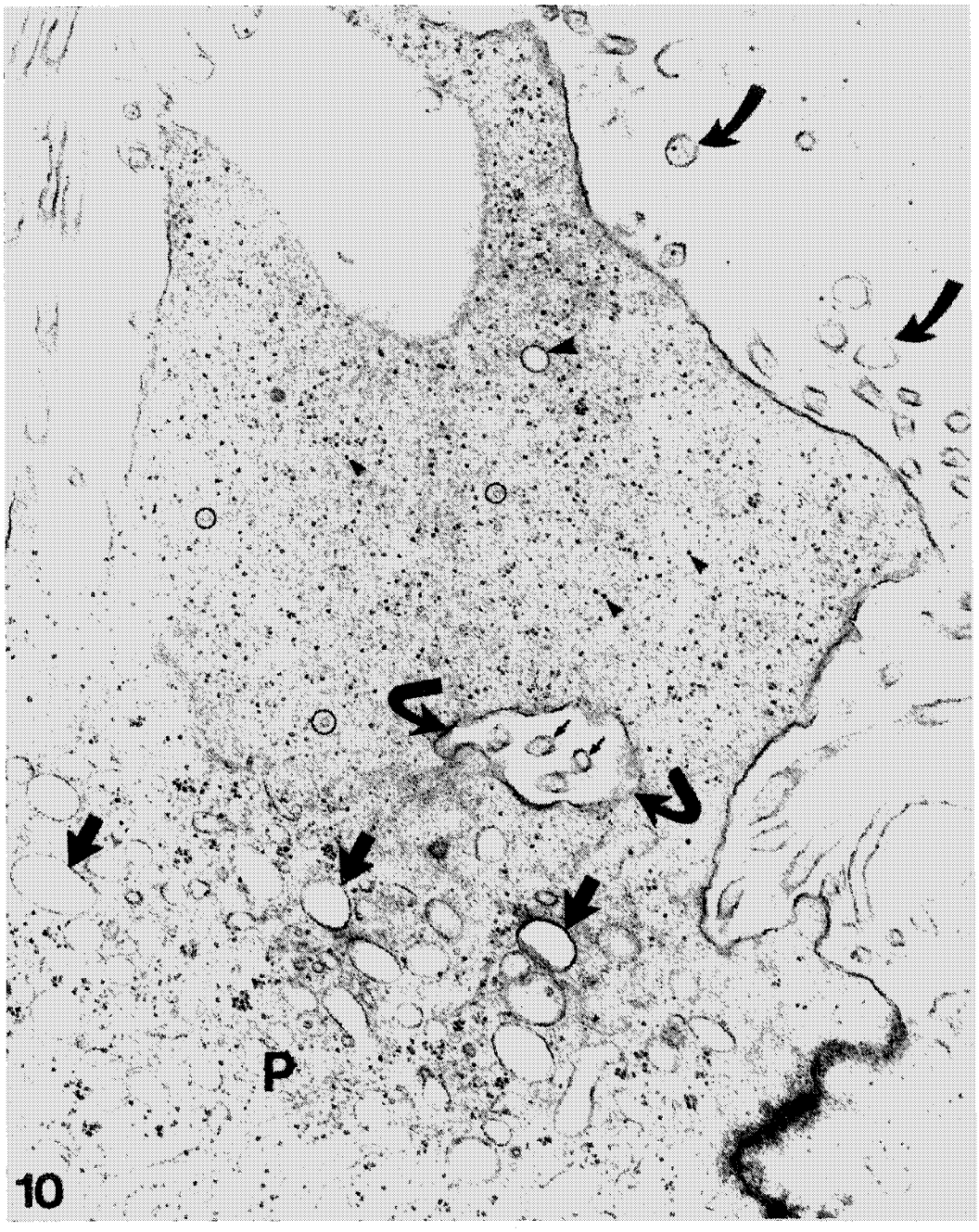

P
10

Figure 11: High power electron micrograph of an apical bleb detaching from a principal cell (P) of the mouse initial segment epididymis. Note the region of detachment (bent arrows). Microvesicles (circle) are found within the bleb, while vesicles are found around the bleb (curved arrow). X29,250

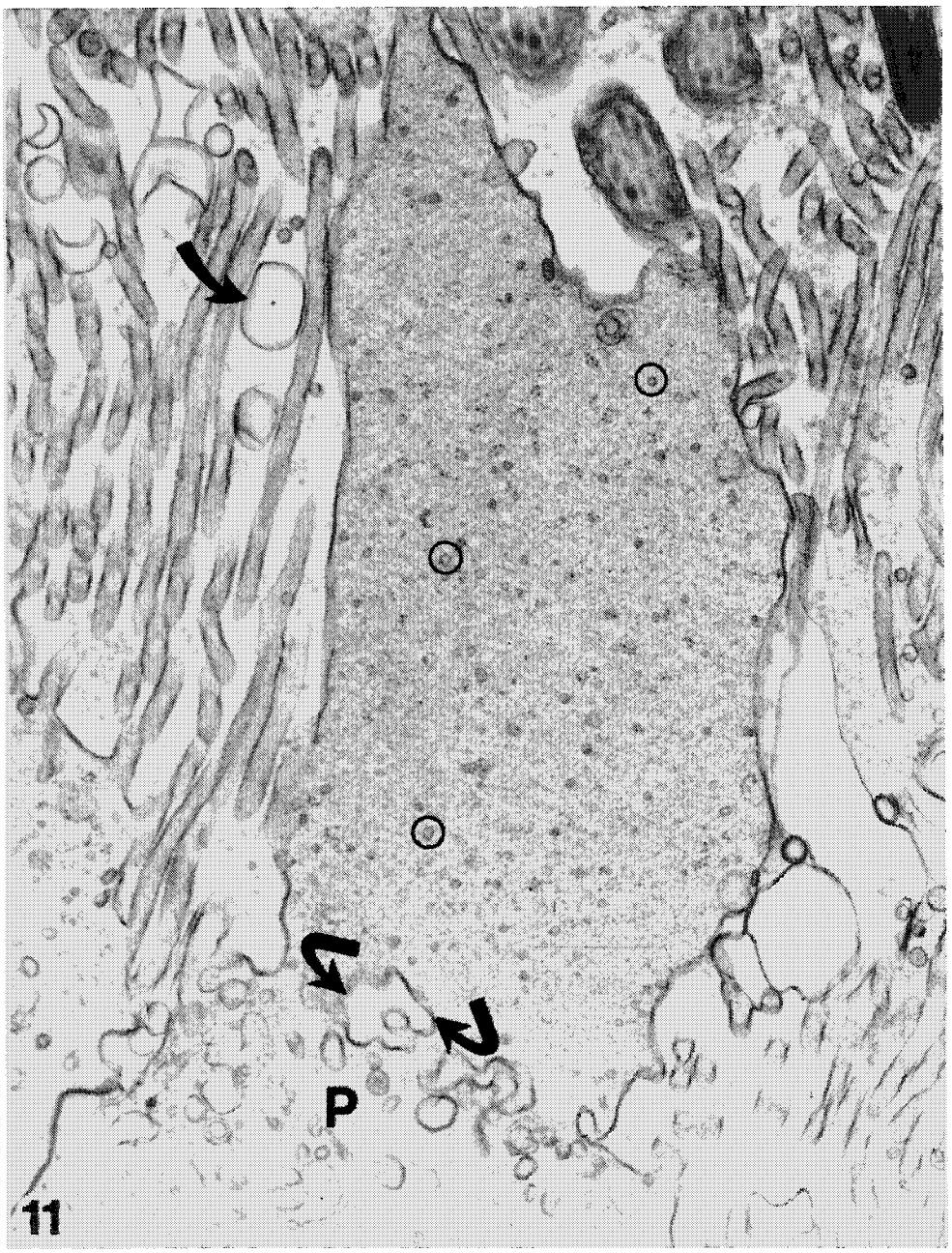
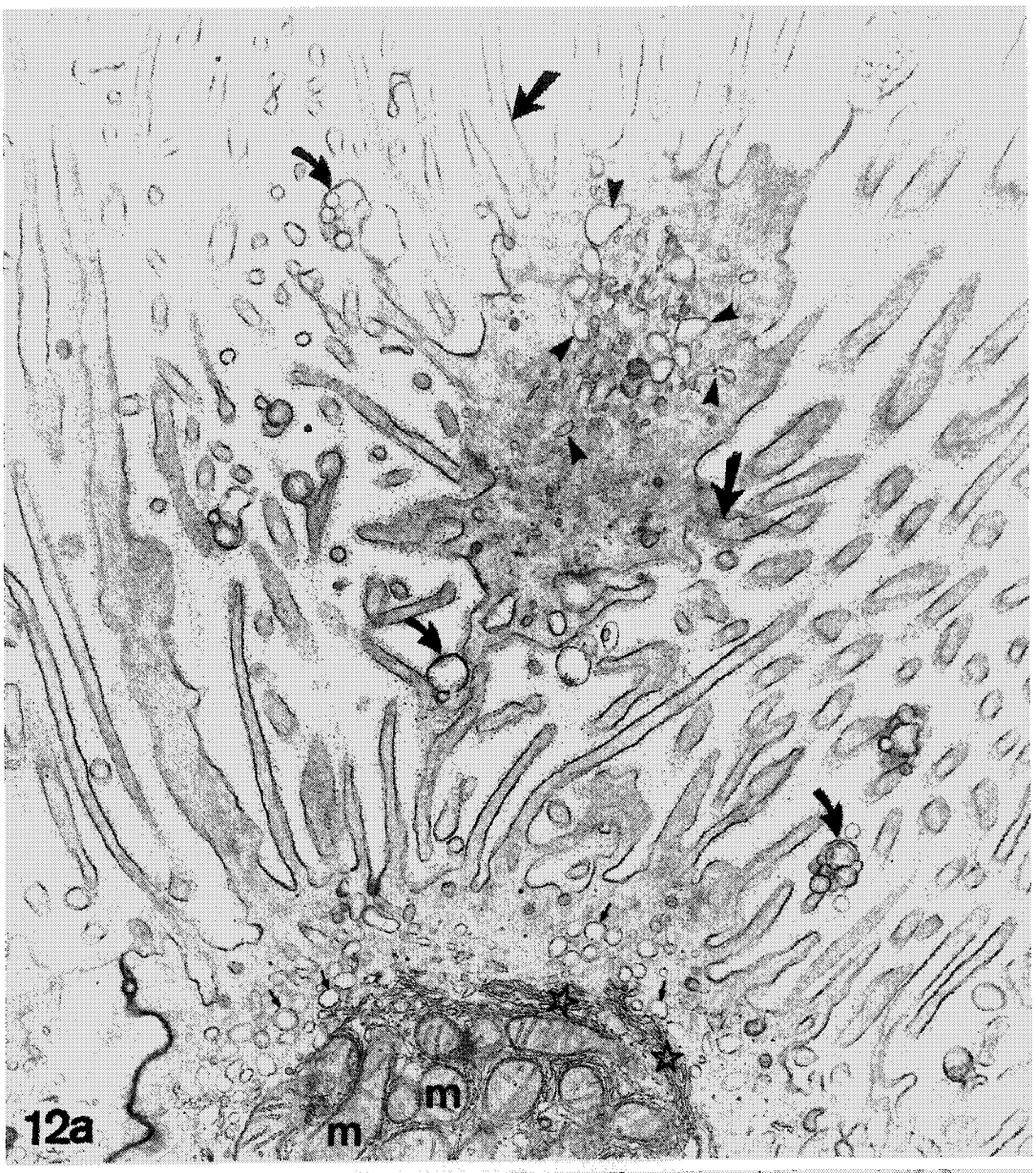

1
2
P
11

Figure 12a: High power electron micrograph of an apical bleb free in the lumen of mouse initial segment epididymis. Vesicles of various sizes are found within the apical bleb (arrowhead) and in the lumen (curved arrow). Microvilli are seen attached to the bleb (slanted arrow). Within the principal cell are mitochondria (m), Golgi (star), and vesicles (small arrow). X35,100

Figure 12b: High power electron micrograph of an apical bleb free in the lumen of the mouse initial segment epididymis. Vesicles of various sizes are found within the bleb (arrowhead) and in the lumen (curved arrow). Microvilli (slanted arrow) are seen attached to the bleb. X38,000


12a
m
m

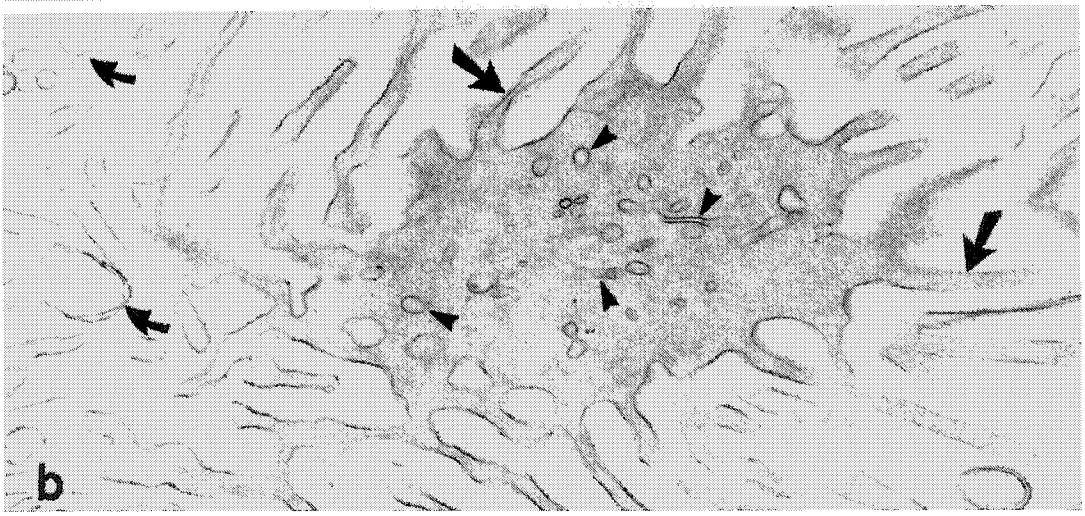
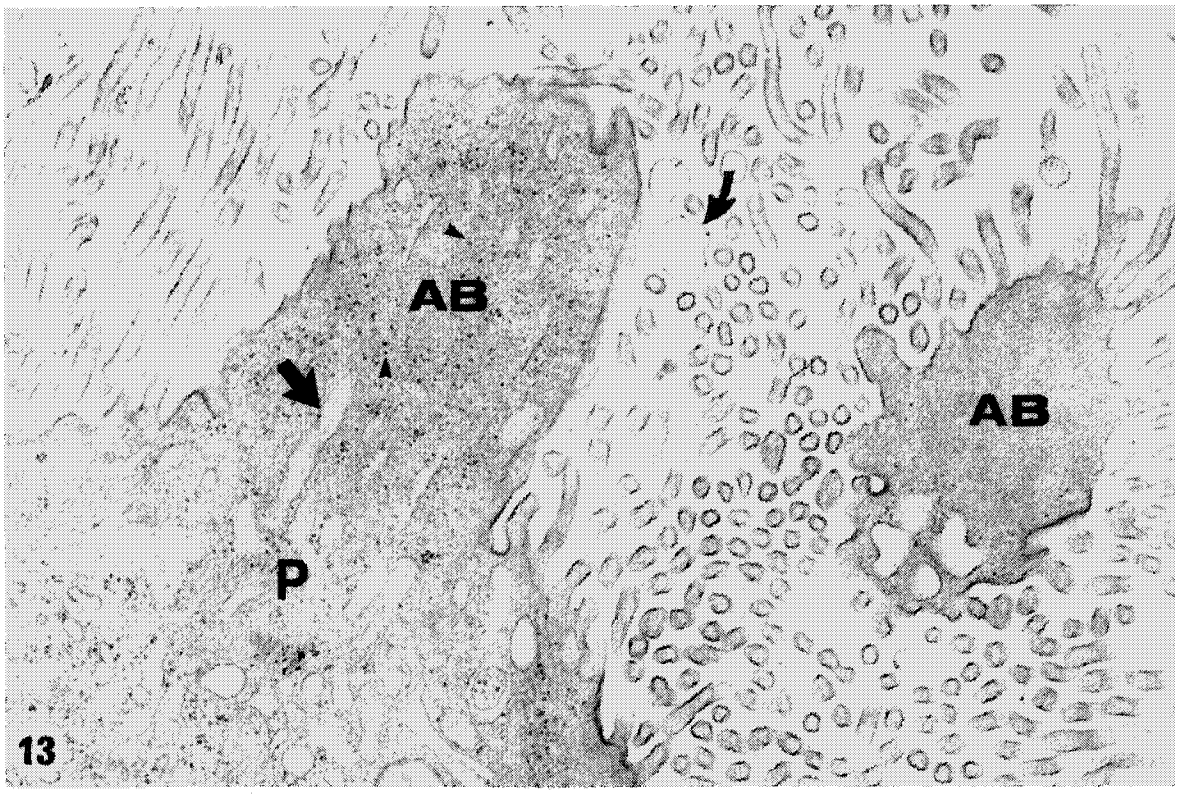

b

Figure 13: High power electron micrograph of an apical bleb (AB) protruding from a principal cell (P) of the mouse caput epididymis and one seen free in the lumen. Vesicles (curved arrow) are found around the apical blebs. Within the apical blebs are ER elements (arrow) and ribosomes (small arrowhead). X29,250

Figure 14: High power electron micrograph of apical blebs (AB) found free in the lumen of the mouse caput epididymis. Found within the bleb are ER elements (arrow), vesicles (large arrowhead), polysomes (triangle), and ribosomes (small arrowhead). Vesicles (curved arrow) are also found around the blebs. X29,250


AB
AB
P
13

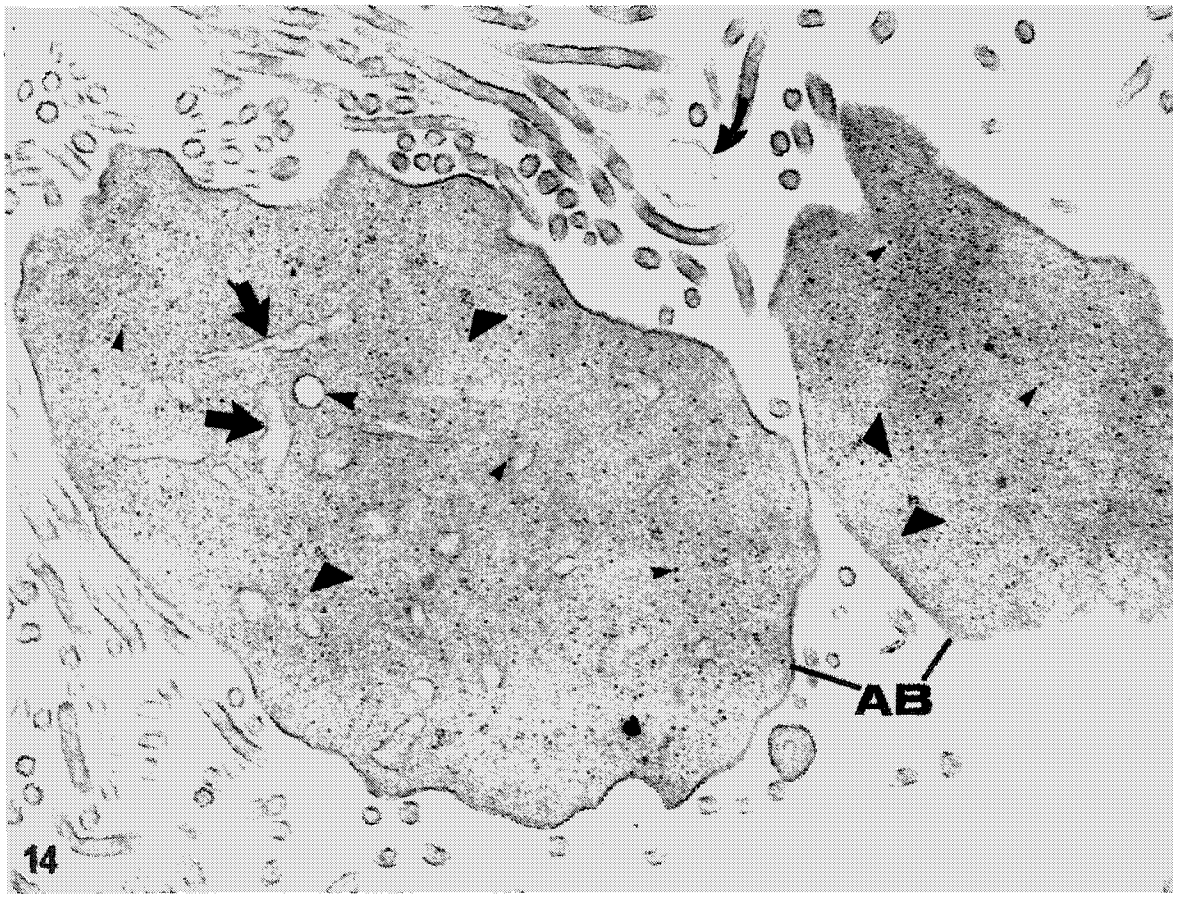

AB
14

Figure 15: High power electron micrograph of apical blebs found free in the lumen of the mouse initial segment epididymis. Found within the blebs are ER elements (arrow), ground substance (star), vesicles (large arrowhead), polysomes (triangle), and ribosomes (small arrowhead). X29,250

Figure 16: High power electron micrograph of apical blebs found free in the lumen of the mouse intermediate zone epididymis. Within the blebs are ground substance (star) and ribosomes (small arrowhead). X29,250

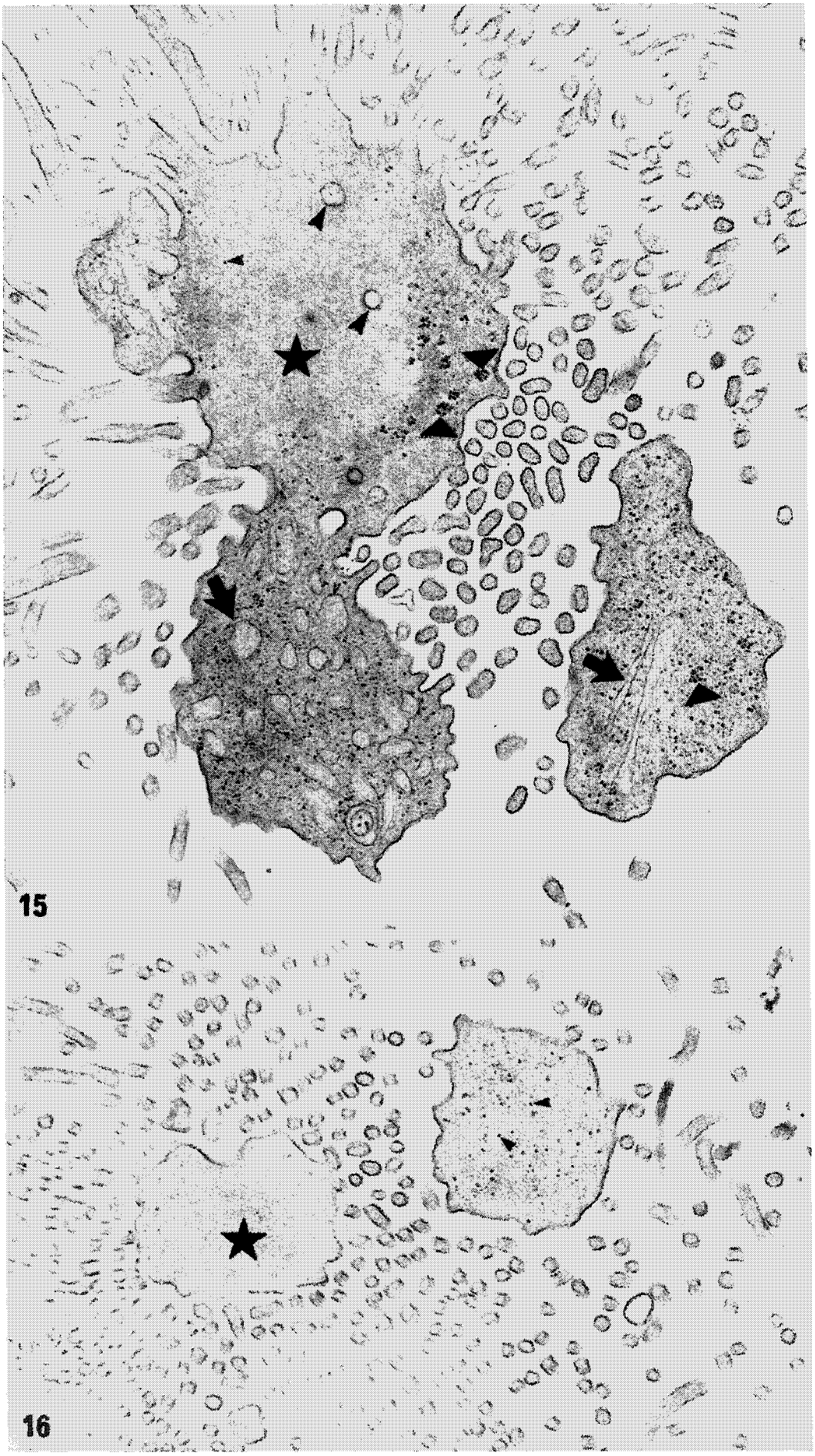

15
16

Figure 17a: High power electron micrograph of an apical bleb (AB) free in the lumen of the mouse initial segment epididymis surrounded by microvilli (Mv). Polysomes (triangle) and microvesicles (circle) are found within the bleb. X29,250

Figure 17b: High power electron micrograph of an apical bleb (AB) that seems to be disintegrating within the lumen of the mouse initial segment epididymis. Vesicles (curved arrow) and microvilli (Mv) are found around the bleb. Microvesicles (circle) are found within the bleb. X35,000

Figure 17c: High power electron micrograph of an apical bleb (AB) that seems to be disintegrating within the lumen of the mouse intermediate zone epididymis. Vesicles (curved arrow) and microvilli (Mv) are found around the bleb. Microvesicles (circle) are found within the bleb. X38,000

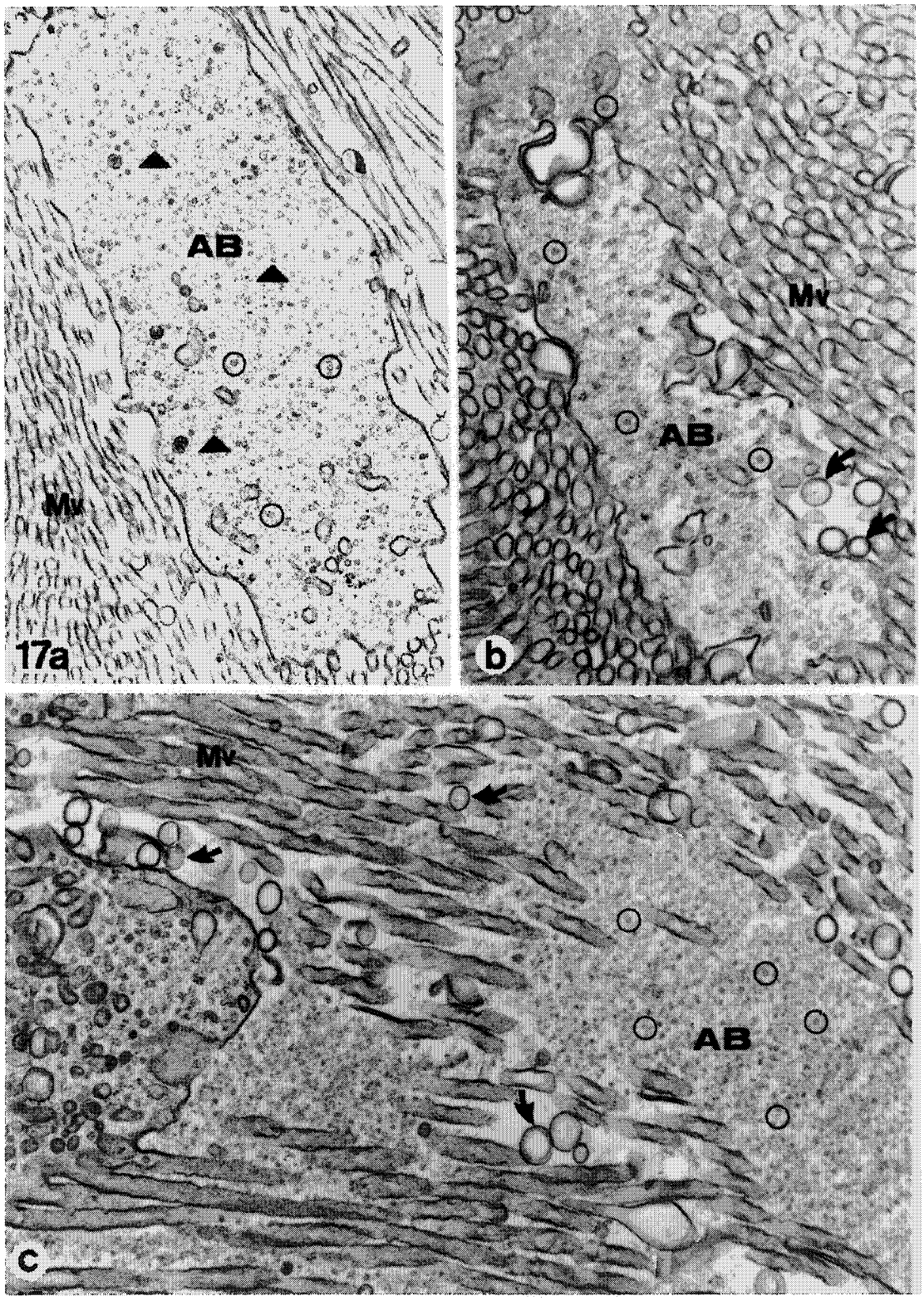
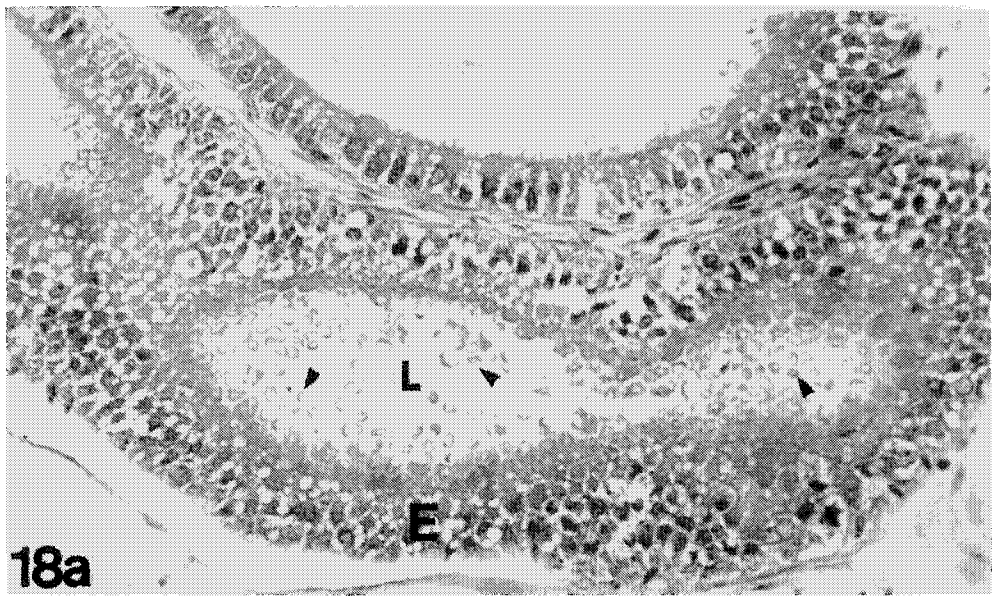
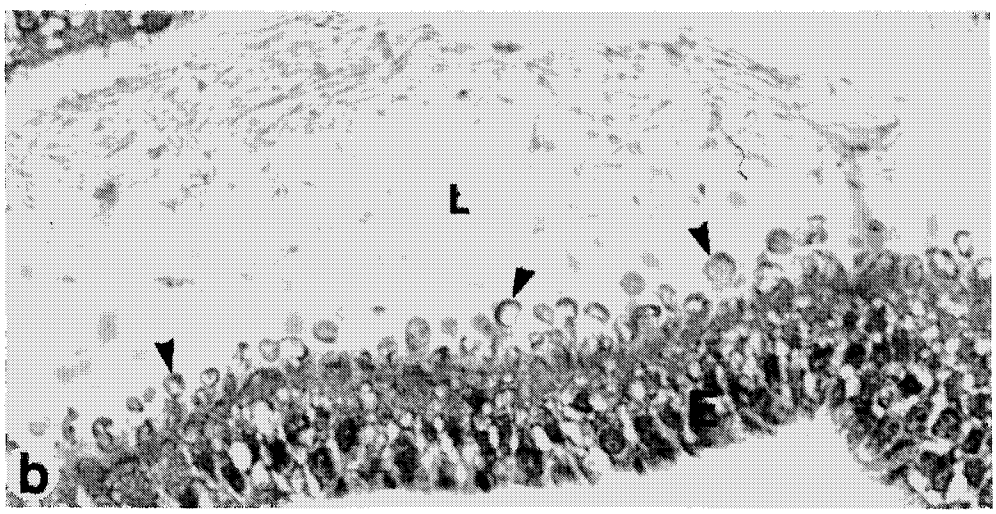

AB
Mv
17a
Mv
AB
b
Mv
AB
c

Figure 18a, b, and c: Light micrographs of the mouse corpus epithelium immunostained with an anti-Ubiquitin antibody. Note the cytoplasmic reaction seen in the epithelium (E) and the reaction also found within apical blebs (arrowhead) that are either attached to the epithelium or found free floating in the lumen (L). X25 (a) X40 (b) X80 (c)


L
E
18a


L
E
b

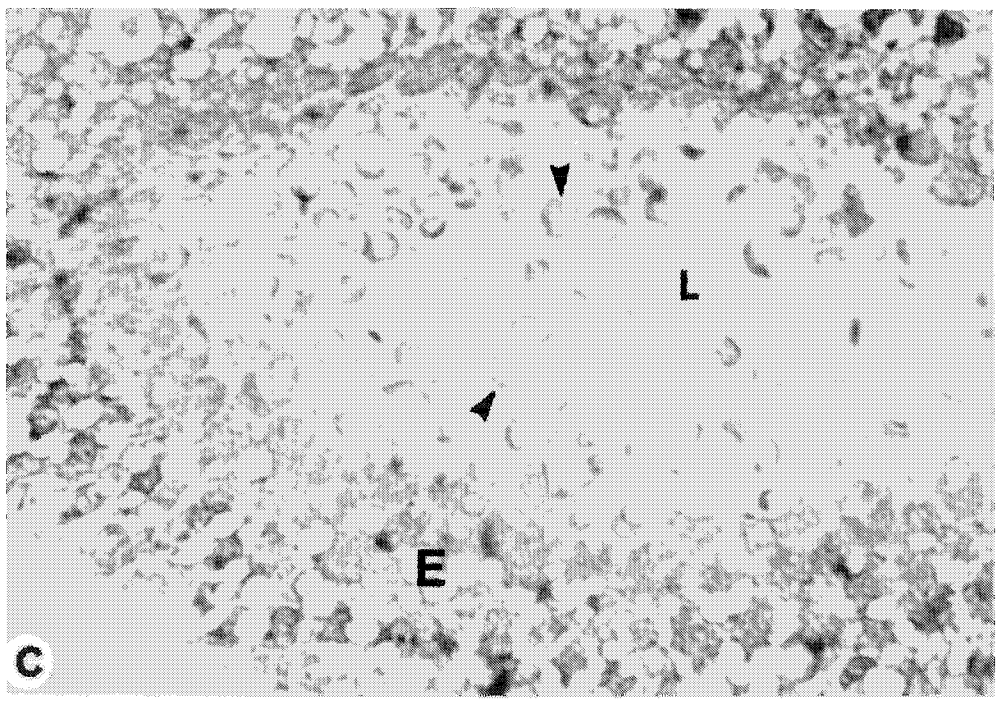

L
E
c

Figure 19 a, b, c: Light micrographs of the rat middle vas deferens epithelium immunostained with an anti-CFTR antibody. Note the cytoplasmic reaction seen in the epithelium (E) and the reaction also found within apical blebs (arrowhead) that are either attached to the epithelium or found free floating in the lumen (L).
X40 (a) X80 (b) X80 (c).

Figure 19d: Light micrograph of the rat distal vas deferens immunostained with an anti-AQP-6 antibody. Note the apical reaction of the epithelium (E) as well as the reaction in the membrane of the apical blebs found in the lumen (L). X160

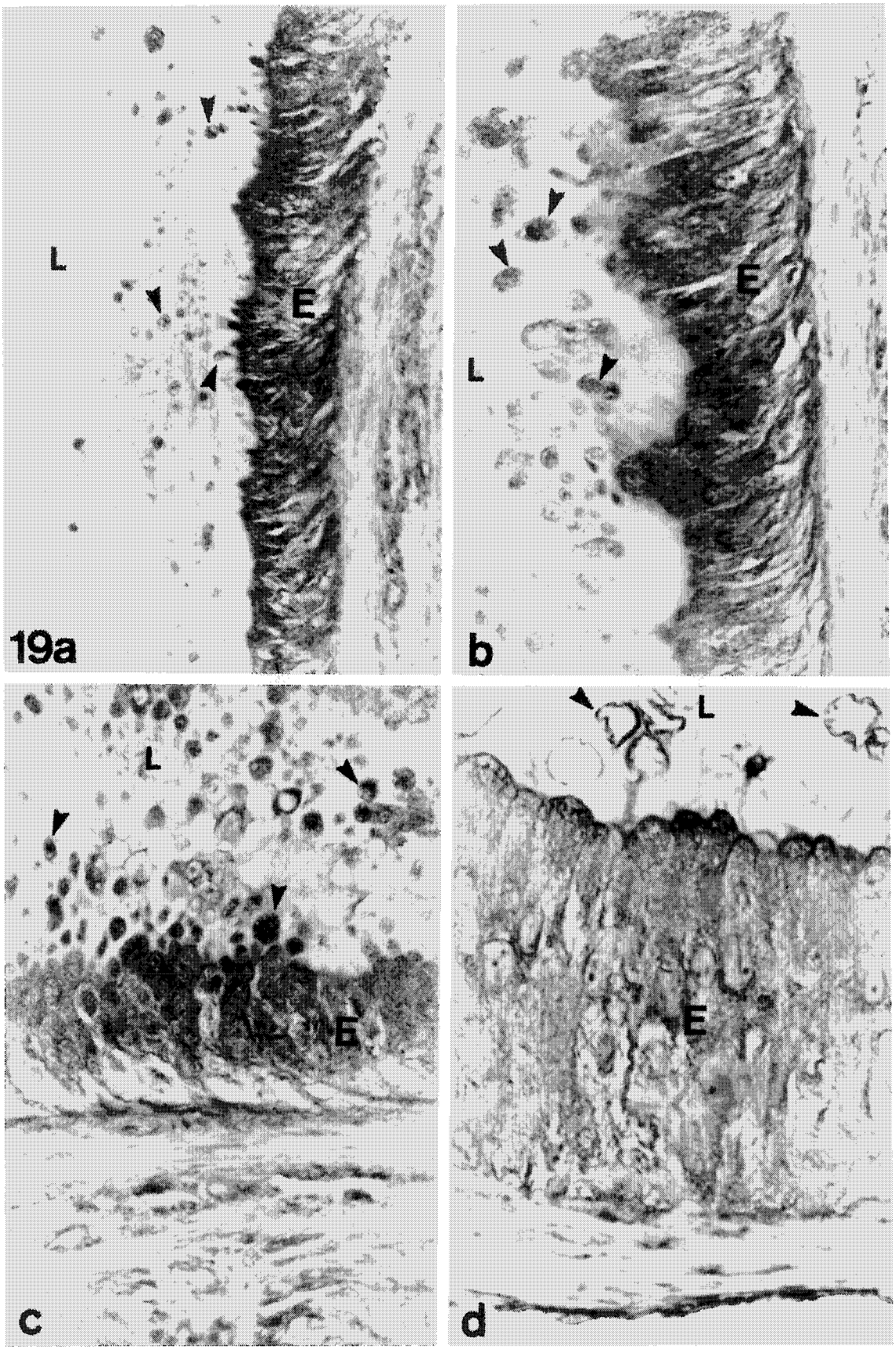

L
E
19a
L
E
b
L
E
c
L
E
d

Figure 20 inset: Light micrograph of the epithelium (E) of the rat distal vas deferens immunostained with an antibody raised against the cytoplasmic droplet of rat sperm. Note reaction over cytoplasmic droplets of sperm in the lumen seen as small vesicular profiles (arrowheads).

Figure 20: Electron micrograph of the lumen of the distal rat vas deferens. Numerous small (curved arrows) vesicular elements are noted amongst the microvilli (Mv) of principal cells (P) as well as large irregular membranous profiles (arrows).

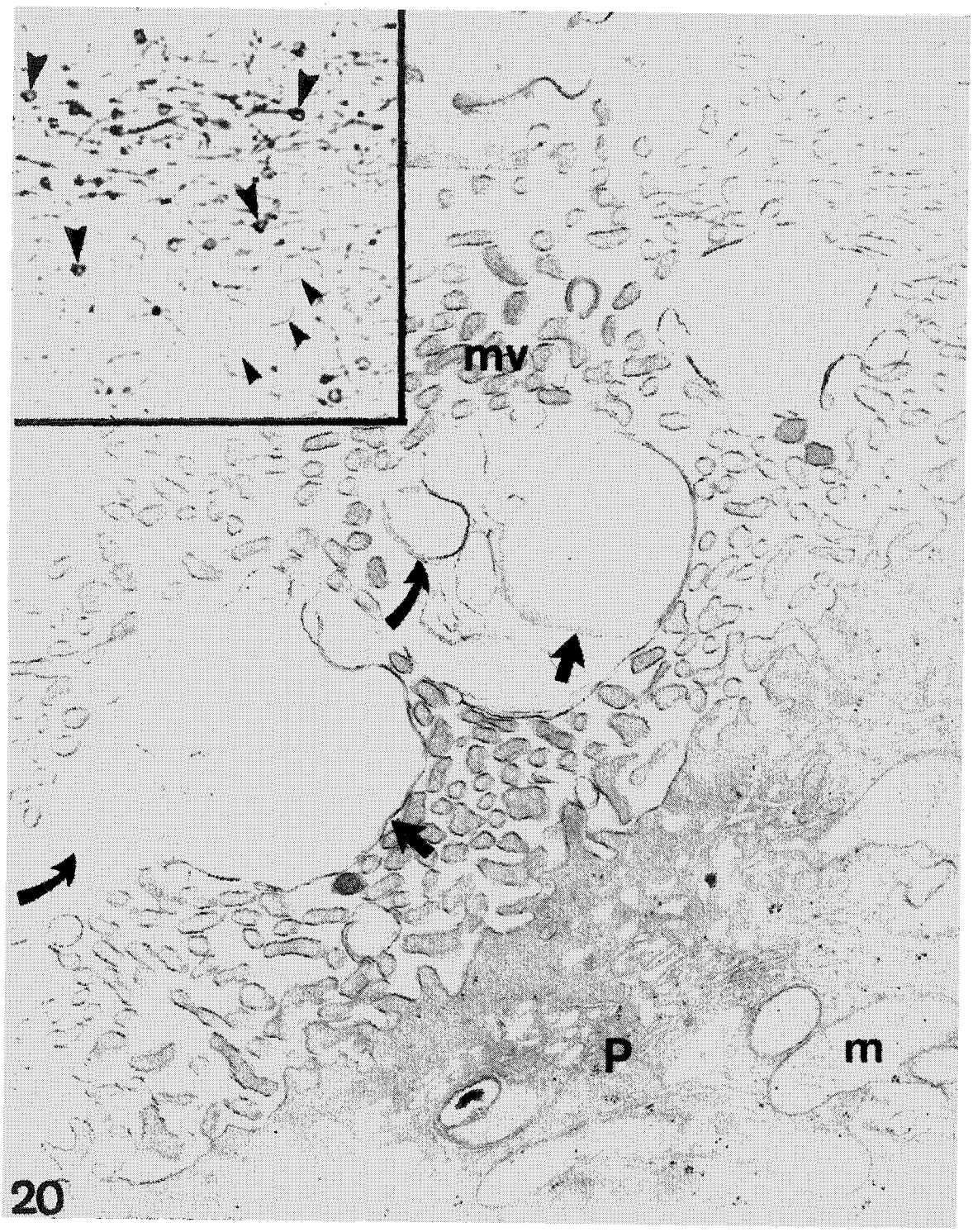

my
P
m
20

Figure 21 inset: Light micrograph of the lumen of the distal rat vas deferens immunostained with an anti-CD antibody. A reaction appears only over the cytoplasmic droplets of sperm and is seen as small vesicular elements (large arrowheads). The larger vesicular profiles are unreactive (small arrowheads). X80

Figure 21: Electron micrograph of the lumen of the rat distal vas deferens. Amongst the microvilli (Mv) are vesicles (curved arrows) and large spherical and flattened membranous profiles (arrows). Mitochondria (m) are found within the principal cells. X30,400

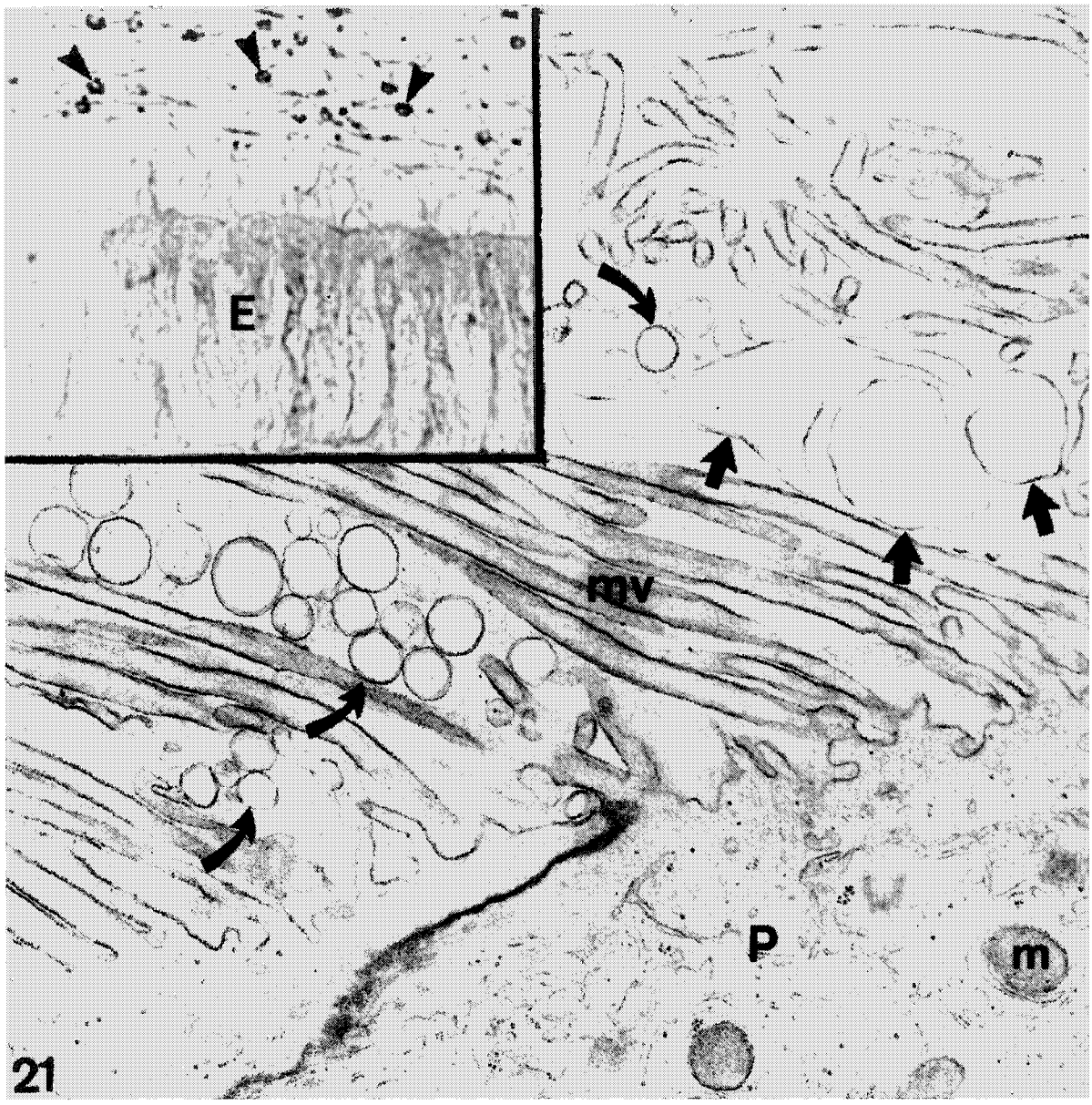

E
mv
P
m
21

Figure 22 a-d: Light micrographs of the human vas deferens. Note numerous apical blebs (arrowhead) protruding from the epithelium (E) while other are found free in the lumen (L). X40 (a) X128 (b,c,d)

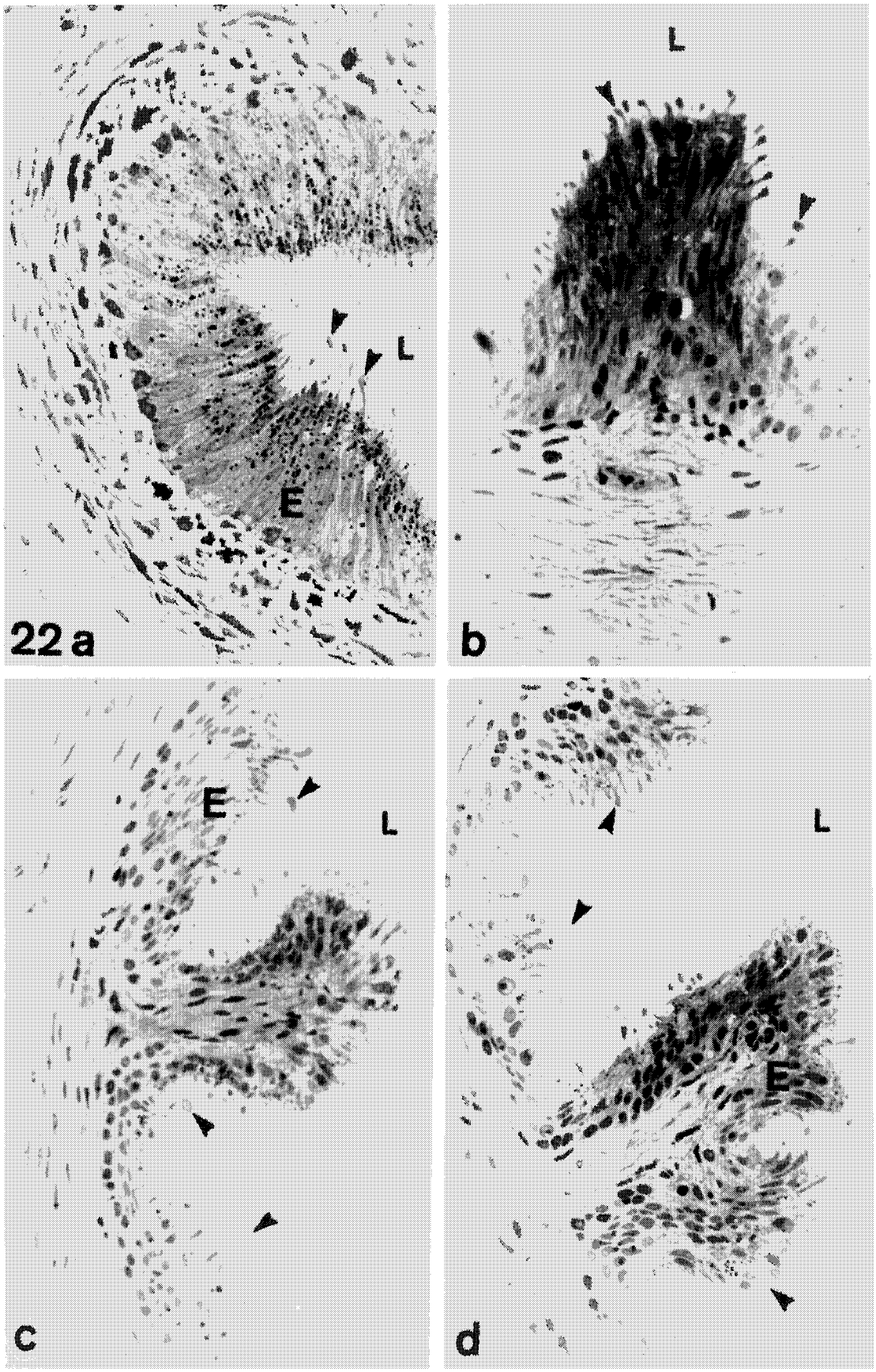

22 a
E
L
b
L
c
E
L
d
L
E

Figure 23: High power electron micrograph of tall principal cells in the human vas deferens. Contain a nucleus (N), nucleolus (n), rough endoplasmic reticulum (open arrow), lysosomes (Ly), lipofuscin granules (LF), and mitochondria (m). A junctional complex is visible at the apical plasma membrane (arrow). X 22,500

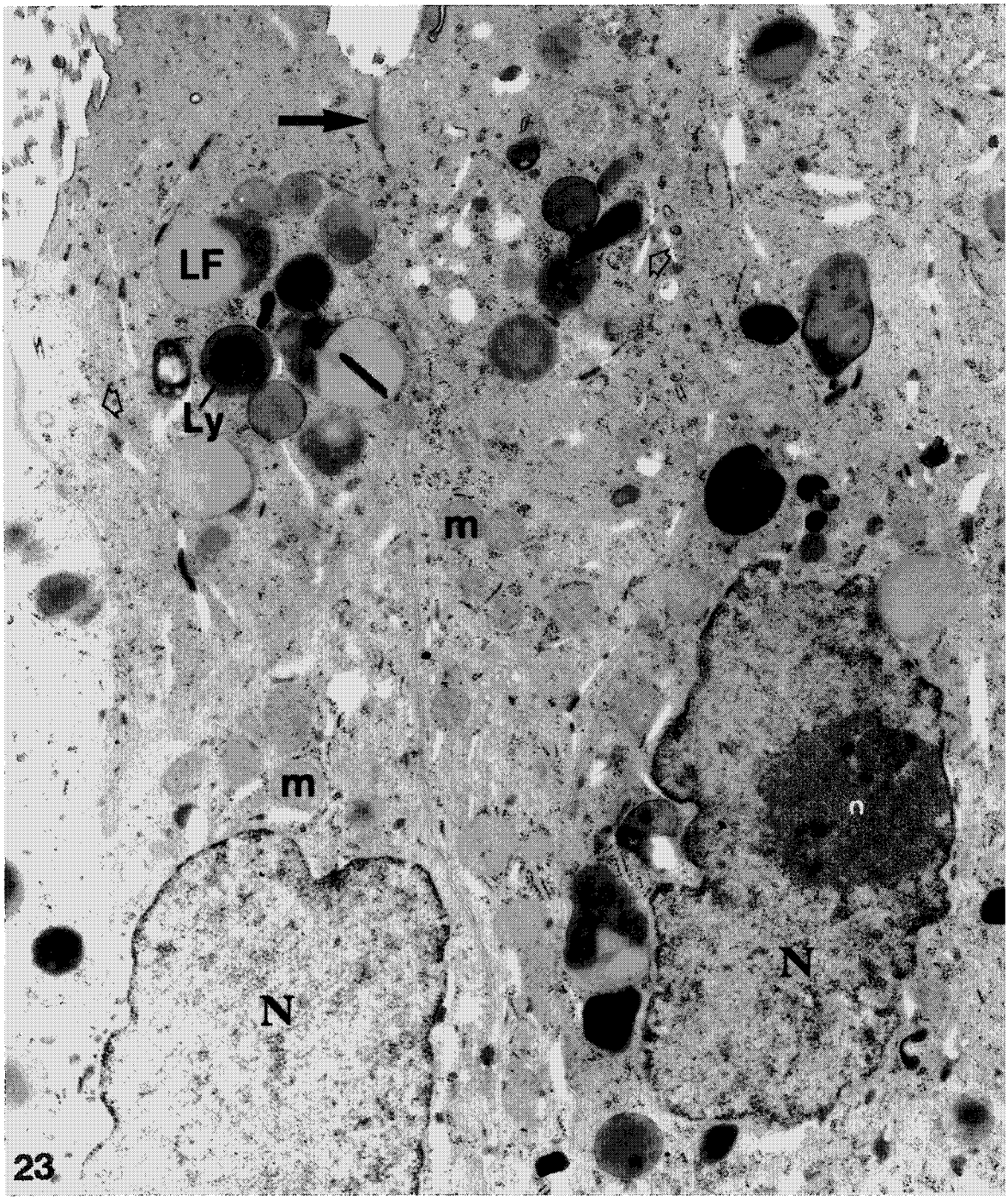

LF
Ly
m
m
N
N
23

Figure 24: High power electron micrograph of apical region of principal cells of the human vas deferens showing regular features: lysosomes (Ly), rough ER (open arrow), nucleus (N). In addition, coated pits (cp) and one bleb (AB) appearing to be detached and free in the lumen (L) are present. X 38,000

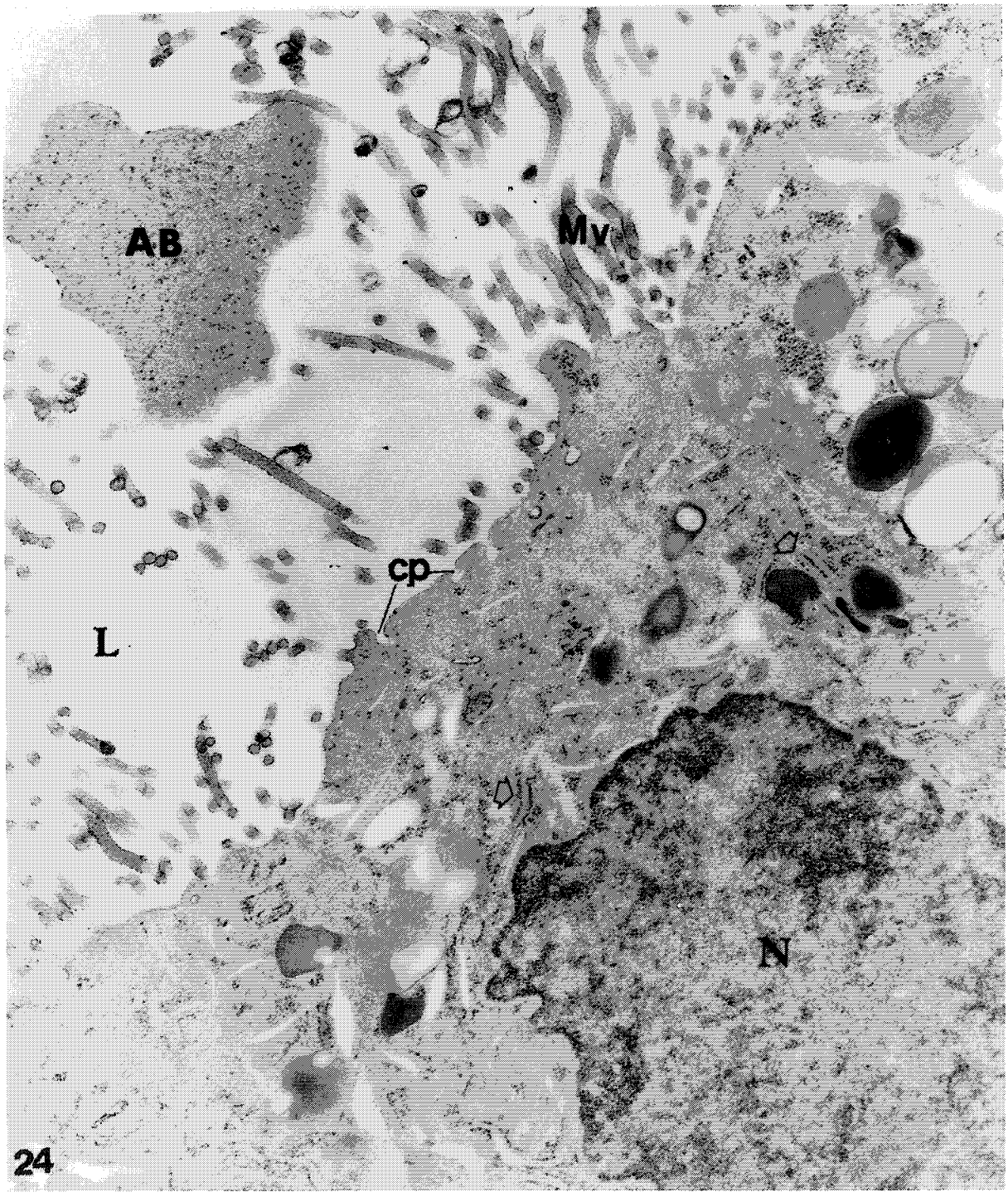
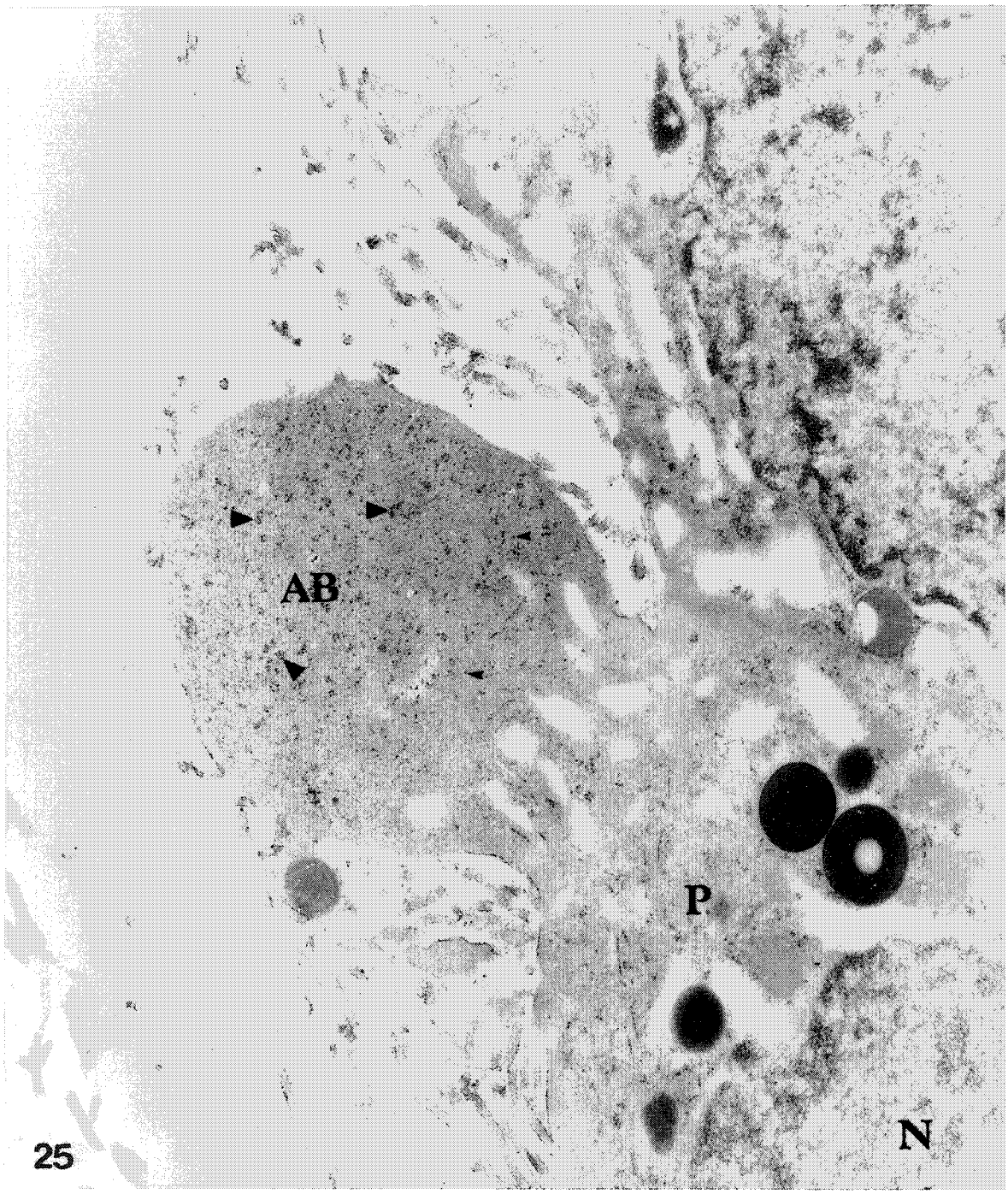

AB
My
cp
L
N
24

Figure 25: High power electron micrograph of an apical bleb (AB) appearing to detach from a principal cell (P) of the human vas deferens. Found within the bleb are polysomes (triangle) and ribosomes (small arrowhead). N, nucleus. X 29,250



25

Figure 26: High power electron micrograph of an apical bleb (AB) emanating from a principal cell (P). The apical region contains only ribosomes (small arrowheads) and polysomes (triangles), while basally, ER elements (arrow) are present. Note also the smooth plasma membrane surrounding the bleb. X 22,500

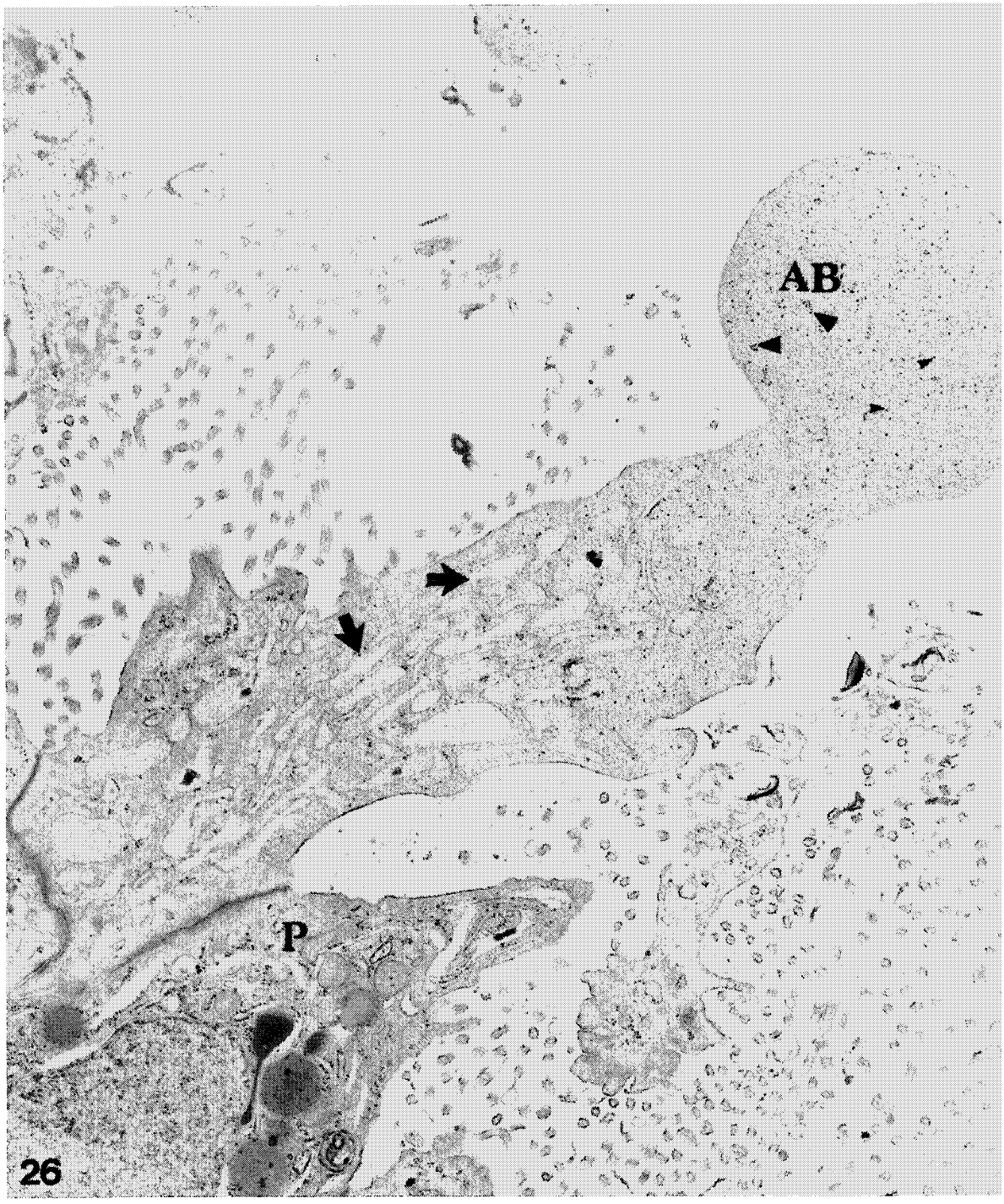
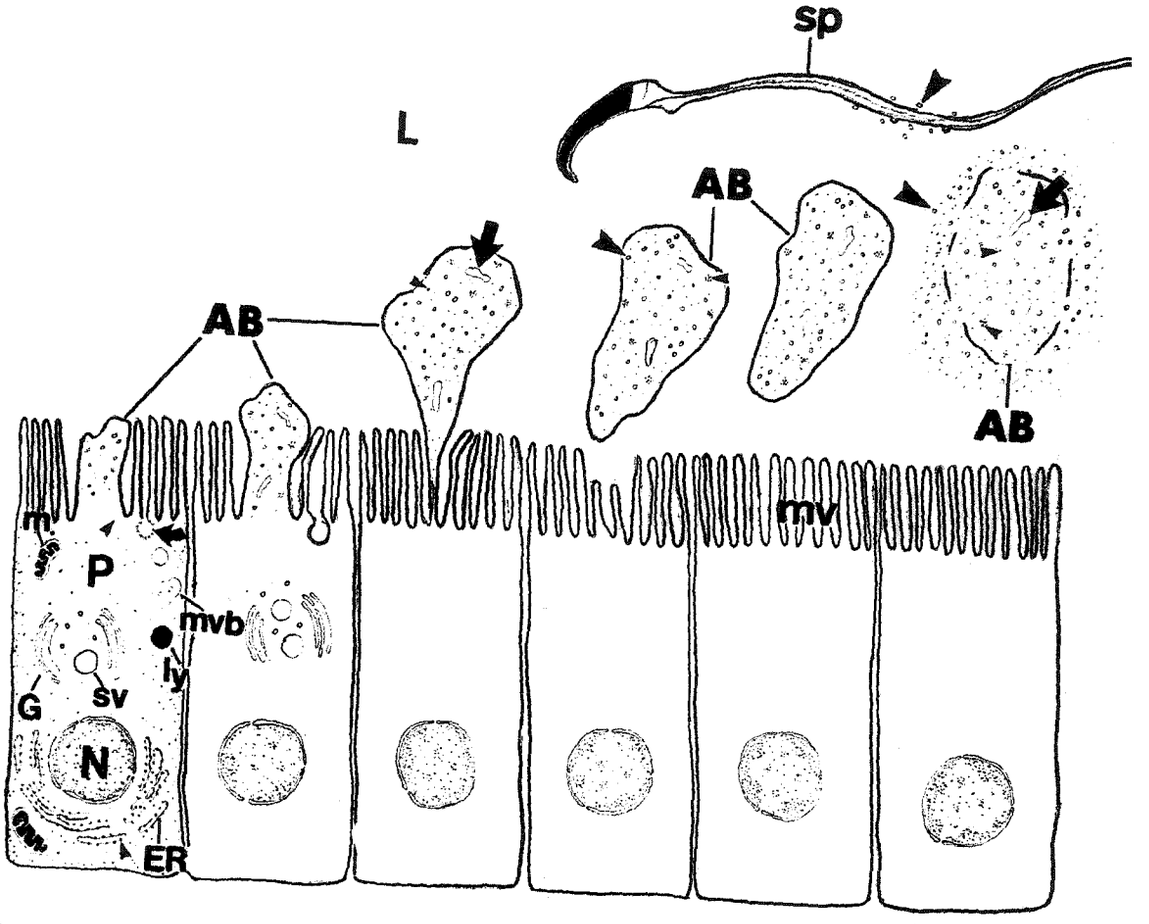

AB
P
26

Figure 27: Schematic drawing of the epididymal epithelium depicting the dynamic process apical bleb (AB) formation. The apical bleb forms at the apical membrane of principal cells (P), then forms a thin stalk and pinches off to be free in the lumen (L), then disintegrates to release its contents into the lumen. Within the apical blebs are ER elements (large arrow), ribosomes and polysomes (small arrowhead), and microvesicles (large arrowhead). Note that microvesicles can also be seen in the lumen in proximity to the apical bleb as well as interacting with sperm (sp). Other organelles remain segregated within the principal cell such as mitochondria (m), lysosomes(ly) and multivesicular bodies (mvb), and Golgi apparatus (G). Also seen within the principal cell is the rough endoplasmic reticulum (ER) studded with ribosomes, free cytoplasmic ribosomes (small arrowhead), secretory vesicles (sv), and a nucleus (N). Note the microvilli (mv) emanating from the apex of the principal cell as well as coated pits (small curved arrow) forming.


sp
L
AB
AB
AB
mv
P
mvb
ly
G
sv
N
ER

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Chapter 3:

Summary

Summary

The present study demonstrates that apical blebs are indeed true structural entities of principal cells of the mouse epididymis, and rat and human vas deferens, and are not fixation artifacts. They are seen in many different species and their morphological characteristics are consistent between species. Apical blebs seem to be involved in a dynamic process of formation, pinching off and liberation into the lumen, and disintergration with the release of their contents. Once liberated, it is suggested that the components of the blebs may interact with sperm in the lumen and perform a variety of functions.