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Endocytosis of Androgen-Binding Protein, Clusterin, and Transferrin in the Efferent Ducts and Epididymis of the Ram

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ABSTRACT: The amount of androgen-binding protein (ABP) in luminal fluid from the central caput epididymidis of the ram, on a per sperm basis, remains the same as that in rete testis fluid (RTF) entering the ductuli efferentes, although >85% of the testicular protein is absorbed in proximal sites. To determine if ABP is spared from endocytosis in proximal sites and if proteins are differentially and selectively absorbed at specific sites in the excurrent ducts, we studied the endocytosis of ABP, clusterin, transferrin, and a 26/35-kd dimer isolated from ovine RTF. Each protein was labeled with colloidal gold and microinjected into the lumen of a ductulus efferens and five specific sites in the ductus epididymidis; uptake was quantified by electron microscopy. Endocytosis of each protein, including ABP, was substantially greater in the ductuli efferentes than in any site in the ductus epididymidis. More ABP was endocytosed in proximal regions of the epididymis than any other protein studied. Endocytosis of the 26/35-kd dimer, like ABP, was greater in proximal sites of the epididymis, whereas endocytosis of clusterin and transferrin was greater in distal sites. Thus, there was a differential absorption, since proteins were endocytosed in one or another specific region of the epididymis, depending on the protein. ABP was endocytosed in the ductuli efferentes and caput epididymidis in amounts similar to or greater than other major testicular proteins, and was not spared from endocytosis in the proximal excurrent ducts. Previously reported maintenance of a similar amount of ABP, on a per sperm basis, throughout the ovine caput epididymidis probably results from recycling of endocytosed ABP or from epididymal secretion of ABP.

Key words: Testicular proteins, uptake, excurrent ducts, colloidal gold, microinjection.

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Dihydrotestosterone (DHT) is presumed to be the primary androgen driving androgen-dependent processes involved in the maturation of sperm in the epididymis, particularly in the caput and corpus epididymidis (Amann, 1987). Rete testis fluid (RTF), which conveys sperm and testicular secretions into the excurrent ducts, is rich in testosterone, but contains little DHT (Ganjam and Amann, 1976; Setchell, 1978). However, epididymal principal cells, particularly those in distal regions of the ovine caput, contain an abundance of 5α-reductase, the enzyme that converts testosterone to DHT (Klinefelter et al, 1982; Amann et al, 1987). Principal cells in these regions are also rich in androgen receptor (Tekpetey and Amann, 1988; Tekpetey et al, 1989). Localization of these androgen-related activities in the ram epididymis is similar to that in the monkey epididymis (Roselli et al, 1991). Androgen-binding protein (ABP), one of several testicular proteins present in RTF, may have a role in transporting testosterone in RTF to principal cells (Feldman et al, 1981; Pelliniemi et al, 1981; Gerard et al, 1988), facilitating conversion of testosterone to DHT. ABP in the epididymal lumen also may have a role in proluminal movement of androgens from blood (Turner, 1988; Turner et al, 1989).

If ABP has a role in androgen transport, it is logical that ABP should remain at a high concentration proximal to sites where 5α-reductase is located. Indeed, in the ram, the amount of ABP in luminal fluid of the central caput epididymidis remains unchanged, relative to the number of sperm, while more than 85% of the protein present in RTF is absorbed in the proximal excurrent ducts (Veeramachaneni et al, 1990). A similar trend toward conservation of ABP during removal of protein in the rete testis and ductuli efferentes has been observed in rats (Turner et al, 1984). It is not known how a similar amount of ABP is maintained throughout the caput epididymidis while most of the bulk protein of testicular origin is endocytosed. We determined if ABP is spared from endocytosis in proximal regions and if several major testicular proteins are selectively endocytosed at specific sites in the excurrent ducts. Endocytosis of gold-labeled ABP, clusterin, transferrin, and a 26/35-kd dimer, isolated from ovine RTF, was evaluated after microinjections in the ductuli efferentes and selected sites in the ductus epididymidis. Clusterin and transferrin are known to be major secretory products of Sertoli cells, and ABP is a minor, but important, protein (Bardin et al, 1988).

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Materials and Methods

Collection and Processing of RTF

During the breeding season, fluid leaving the testis was collected from 10 adult Suffolk or western range rams, by cannulation of the rete testis (Fig 1) as described by Suominen and Setchell (1972), except that an L-shaped cannula was used. Fluid was pumped, drop-by-drop, from the distal end of a 15-cm long cannula into a tube maintained at 5°C. Every 12 hours, accumulated fluid was centrifuged at 350g for 15 minutes to remove sperm. The supernatant was further centrifuged at 10,000g for 15 minutes; this RTF was stored at −70°C. Frozen samples of RTF from several rams were thawed, pooled, divided into aliquots, snap-frozen, and stored at −70°C. Aliquots were used for fractionation and isolation of proteins.

Isolation of Proteins

Aliquots of pooled RTF were thawed and loaded onto a Mono-Q HR5/5 anion exchange high-pressure liquid chromatography column (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with 20 mmol/L Tris (pH 7.4) and eluted using a gradient (2.14% [v/v]/minute) of 600 mmol/L NaCl in 20 mmol/L Tris (six runs totaling 35 ml; about 10 mg protein/run). Twelve fractions were collected during each run and pooled across runs. Three fractions, an unidentified protein (fraction 1), a protein thought to be transferrin (fraction 4), and one thought to be clusterin (fraction 7), were rechromatographed using a gradient of 1.86% (v/v)/minute (Fig 2a). Eluates were concentrated by ultrafiltration (Amicon YM-10; Amicon Corp, Danvers, MA) and stored at −70°C in 20 mmol/L Tris. Aliquots of concentrated proteins were subjected to one-dimensional sodium dodecyl sulfate (SDS)-gel electrophoresis (10% gel, reducing conditions; Hoefer Scientific, San Francisco, CA)) followed by silver staining (Sammons et al, 1981). Fraction 1 migrated as two bands in electrophoresis, present in approximately equal amounts; their apparent molecular weights were 26 and 35 kd. Fraction 4 contained a trace contaminant of about 95 kd in addition to the predominant band of transferrin at 78 kd. Elution time of transferrin from the anion exchange column was identical to that of authentic human transferrin, and the apparent molecular weight was appropriate. Clusterin (fraction 7) migrated as a single band of 38 kd (data not shown) and bound antibody against the rat molecule (rabbit anti-rat SGP-2 antibody, kindly provided by Dr. M. D. Griswold).

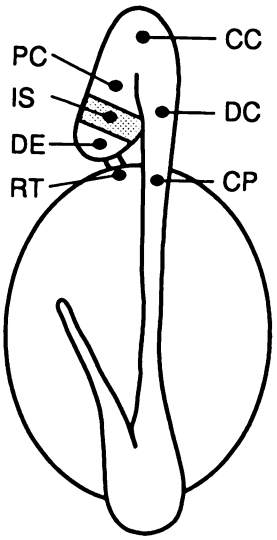

FIG. 1. Sites in the ram excurrent ducts at which cannulation or microinjections were performed. Caput epididymidis is depicted partially freed from the testis for clarity. RT = rete testis; DE = ductuli efferentes; IS = initial segment of the epididymis; PC = proximal; CC = central; DC = distal caput epididymidis; CP = proximal corpus epididymidis.

ABP was isolated by passing RTF through a DHT-Sepharose affinity column, followed by elution of the retained ABP with DHT and purification of ABP by anion exchange. A volume of 1,360 ml pooled RTF was dialyzed against TDK buffer (20 mmol/L Tris, 10% dimethylformamide, 1 mol/L KCl) at 5°C, and passed onto an affinity column (DHT-hexanoic acid coupled to Sepharose; Musto et al, 1980). After 18 hours, the column was eluted with TDK buffer containing DHT (20 mg/L); a second elution followed 12 hours later. Eluates were mixed 1:1 with TG buffer (20 mmol/L Tris, 10% glycerol) and concentrated using an Amicon YM-10 filter. Concentrated ABP was loaded onto a Mono-Q HR5/5 anion exchange column equilibrated with 20 mmol/L Tris, washed with the same buffer, and eluted using a sodium chloride gradient (Cheng et al, 1985). Ovine ABP eluted at about 260 mmol/L sodium chloride and was rechromatographed (Fig 2b). Isolated ABP was concentrated using a YM-10 filter and stored at −70°C in TG buffer. When subjected to one-dimensional SDS-gel electrophoresis as above, ABP migrated as a diffuse band (two monomer bands of the dimeric protein) at 48 kd; no contaminant was evident. Other aliquots of this ABP bound [^{3}H] DHT.

Preparation of Protein–Colloidal Gold Conjugate

A monodisperse suspension of colloidal gold particles with diameters of 8 to 10 nm was prepared (Slot and Geuze, 1985). In brief, a reducing mixture containing 4 ml of 1% trisodium citrate dihydrate and 80 μl of 1% (w/v) tannic acid plus 15.92 ml of distilled water was heated to 60°C and added, while stirring, to a similarly heated solution of Au^{3+} prepared with 1 ml of 1% (w/v) hydrogen tetrachloroaurate trihydrate in 79 ml of water. After sol formation was completed, as indicated by red coloration, the solution was brought to a boil and cooled on ice to 20°C. The pH of the colloidal suspension was adjusted to 7.4 with 0.1 mol/L potassium carbonate.

Concentration of protein in individual fractions was determined using the BioRad (Richmond, CA) procedure as modified for microtiter plates. For each fraction, a known amount of protein was conjugated to gold particles present in colloidal suspension at a ratio of 1 mg protein/10 ml of colloid (Jennes et al, 1986). Protein–gold conjugate was concentrated by centrifuging at 20,000g for 1 hour, and resuspended in 1 ml of 0.01 mol/L potassium phosphate-buffered saline (0.15 mol/L; pH 7.4) containing polyethylene glycol and thimerosal (each at 0.01% w/v). Protein–gold conjugates were 300 to 310 mOsm/kg at a final concentration of 1 μg protein/1 μl conjugate.

Microinjection Procedure

Eight adult Suffolk or western range rams were used so that there were four rams/site/treatment. Excurrent ducts on both sides were

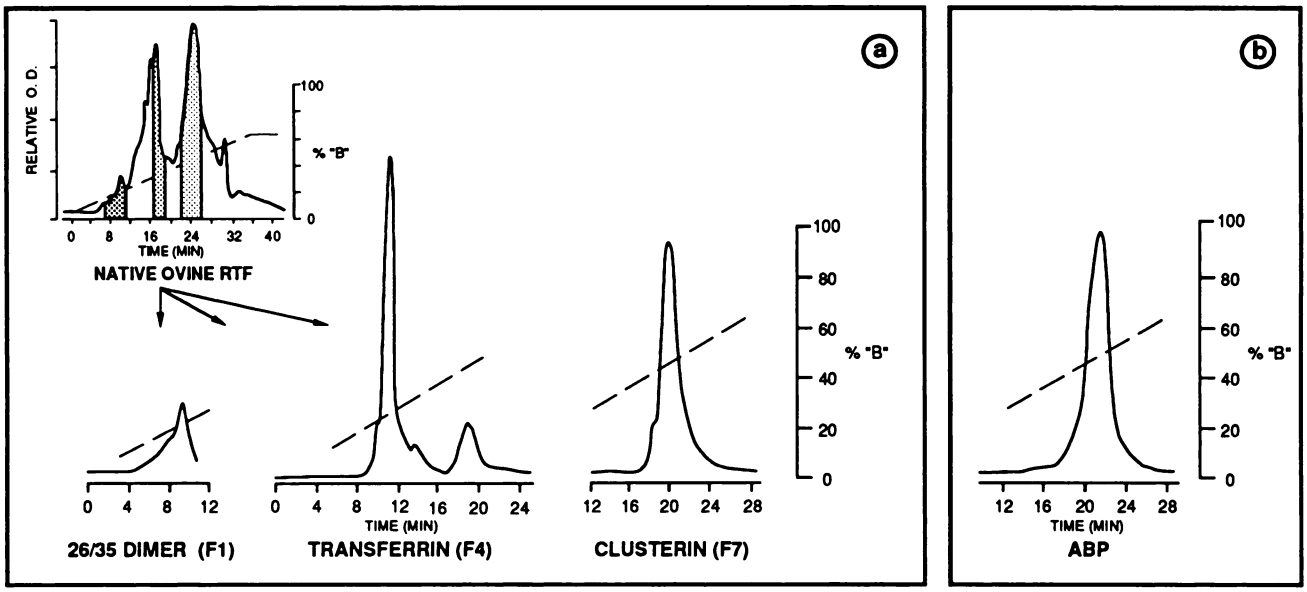

FIG. 2. Fractionation and isolation of proteins from ovine rete testis fluid (RTF). Panel A: After initial fractionation of RTF (upper left), 12 fractions were pooled across six runs and three fractions of interest (shaded areas) were rechromatographed using a Mono-Q HR5/5 anion exchange high-pressure liquid chromatography column to isolate a 26/35-kd dimer, transferrin, and clusterin (buffer A: 20 mmol/L Tris; buffer B: 20 mmol/L Tris plus 600 mmol/L NaCl). Absorbance is shown in arbitrary, but linear, optical density units. Panel B: After DHT-Sepharose affinity and anion exchange chromatography, androgen-binding protein was rechromatographed as described previously.

used; two treatments were assigned randomly to each ram, one treatment/side. Surgical preparation and microinjection procedures have been described (Veeramachaneni et al, 1990). In brief, micropipettes with a tip diameter of 30 or 40 μm were fabricated, calibrated to the 10-μl level, and filled with protein–gold conjugate. A ram was anesthetized and the excurrent ducts were prepared for microinjection. Using a surgical microscope and a micropipette held in a Leitz micromanipulator (Rockleigh, NJ), intraluminal injections (10 μl) were made in a random order at designated sites in a ductulus efferens and the ductus epididymidis (Fig 1). Injections required 1 to 2 minutes, depending on the flow rate of endogenous fluid.

Tissue Preparation and Electron Microscopy

Beginning 25 minutes after injection, approximately 1 cm of duct distal to and including the injection site was teased loose; at 30 minutes, the tissue was excised and immersed in 4% glutaraldehyde (v/v in 0.1 mol/L sodium cacodylate). A single time point was used because our goal was to determine relative uptake rather than study the fate of material that was endocytosed. After 2 hours, the initial fixative was replaced with 2% glutaraldehyde. Tissues were kept in 2% glutaraldehyde overnight and then postfixed in 1% osmium tetroxide (w/v in 0.1 mol/L sodium cacodylate) for 1 hour, washed in cacodylate buffer, dehydrated in acetone, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ductal tissue distal to the injection site was cut and cross-sectional profiles were selected. Thin sections (600 to 800 nm) were cut and stained with uranyl acetate and lead citrate. For each site (four sections/site/treatment), a continuous sheet of full profiles of longitudinally cut epithelial cells occupying one square mesh was identified at low magnification without regard to the presence or absence of gold particles, using a JEOL-1200EX electron microscope (Peabody, MA). The area of the mesh, as determined by Bioquant System IV (R & M Biometrics, Nashville, TN), was 6,400 μm^2. Gold particles present in different organelles of epithelial cells were enumerated at higher magnifications and were recorded as the number of gold particles endocytosed/6,400 μm^2 epithelial area. The areas examined also included some cells that did not endocytose protein–gold. Representative portions of cells with endocytosed protein–gold conjugate were photographed.

Data were analyzed using analysis of variance and Duncan's multiple range test (SAS, 1982). Data for ductuli efferentes were excluded from statistical analysis of regional differences in endocytosis of individual proteins because of the well documented structural and functional differences between the ductuli efferentes and the ductus epididymidis.

Results

Endocytosis of each protein injected, including ABP, was substantially greater in the ductuli efferentes than in any site in the ductus epididymidis (Table 1, Figs 3 and 4). ABP was endocytosed in the ductuli efferentes and the caput epididymidis in amounts similar to or greater than other proteins. More ABP was endocytosed in the initial segment and the proximal caput epididymidis than any other protein studied ($P < 0.05$). Endocytosis of the 26/35-kd dimer, like ABP, was greater in proximal than in distal sites of the epididymis. There was little uptake of transferrin in the epididymis except in the proximal corpus (Fig 5), whereas

Table 1. *Endocytosis of proteins isolated from ovine RTF in the ductuli efferentes and ductus epididymidis*

Region	Number of gold particles endocytosed per 6400-μm^2 epithelial area			
	ABP	26/35 Dimer	Transferrin	Clusterin
Ductuli efferentes	1825 ± 265^{A}	1673 ± 322^{A}	1288 ± 197^{A}	1595 ± 505^{A}
Caput epididymidis				
Initial	78 ± 8^{Aa}	32 ± 17^{Ba}	12 ± 7^{Ba}	35 ± 22^{Ba}
Proximal	73 ± 15^{Aa}	23 ± 12^{Ba}	4 ± 4^{Ba}	6 ± 5^{Ba}
Central	51 ± 9^{Ab}	15 ± 4^{Ba}	6 ± 3^{Ba}	62 ± 12^{Ab}
Distal	27 ± 8^{Ac}	7 ± 4^{Bb}	7 ± 5^{Ba}	31 ± 5^{Aa}
Corpus epididymidis				
Proximal	24 ± 8^{Ac}	12 ± 3^{Ab}	30 ± 7^{Ab}	72 ± 16^{Bb}

Data are expressed as mean ± SD.

Mean values within a row with different capital superscript letters for proteins, or within a column with different lowercase superscript letters for epididymal sites, are different ($P < 0.05$).

the uptake of clusterin was bimodal in the central caput and proximal corpus epididymidis.

Endocytosis occurred both in nonciliated and ciliated cells of the ductuli efferentes, but it was greater in the former cell type (see cover illustration). In nonciliated cells, protein–gold was found in coated pits and vesicles, endosomes, apical dense tubules, specific vacuoles, and occasionally in lysosomes. Protein–gold also was found, to a lesser extent, in uncoated pits and vesicles. In ciliated cells, protein–gold was found in coated pits, vesicles, and multi-

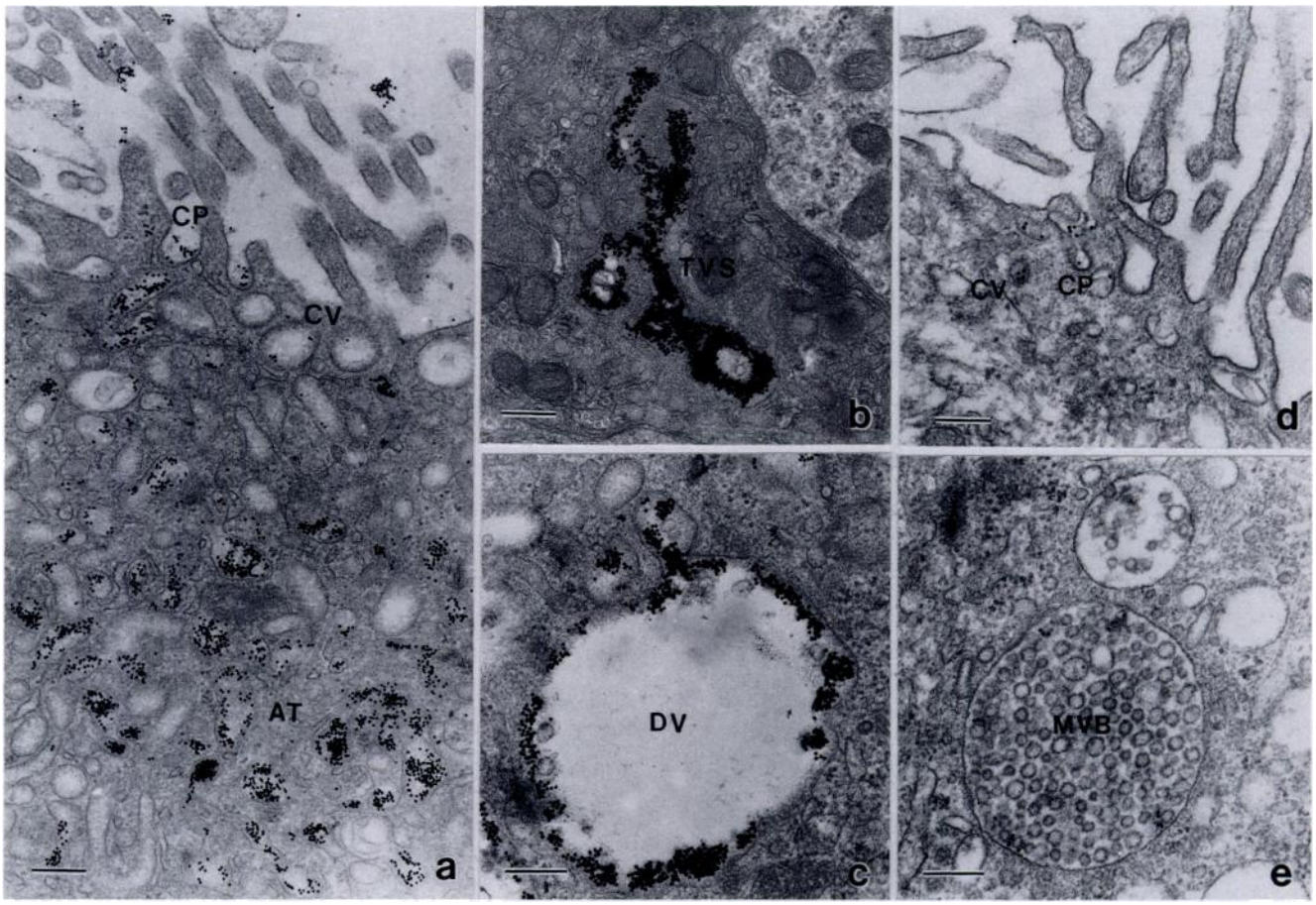

FIG. 3. Endocytosis of androgen-binding protein labeled with colloidal gold (ABP-gold) in the ram excurrent ducts 30 minutes after intraluminal injections. Panels A–C: Portions of nonciliated epithelial cells in the efferent ducts. Panels D, E: Portions of principal cells in the initial segment of the epididymis. AT = apical tubules; CP = coated pit; CV = coated vesicle; DV = dilated vacuole containing flocculent material with ABP–gold and a few vesicles; MVB = multivesicular body; TVS = tubular–vesicular system. Bar = 200 nm.

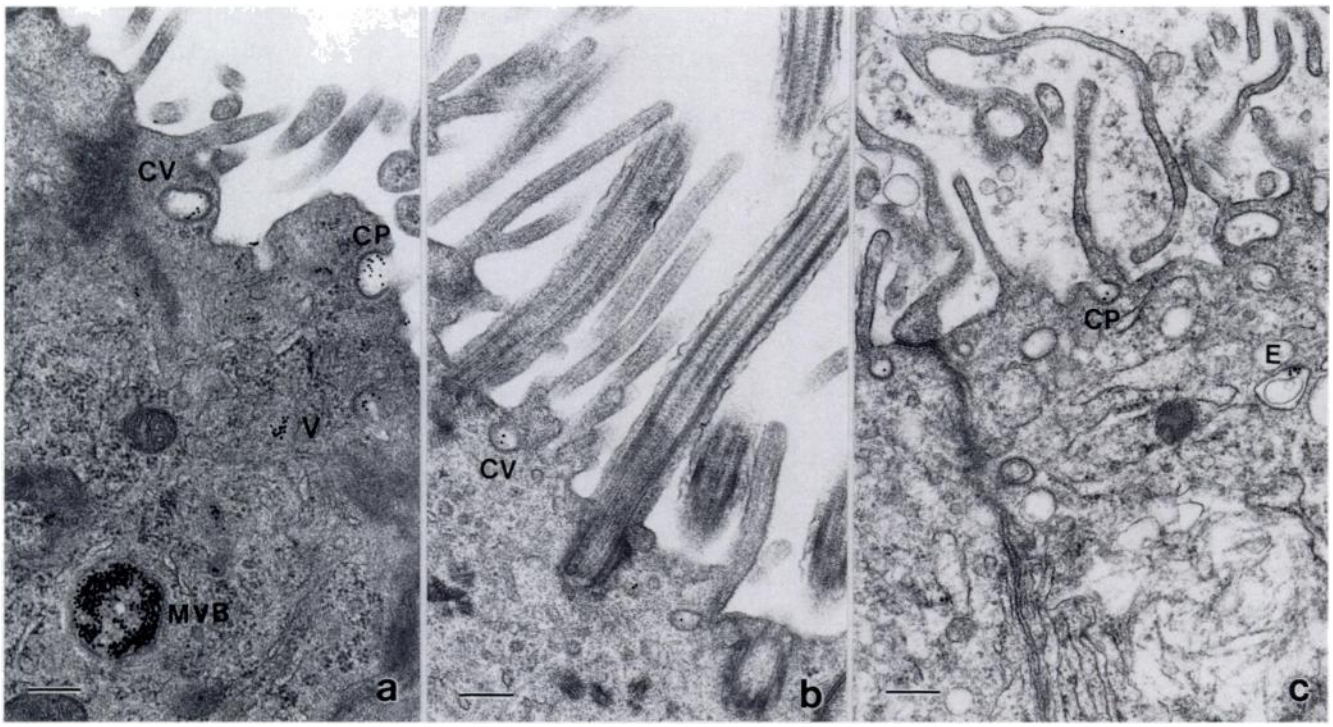

FIG. 4. Endocytosis of clusterin–gold in the ram excurrent ducts 30 minutes after intraluminal injections. Apical portions of (panel A) nonciliated and (panel B) ciliated epithelial cells in the efferent ducts, and (panel C) principal cells in the initial segment of the epididymis. CP = coated pit; CV = coated vesicle; E = endosome; MVB = multivesicular body; V = vesicle. Bar = 200 nm.

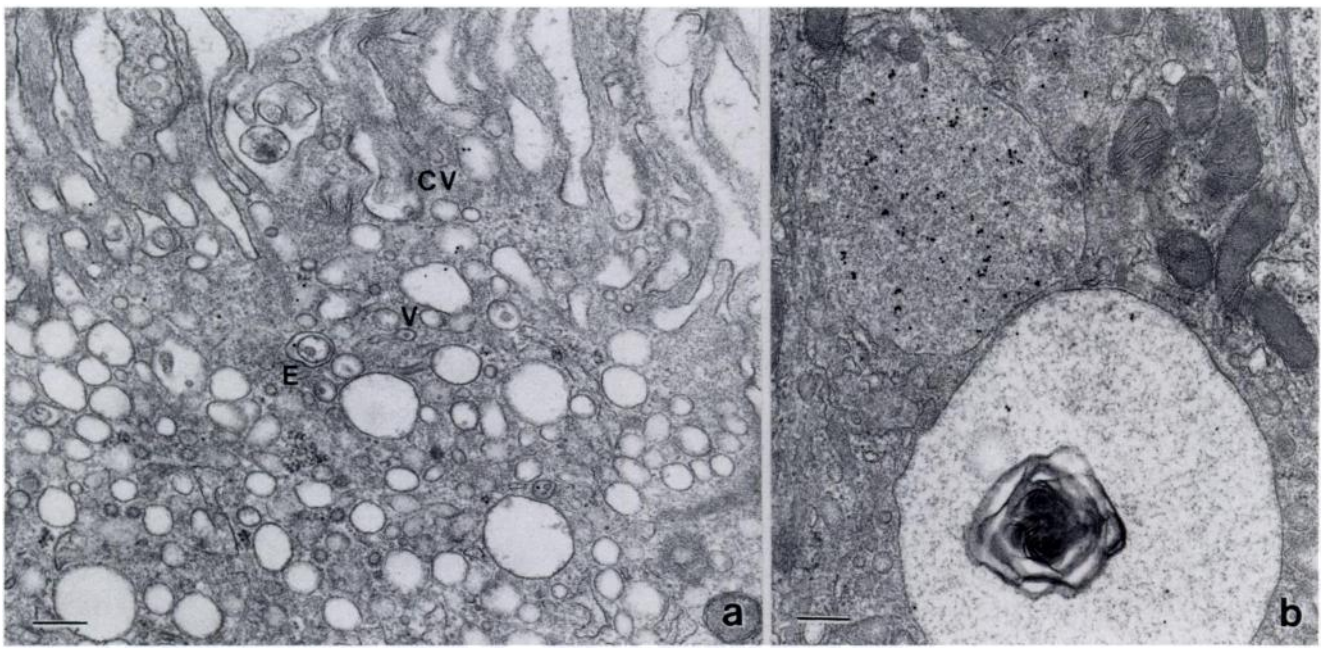

FIG. 5. Endocytosis of transferrin–gold in the ram excurrent ducts 30 minutes after intraluminal injections. Panel A: Apical portion of a principal cell in the corpus epididymidis. Panel B: Lysosomes in a nonciliated epithelial cell of a ductulus efferens. CV = coated vesicle; E = endosome; V = vesicle. Bar = 200 nm.

vesicular bodies. In the epididymis, endocytosis was predominant in principal cells in which protein–gold was found in coated pits, coated vesicles, endosomes, tubular–vesicular system, and multivesicular bodies. The organelles involved in the process of endocytosis in each region were essentially similar for all proteins.

Discussion

A substantial amount of ABP was endocytosed in the ductuli efferentes, as was true for other testicular proteins. Indeed, substantial endocytosis of exogenous ABP occurred despite the fact that the epithelium had not been deprived of endogenous ABP, and the relatively short time that the injected ABP–gold probably resided at the face of the epithelium examined. It should be recognized, however, that it is impossible to control either the concentration of endogenous plus injected protein or the residence time, so that availability of each labeled protein is identical for cells at each site. Fluid flow, as monitored by the movement of the injected bolus, is rapid and continuous in the ductuli efferentes, making the duration of contact relatively short compared to that in the ductus epididymidis, where the fluid flow is rather slow and peristaltic in nature. Endocytosis of ABP continued to be greater than that of any other protein in proximal regions of the epididymis. Thus, it is evident the ABP was not selectively "spared" from endocytosis in the proximal excurrent ducts.

Although the extent of nonspecific endocytosis was not quantified in the current study, data on the uptake of colloidal gold alone are available from a contemporaneous study (Veeramachaneni et al, 1990). Similar injection sites and procedures were used in this study; values for sites in Figure 1 were DE: 334, IS: 17, PC: 6, CC: 5, and DC: 6. Based on those data, we conclude that endocytosis of proteins in the current study, especially that of ABP, was specific. The presence of protein–gold in coated pits and coated vesicles, the organelles known to be involved in receptor-mediated endocytosis, is further evidence that endocytosis was specific. ABP (Gerard et al, 1988), alpha$_2$-macroglobulin (Djakiew et al, 1985), transferrin (Djakiew et al, 1986), and oxytocin (Veeramachaneni and Amann, 1990) have been shown to be endocytosed *in vivo* in the excurrent ducts by a receptor-mediated process. However, it is likely that protein–gold also underwent endocytosis by fluid phase and/or adsorptive processes. Such endocytotic processes are known to occur in the ductuli efferentes (Goyal and Hrudka, 1980; Morales and Hermo, 1983; Hermo et al, 1985). Pulse-chase experiments using injections of unconjugated proteins just prior to injections of protein–gold conjugates could establish the extent and nature of uptake. For instance, in the ductuli efferentes, injection of an excess of unlabeled oxytocin 1 minute before the injection of oxytocin–gold substantially reduced the uptake of oxytocin–gold (Veeramachaneni and Amann, 1990). The amount of uptake of oxytocin–gold following unlabeled oxytocin was about 80% less than that endocytosed without the chase, indicating that the uptake was largely specific and receptor-mediated.

Endocytosis of ABP or the 26/35-kd dimer was greater in proximal than in distal sites of the epididymis, whereas more clusterin or transferrin was endocytosed in distal sites. Thus, each protein was endocytosed in a differential and site-specific manner. The biologic significance of endocytotic patterns of clusterin, transferrin, or the 26/35-kd dimer is not clear. The endocytotic pattern of testicular clusterin conforms with observations that concentrations of clusterin are high in RTF and cauda epididymal plasma of the ram (Tung and Fritz, 1985), and that epididymal clusterin binds to sperm in proximal regions of the rat epididymis, whereas in distal regions it dissociates from sperm membranes and is endocytosed by the epithelium (Mattmueller and Hinton, 1991). Although we found that uptake of transferrin was greater in the proximal corpus epididymidis than in any region in the caput of rams, no such difference was found between the proximal caput and corpus epididymidis in rats (Djakiew et al, 1986). Presumably, in rats, transferrin receptors on principal cells were not saturated with endogenous transferrin, since removal of endogenous transferrin by efferent duct ligation did not alter the pattern of transferrin–gold uptake. We suspect that the relatively low amounts of transferrin–gold uptake in the caput epididymidis in the current study are due to inherent regional differences, rather than the saturation of available receptors for transferrin with endogenous ligand.

In conclusion, in rams, ABP is endocytosed in the ductuli efferentes and caput epididymidis in amounts similar to or greater than other major testicular proteins, and it is not selectively spared from endocytosis in the proximal excurrent ducts. Therefore, recycling of endocytosed ABP must be responsible for the maintenance of the luminal content of ABP through the distal caput epididymidis at the same level, on a per sperm basis, as that in RTF entering the ductuli efferentes, as previously reported (Veeramachaneni et al, 1990). However, secretion of ABP by the epithelium in the proximal excurrent ducts cannot be ruled out. Studies including time-course tracing of endocytosed labeled ABP and *in situ* hybridization using cDNA probes for ABP should clarify the involvement of such phenomena.

Acknowledgments

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