Localization of glucose-6-phosphatase activity and carbohydrates in boar caput epididymal principal cells

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Unidentified tubulovesicular profiles have been reported in the apical cytoplasm of boar caput epididymal principal cells in addition to vesicles considered to be involved in endocytosis and secretion. The main aim of the present study was to clarify the character of these organelles and to differentiate them from the endocytic apparatus. Glucose-6-phosphatase (G6Pase) activity was determined as the reporter enzyme of the endoplasmic reticulum (ER) and phosphotungstic acid was used to visualize carbohydrate moieties in both the proximal and distal caput. Phosphotungstic acid revealed the glycocalyx of the endocytic apparatus, which was similar in both regions studied, and also stained specific granules of the proximal caput. Glucose-6-phosphatase showed the tubulovesicular profiles to be sparsely granulated ER that was poorly developed in the proximal caput and very abundant in the apical cytoplasm of the distal caput principal cells. The function of such large amounts of sparsely granulated ER with corresponding G6Pase activity in caput epididymal principal cells is unknown.

Introduction

The crucial role played by the epididymis in promoting the fertilizing ability of spermatozoa is now generally recognized (Cooper, 1986). The complexity of the subtle process of sperm maturation is reflected by regional differences in boar epididymal duct epithelial ultrastructure (Stoffel and Friess, 1994). The presence of abundant vesicles in the apical cytoplasm of caput epididymal principal cells is thought to reflect endocytic and secretory processes. However, small tubulovesicular profiles that are particularly abundant in the distal caput do not seem to fit either of these two categories and are thought to constitute oligogranular ER. Therefore, the present study was undertaken to determine the nature of these particular organelles and to differentiate them from the endocytic apparatus.

Glucose-6-phosphatase is the reporter enzyme for the ER (Nichols *et al.*, 1984; Thorne-Tjomsland *et al.*, 1991). Although this enzyme has recently been purified to enable production of monospecific antibodies (Hume *et al.*, 1994), cytochemical demonstration of its hydrolytic activity (Robinson and Karnovsky, 1983; Van Norden and Frederiks, 1993) is currently the most widely used method for its detection. This technique has been used extensively to demonstrate G6Pase activity in liver, blood cells, testes and pancreatic islets (Leskes *et al.*, 1971; Colilla *et al.*, 1975; Nichols *et al.*, 1984; Waddell and Burchell, 1988; Thorne-Tjomsland *et al.*, 1991) and in epithelia lining the proximal convoluted tubules of

the kidney, ciliary body, small intestine, gall bladder, oviduct and epididymis (Hugon *et al.*, 1971; Kanai *et al.*, 1981, 1983; Thiéry *et al.*, 1983; Asaka *et al.*, 1991, 1993). In the present study, G6Pase cytochemistry was used to identify the ER. In addition, carbohydrates were stained with phosphotungstic acid to allow differentiation between elements of the endocytic apparatus and other organelles. This permitted ultrastructurally similar profiles to be distinguished.

Materials and Methods

Sampling sites

The caput epididymidis comprises the efferent ductules, an initial segment and the two segments called the proximal and distal caput. Tissue designated as proximal caput was collected from the bend of the caput epididymidis while samples originating from the lateral limb of the caput epididymidis are referred to as distal caput (Stoffel and Friess, 1994).

Tissue fixation

Epididymides from mature large white boars, *Sus scrofa* f. dom. (approximately 110 kg body weight), were obtained at castration. The spermatic cord was cut as proximally as possible to preserve all the connections between the testicular and epididymal blood vessels. A bulb-headed cannula was directly inserted into the Ramus epididymalis

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proximalis within 5 min of removing the testes (Stoffel *et al.*, 1990). The epididymides were perfused with 50 ml lukewarm Hank's balanced salt solution (Gibco BRL, Paisley) until they were cleared of blood. Perfusion with 50–100 ml of fixative (2.5% glutaraldehyde in 43 mmol Na_2SO_4 l^{-1} , 16 mmol NaHCO_3 l^{-1} , 10 mmol sodium acetate l^{-1} , 3.5 mmol KCl l^{-1} , 1 mmol CaCl_2 l^{-1} , 1 mmol MgCl_2 l^{-1} , 1 mmol D(+)-glucose l^{-1} , 1.6 mmol Na_2HPO_4 l^{-1} , 0.4 mmol NaH_2PO_4 l^{-1} and 33 mmol D(+)-sucrose l^{-1} , pH 7.4; modified after Xu and Ling, 1994) followed immediately and was maintained for 10 min. For G6Pase cytochemistry, small blocks of tissue were excised, immersed in fixative at 4°C for 20 min and then washed at 4°C in 0.1 mol cacodylate l^{-1} buffer containing 6.8% (w/v) sucrose and 0.2 mol glycine l^{-1} , pH 7.4. Tissue blocks used for staining with phosphotungstic acid were transferred to fixative for a further 2 h. Diluted Karnovsky's fixative (1% (w/v) paraformaldehyde, 1.25% glutaraldehyde in 0.1 mol cacodylate l^{-1} buffer with 2 mmol CaCl_2 l^{-1} , pH 7.4) was used for tissue stained with phosphotungstic acid.

Glucose-6-phosphatase

Cryostat sections of about 8–9 μm were transferred to poly-L-lysine-coated slides and incubated for 1 h at room temperature in the following reaction medium (modified after Kanai *et al.*, 1983): 3.7 mmol glucose-6-phosphate l^{-1} (Sigma, Buchs), 3.6 mmol $\text{Pb}(\text{NO}_3)_2$ l^{-1} (Merck AG, Zürich), 230 mmol sucrose l^{-1} in 80 mmol cacodylate l^{-1} buffer, pH 6.5. Substrate was omitted from control reactions. After thorough washing in distilled water, staining was enhanced by incubating the sections in 1% (v/v) ammonium sulphide for 2 min.

For electron microscopy, thin slices of about 150–200 μm were produced under a dissecting microscope with razor blades. The cytochemical reaction was obtained by preincubating for 1 h at room temperature and subsequently for 1 h at 37°C in the following medium (modified after Kalicharan *et al.*, 1985): 4 mmol glucose-6-phosphate l^{-1} and 2 mmol cerium chloride l^{-1} (Fluka Chemie AG, Buchs) in 50 mmol Tris-maleate l^{-1} buffer, pH 6.5. Substrate was omitted from control incubations. Tissue was then postfixed with 1% (w/v) OsO_4 in 0.1 mol cacodylate l^{-1} buffer, dehydrated in an ascending ethanol series and embedded in an epon-araldite mixture. Ultrathin sections were examined in a Zeiss 109 electron microscope.

Phosphotungstic acid

After aldehyde fixation, tissue blocks were embedded in K4M (Fluka Chemie AG, Buchs) at 0°C. Incubations with phosphotungstic acid were carried out according to Rambourg (1967). Ultrathin sections of about 90 nm were floated for 2 min on a solution of 1% (w/v) phosphotungstic acid in 10% (w/v) chromic acid (E. Merck AG, Zürich) at pH 0.3 using self-made polyethylene rings for transfer (Farragiana and Marinozzi, 1979). After several washes in distilled water, sections were picked up on copper grids and examined in a Zeiss 109 electron microscope.

Results

Glucose-6-phosphatase

Reaction product on paraffin wax sections was localized in the upper half of the epithelium except for a faint staining along the basement membrane (Figs 1a and 2a). Lead precipitate was particularly abundant along the apical plasmalemma. This strongly positive apical band was more prominent in the distal caput than in the proximal caput. In both regions the band was separated from a positive supranuclear zone by a narrow non-reactive belt. Incubation without substrate resulted in a low background staining along the basement membrane and occasionally along the luminal cell border (Figs 1b and 2b).

In the electron microscope, cerium phosphate was identified as a coarse extracellular deposit along the stereocilia in the proximal caput only and intracellularly as a finely granular reaction product in both epididymal regions studied. Precipitate was observed along the nuclear membrane, in Golgi stacks, and in the trans-Golgi network (Figs 1c,d and 2c). Long parallel strands in the infra-, peri- and supranuclear cytoplasm were also observed (Figs 1d and 2c). Small tubulovesicular profiles filled with precipitate were detected in the apical cytoplasm. These were scarce in the proximal caput (Fig. 1c) and abundant in distal caput principal cells, where they occupied the apical protrusions up to the plasma membrane (Fig. 2d,e). However, no communication was observed between vesicular membranes and plasmalemma. Control reactions were negative (Fig. 3).

Phosphotungstic acid

Phosphotungstic acid stained the apical plasmalemma including spherical and tubular pits in both the proximal and distal caput (Fig. 4a,c), and gave a faint reaction on the lateral cell membranes (Fig. 4b) and the basement membrane. In addition, the membranes of numerous electron-lucent tubules and vesicles in the apical cytoplasm were positive (Fig. 4a,c). Continuity between the membrane of the tubulovesicular profiles and the apical plasmalemma was frequently observed (Fig. 4a). However, phosphotungstic acid staining became fainter with increasing distance from the apical cell border. The granules characteristic of the proximal caput were positive (Fig. 4b). Golgi stacks were also positive; nuclei, mitochondria and ER were completely negative.

Discussion

The main purpose of the present study was to identify ultrastructurally similar vesicular profiles in the apical cytoplasm of caput epididymal principal cells. Glucose-6-phosphatase activity was used as a marker for the ER and phosphotungstic acid identified the endocytic apparatus. Vesicular profiles negative for both reactions were considered to be part of the secretory pathway.

Phosphotungstic acid revealed the glycocalyx of the apical

Fig. 1. Glucose-6-phosphatase reaction in boar proximal caput epididymal epithelium. (a) Light microscopy reveals a moderately positive apical band separated from the supranuclear reactive zone by a narrow unstained strip. (b) Control reaction without substrate showing very faint background staining along the basement membrane. (c) In the apical zone, a coarse reaction product is observed on the stereocilia, while precipitate in the rough endoplasmic reticulum is finely granular. A similar deposit is present in a loosely arranged network of tubulovesicular profiles (arrowheads). (d) Prominent staining of parallel strands of rough endoplasmic reticulum, Golgi apparatus and nuclear membrane. bm, basement membrane; l, lumen; m, mitochondria; n, nucleus; rER, rough endoplasmic reticulum; sg, specific granules; st, stereocilia. Scale bars represent 10 μm in (a) and (b) and 1 μm in (c) and (d).

plasmalemma and also labelled the membranes of the tubulovesicular profiles involved in absorption. This allowed the organelles belonging to the endocytic apparatus and sparsely granulated ER to be distinguished on the basis

of their shape and distribution pattern. On the basis of the number of vesicles present, proximal and distal caput appear to be equally involved in absorption. Phosphotungstic acid also stained the granules that are characteristic of the

Fig. 2. Glucose-6-phosphatase reaction in boar distal caput epididymal epithelium. (a) A strongly positive apical zone is narrowly separated from moderately stained supranuclear cytoplasm. (b) Control reaction without substrate showing very faint background staining along the basement membrane and luminal border. (c) Dark staining of nuclear membrane, rough endoplasmic reticulum and Golgi apparatus. (d,e) Extensive tubulovesicular profiles in the apical cytoplasm in close proximity to, but lacking continuity with, the plasmalemma. bm, basement membrane; g, Golgi apparatus; l, lumen; m, mitochondria; n, nucleus; rER, rough endoplasmic reticulum. Scale bars represent 100 μm in (a) and (b) and 1 μm in (c)–(e).

Fig. 3. Control incubation of boar proximal caput epididymal principal cells for glucose-6-phosphatase activity in the absence of substrate. No reaction product is observed. Scale bar represents 1 μ m.

proximal caput, which have been shown to contain antagglutinin (Dacheux and Dacheux, 1987). Antagglutinin is a secretory product of the caput epididymidis in boars. Immunocytochemical detection of antagglutinin in rough ER, Golgi apparatus, vesicles and epididymal lumen suggests it is a glycoprotein, which explains staining of the granules with phosphotungstic acid. However, the granules are thought to be sites of degradation rather than being part of the secretory pathway (Dacheux and Dacheux, 1987).

Glucose-6-phosphatase is a reporter enzyme for the ER (Allen, 1961; Nichols *et al.*, 1984; Thorne-Tjomsland *et al.*, 1991; Burchell *et al.*, 1994) and is a useful tool for determination of complex membrane systems (Leskes *et al.*, 1971; Robinson and Karnovsky, 1983). However, glucose-6-phosphate is not only hydrolysed by the specific enzyme

G6Pase but also by other phosphatases (Allen, 1961; Nordlie, 1979; Nichols *et al.*, 1984). In addition to different pH optima, these enzymes differ substantially in lability. While G6Pase is susceptible to inactivation by fixation, both acid and alkaline phosphatases remain cytochemically active even after prolonged exposure to aldehyde fixatives (Sabatini *et al.*, 1963). The presence of a coarse precipitate along the apical plasmalemma in proximal caput principal cells and along the cell membranes of basal cells in both epididymal regions studied (not shown) was independent of any fixation parameter. Inayama *et al.* (1995) showed alkaline phosphatase to be a marker for basal cells in rabbit airways. In addition, the presence of this enzyme has been reported in the epididymides of bulls (Sinowatz, 1981) and boars (Wrobel and Fallenbacher, 1974). In both species, basal cells throughout the epididymal duct are positive for alkaline phosphatase, whereas stereocilia in the distal caput display little or no enzymic activity. This finding is in agreement with the present findings and thus the staining in these areas is considered to be due to non-specific hydrolysis by alkaline phosphatase.

The finely granular reaction product within principal cells was only observed after minimal fixation. Indeed, adequate preservation of ultrastructure without substantial loss of intracellular enzymic activity was a problem. The precipitate observed in rough ER and nuclear envelope was taken as a reference for the specific cytochemical reaction. Therefore, the presence of an identical reaction product in the Golgi apparatus and in numerous irregular tubulovesicular profiles was considered to reflect G6Pase activity. Although Golgi elements are considered to be G6Pase-negative, there is increasing evidence that this enzyme is a constituent of this organelle in some cells. Glucose-6-phosphatase activity has been demonstrated in Golgi stacks and in the trans-Golgi network of principal cells in the initial segment of the rat epididymal duct (Hermo *et al.*, 1991) and in spermatocytes, spermatids and Leydig cells but not Sertoli cells of rat testis (Thorne-Tjomsland *et al.*, 1991).

Glucose-6-phosphatase-positive tubulovesicular profiles of sparsely granulated ER were detected close to the Golgi apparatus and also intermingled with other vesicular structures at the very apex of the cells. Close apposition of oligogranular ER with both cis- and trans-Golgi elements has been described in detail in the rat epididymis (Hermo *et al.*, 1991). Whereas close proximity of sparsely granulated ER to cis-Golgi elements is explained by intracellular traffic, the functional basis for the proximity of sparsely granulated ER to the trans-Golgi is unknown. However, the most interesting observation of this study was the large amount of G6Pase-positive sparsely granulated ER in the apical cytoplasm of distal principal cells. This is particularly interesting because the intracellular distribution of smooth and oligogranular ER appears to be very specific. In ruminant Sertoli cells, for example, the smooth ER is localized basally and in close association with spermatids (Wrobel *et al.*, 1995). A secretory mechanism that bypasses the Golgi apparatus has been suggested on the basis of the intimate topographical relationship between sparsely granulated ER and apical plasmalemma in the initial segment of the rat epididymis (Hoffer *et al.*, 1973). Although

Fig. 4. Staining of boar (a,b) proximal and (c) distal caput epididymal principal cells with phosphotungstic acid. (a) Stereocilia, vesicular and tubular pits (*) as well as the endocytic apparatus (arrows) are clearly outlined. (b) Granules that are characteristic of proximal caput principal cells are reactive. (c) The apical plasmalemma and numerous endocytic apparatus profiles (arrow) are stained. Scale bars represent 1 μm .

the transport carrier from the Golgi apparatus to the lumen has been tentatively identified (Hermo *et al.*, 1994), autoradiographic and immunocytochemical studies have shown that synthesis and secretion of epididymal proteins involves the ER and the Golgi apparatus (Flickinger, 1985; Dacheux and Dacheux, 1987).

In the liver, G6Pase catalyses the last step of both glycogenolysis and gluconeogenesis and thus plays a key role in blood glucose homeostasis (Nordlie, 1985). The presence of G6Pase activity in other organs is usually considered to be a mechanism by which glucose is released into a luminal environment, for example, the aqueous humour (Asaka *et al.*, 1991), oviductal fluid (Asaka *et al.*, 1993) and epididymal fluid (Kanai *et al.*, 1983). In fact, glucose has been shown to occur in the luminal environment after its normal contents are removed (Cooper and Waites, 1979) and it has been suggested that sperm cells prevent accumulation of reducing sugars in epididymal duct fluid by using glucose as a substrate (Brooks, 1979a). Therefore, such a function would be compatible with the apical localization of G6Pase in caput epididymal principal cells. However, rapid transport of glucose from the bloodstream across the epididymal epithelium via a facilitated diffusion mechanism is an alternate route by which glucose can be supplied to luminal fluid (Brooks, 1979b; Hinton and Howards, 1982).

Glucose-6-phosphatase may be involved in generating NADPH via the hexose-monophosphate shunt (Colilla *et al.*, 1975). NADPH, in turn, is needed to reduce testosterone to dihydrotestosterone. Dihydrotestosterone is about twice as potent as the substrate and is crucial in maintaining high levels of androgen activity within tissues (Luke and Coffey, 1994). In boars, luminal concentrations of dihydrotestosterone exceed those of testosterone and the proximal epididymis contains more androgens than the distal region (Aafjes and Vreeburg, 1972). In addition, the activity of epididymal G6Pase has been shown to be controlled by circulating testosterone in mice (Kanai *et al.*, 1983). The irreversible interconversion of testosterone to dihydrotestosterone is catalysed by 5 α -reductase. However, 5 α -reductase activity is known to be highest in the central caput of the bovine epididymis (Hammerstedt and Amann, 1976), a region equivalent to the segment referred to as the proximal caput in the present paper. In rats, immunocytochemical labelling of 5 α -reductase was most intense in discrete lobules of the initial segment, in which it was restricted to the infranuclear zone of principal epididymal cells. Intense supranuclear staining for the enzyme was typical for the proximal caput, whereas weak staining was observed throughout the cytoplasm in distal regions (Viger and Robaire, 1994; Robaire and Viger, 1995). Therefore, the distribution pattern of 5 α -reductase in rats is not the same as the localization of G6Pase activity in the corresponding segments of the boar epididymis.

Further investigations are required to determine the functional implications of abundant profiles of sparsely granulated ER with corresponding G6Pase activity in boar caput epididymal principal cells.

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