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ROLE OF V-ATPase-RICH CELLS IN ACIDIFICATION OF THE MALE REPRODUCTIVE TRACT

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Summary

Specialized proton-secreting cells play important physiological roles in a variety of tissues. On the basis of the immunocytochemical detection of carbonic anhydrase and V-ATPase in distinct epithelial cells of the epididymis and vas deferens, we predicted that the vacuolar V-ATPase that is located on the apical membrane of these cells should be a major contributor to luminal acidification in parts of the male reproductive tract. Physiological studies using the proton-selective vibrating probe in the vas deferens confirmed this hypothesis. As discussed recently, maintenance of the pH of the reproductive tract is probably under tight physiological control, by analogy with the situation in the kidney. Manipulation of luminal pH might, therefore, provide a point of intervention for the regulation of male fertility. In addition, it is possible that some cases of unexplained male infertility might result from defective acidification, resulting either from pathological states or potentially from environmental factors that may inhibit proton secretory pathways.

Key words: epididymis, vas deferens, pH regulation, immunocytochemistry, bafilomycin.

Introduction

Many transporting epithelia contain a subpopulation of specialized cells that contain high levels of a vacuolar-type proton-pumping H^+ ATPase (V-ATPase) on their plasma membrane and on intracellular vesicles (Brown and Breton, 1996). These cells are involved in the physiologically regulated transepithelial transport of protons and, in the kidney, they play a role in acid–base homeostasis. We have found that the epithelium lining parts of the male reproductive tract – the epididymis and the vas deferens – also contains similar V-ATPase-rich cells (Breton *et al.* 1996; Brown *et al.* 1992). In some parts of the epididymis, immunocytochemical staining with antibodies against V-ATPase subunits shows that up to 40 % of the epithelial cells are V-ATPase-rich (Fig. 1). As will be discussed below, we have used the proton-selective vibrating probe to show that the bafilomycin-sensitive V-ATPase is a major pathway for proton secretion in this tissue. This brief review outlines the role of V-ATPase-rich cells in acidification in various epithelia and highlights their role in reproductive physiology.

Tissue distribution of V-ATPase-rich cells

Several transporting epithelia contain a population of characteristic cells that are specialized for proton secretion. These cells have been extensively studied in the turtle urinary bladder, toad bladder and amphibian skin, as well as in the kidney collecting duct, where they are known as intercalated cells (Brown and Breton, 1996; Brown *et al.* 1978, 1988; Madsen and Tisher, 1986; Schuster, 1993; Schwartz *et al.* 1985; Steinmetz, 1986). Osteoclasts, involved in bone remodeling, represent a non-epithelial type of V-ATPase-rich cell (Baron, 1989; Gluck, 1992). These cells, as well as other V-ATPase-rich cells present in a variety of invertebrate epithelia (Brown and Breton, 1996; Harvey, 1992; Wieczorek *et al.* 1992) have been reviewed recently (Harvey and Wieczorek, 1997; Ehrenfeld and Klein, 1997; Dow *et al.* 1997). These proton-pumping cells all contain high levels of cytosolic carbonic anhydrase (CAII), they have plasma membrane-associated V-ATPase and they undergo distinctive morphological alterations during an increase in H^+ secretion by their respective epithelia (Brown and Breton, 1996).

V-ATPase-rich cells contain specialized ‘stud-coated’ vesicles

The change in appearance of V-ATPase-rich intercalated cells upon stimulation results from the exocytotic fusion of specialized acidic intracellular vesicles with the plasma membrane. These vesicles are also involved in endocytosis of V-ATPase-rich segments of the plasma membrane (Brown *et al.* 1987*a*,*b*; Gluck *et al.* 1982; Madsen *et al.* 1991; Schwartz and Al-Awqati, 1985; Van Adelsburg and Al-Awqati, 1986).

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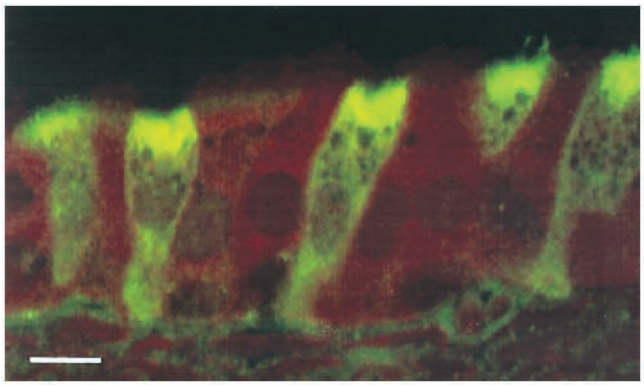

Fig. 1. Section of rat epididymis (tail region) stained to show the mosaic of V-ATPase-rich cells (stained green) that are involved in acidifying the luminal fluid in this region of the male reproductive tract. The section was examined using a BioRad 600 confocal microscope. In some parts of the epididymis, these cells comprise up to 40 % of the total epithelial cells. The antibody used to stain these cells was prepared against the 56 kDa subunit ('kidney' isoform) of the V-type H^+ ATPase (kindly provided by Dr Steven Gluck, Washington University, St Louis, USA). The red background is Evan's Blue counterstain. Scale bar, 10 μm.

Thus, V-ATPase molecules are recycled between the plasma membrane and intracellular vesicles in intercalated cells by a highly specialized population of vesicles. This recycling activity results in an extremely high rate of endocytosis as detected using fluorescent markers linked to dextran (Lencer *et al.* 1990; Schwartz *et al.* 1985) or electron-dense markers such as horseradish peroxidase (Brown *et al.* 1987*b*; Schwartz *et al.* 1985).

Using specific antibodies, we showed that the coating of electron-dense material on the cytoplasmic side of these vesicles and some domains of the plasma membrane does not contain clathrin (Brown and Orci, 1986), a protein associated with vesicles involved in receptor-mediated endocytosis (Brown and Goldstein, 1986; Pearse and Robinson, 1990), but is instead composed of the cytoplasmic subunits of a V-ATPase (Brown *et al.* 1987*a*). High-resolution images of the proton pump using the rapid-freeze, deep-etch technique showed that the structure of the membrane coat was identical to that of immunoaffinity-purified proton pumps (Brown *et al.* 1987*a*). By analogy with other previously identified coated intracellular transporting vesicles, the 'studs' of V-ATPase-rich vesicles must contain proteins that are involved in regulating and defining their intracellular targeting. The other known types of coated transport vesicles are clathrin-coated vesicles, which are predominantly (but not exclusively) involved in receptor-mediated endocytosis (Pearse and Robinson, 1990), coatomer protein (COP)-coated vesicles involved in transport within and among Golgi cisternae and the rough endoplasmic reticulum (RER) (Orci *et al.* 1986; Rothman and Orci, 1990) and caveolae, the pinocytotic membrane invaginations whose coat contains a protein called caveolin (Rothberg *et al.* 1992). These or similar proteins have not so far been detected on V-ATPase-coated vesicles in proton-secreting cells (S. Breton and D. Brown, unpublished results). An important direction for future research will, therefore, be to identify vesicle-associated proteins that are involved in V-ATPase vesicle targeting in these cells.

Regulation of proton secretion by V-ATPase-rich cells

How is the proton pumping capacity of V-ATPase-rich cells regulated? It has been clearly demonstrated that systemic acidosis in animals stimulates exocytosis of the V-ATPase-containing vesicles and that the amount of V-ATPase in the apical plasma membrane increases rapidly and dramatically under these conditions (Bastani *et al.* 1991; Sabolic *et al.* 1996). A similar effect can be provoked in isolated epithelia by increasing the partial pressure of CO_2 (acid loading) in the basolateral bathing medium (Schwartz and Al-Awqati, 1985). The situation is complicated in cortical collecting ducts of the kidney because at least two subtypes of intercalated cells are present. 'A-cells' have apical proton pumps and a basolateral Cl^-/HCO_3^- anion exchanger (AE1), whereas 'B-cells' are the mirror-image and have a diffuse or basolateral localization of proton pumps (Alper *et al.* 1989; Schuster *et al.* 1986) and an apical anion exchanger. These latter cells are involved in secretion of bicarbonate into the tubule lumen (Schuster, 1993). This functional duality is not the case in the male reproductive tract, where all V-ATPase-rich cells appear to be oriented to participate in proton secretion (Breton *et al.* 1996; Brown *et al.* 1992).

Luminal acidification in several epithelia can be modified by various factors in addition to acidosis and alkalosis, including specific (bafilomycin) and relatively non-specific (*N*-ethylmaleimide, NEM) inhibitors of the V-ATPase (Sabolic *et al.* 1994), aldosterone (Hays *et al.* 1986; Stone *et al.* 1983*b*), adrenergic agonists (Emmons and Stokes, 1994; Ikeda *et al.* 1993), perturbation of microtubules (Stetson and Steinmetz, 1983) and inhibition of cotransport of ions including Cl^- (Stone *et al.* 1983*a*). Future work will determine whether these agents also modify luminal pH in the reproductive tract.

Acidification in the excurrent duct system

The luminal fluid along much of the male reproductive tract is maintained at an acidic pH (Carr *et al.* 1985; Levine and Marsh, 1971; Rodriguez *et al.* 1990). It has been proposed that low pH is required for sperm maturation and that it is involved in maintaining sperm in an immotile state during their passage through the epididymis and vas deferens, probably in conjunction with factors including specific proteins, weak acids and other ions (Carr *et al.* 1985; Hinton and Palladino, 1995; Usselman and Cone, 1983; Wong *et al.* 1980). During ejaculation, an increase in intracellular pH (pH_i) is a key factor in the complex series of events that triggers sperm motility, although this alone may not be sufficient for motility to occur (Acott and Carr, 1984; Carr and Acott, 1989; Carr *et al.* 1985). This pH change results from mixing of the epididymal fluid

with alkaline prostatic and seminal vesicle fluid. The bicarbonate-rich semen also serves to neutralize the acidic environment that exists in the proximal portions of the female reproductive tract.

Despite the physiologically important consequences of the luminal acidification process, the mechanism of acidification and its physiological regulation have been addressed in relatively few studies. Previous work on the perfused epididymis implicated a Na^+/H^+ exchanger in the process of acidification (Au and Wong, 1980). This was based on the Na^+-sensitivity of the proton flux. These data could also be interpreted to show the involvement of a basolateral Na^+/HCO_3^- cotransporter in the epithelial cells. In the same report, it was shown that systemic acidosis in rats resulted in a decrease in luminal pH in the epididymis, and alkalosis resulted in less acidification (Au and Wong, 1980).

As mentioned above, we have identified a specialized cell type in the epithelium of rat epididymis and the vas deferens that expresses high levels of V-ATPase on its luminal plasma membrane as well as in intracellular vesicles (Breton *et al.* 1996; Brown *et al.* 1992). An earlier histochemical study showed a distinct population of epithelial cells that contained large amounts of carbonic anhydrase (CAII) (Cohen *et al.* 1976) and we have now shown that proton pumps and CAII are co-localized in the same cell type (Breton *et al.* 1996). These cells are the 'apical' cells or 'narrow' cells in the head (caput) portion of the epididymis, and the 'light' cells or 'clear' cells in the body (corpus) and tail (cauda) (Moore and Bedford, 1979*a*,*b*; Reid and Cleland, 1957; Sun and Flickinger, 1979). The morphology of apical and clear cells is quite distinct, although both share an extremely high level of apical endocytotic activity, similar to that of renal intercalated cells (Brown *et al.* 1987*b*; Moore and Bedford, 1979*b*; Schwartz *et al.* 1985). The presence of CAII in the epididymis prompted studies to determine whether the CA inhibitor acetazolamide would inhibit luminal acidification. Conflicting data were obtained. In one study, acetazolamide did partially inhibit acidification (Au and Wong, 1980), whereas subsequent work showed no effect on epididymal pH (Caflisch and DuBose, 1990), possibly as a result of a failure to deliver an appropriate dose of the inhibitor to the CAII-rich cells. However, anti-androgen treatment (flutamide) resulted in an increase in epididymal pH (Caflisch, 1993).

Because the 'apical' and 'clear' cells in the reproductive tract contain large numbers of proton pumps on their apical plasma membranes, we hypothesized that they are analogous in function to renal intercalated cells and that they are primarily responsible for luminal proton secretion into the excurrent duct system. The following section describes how the proton-selective vibrating probe was utilized to implicate V-ATPase-driven proton transport as the major source of luminal

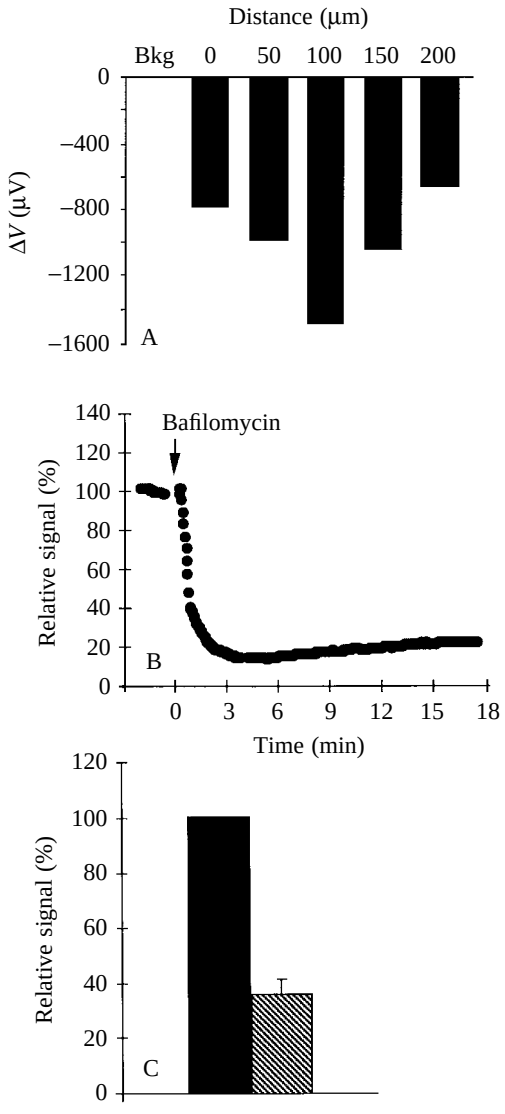

Fig. 2. (A) An example illustrating the detection of an acidification 'hot-spot' in the epithelium of the vas deferens. The proton-selective vibrating probe was scanned over the surface of the tissue in 50 μm lateral increments. At each of several positions over the tissue (five individual points in this example), the proton gradient in a plane perpendicular to the surface of the tissue was measured as a change in voltage, ΔV, by constantly oscillating the tip of the electrode between one location close to the tissue and a second location 50 μm away from the tissue surface. The highest rate of proton secretion found during the scan is presumed to reflect the positioning of the electrode tip over the apical pole of a proton-secreting epithelial cell. The column marked Bkg (background) shows that there is no measurable ΔV (i.e. no proton flux) when the tip of the electrode is oscillated in the bath solution far away (about 1 cm) from the surface of the tissue. (B) An example showing 'real-time' data obtained using the signal from the proton-selective vibrating probe to monitor the rapid and marked inhibition of proton flux over a 'hot-spot' in the vas deferens by 1 μmol l^{-1} bafilomycin, a specific inhibitor of the V-ATPase. (C) Histogram showing the mean effect of 1 μmol l^{-1} bafilomycin on proton secretion in seven preparations of vas deferens. Filled column, control value; hatched column, value after treatment with bafilomycin expressed as mean percentage reduction in signal + S.E.M. compared with the control value, which was normalized for each experiment. Each measurement was performed over an acidification 'hot-spot' such as the one shown in A. Figure modified from Breton *et al.* (1996).

acidification in the vas deferens. The vas deferens was chosen as an experimental model because of its large lumen, allowing easy access of the proton-selective electrode to the apical surface of the epithelium.

Detection of a bafilomycin-sensitive proton flux in vas deferens using the proton-selective vibrating probe

Proton flux from the apical surface of the isolated vas deferens was measured using a proton-selective vibrating probe. Previous studies detecting proton or Cl^- fluxes in epithelia used whole-current vibrating probe measurements coupled with pharmacological studies to identify the origin of the flux as proton transport, for example in the stomach, turtle urinary bladder and frog skin (Demarest *et al.* 1986; Foskett and Ussing, 1986; Katz and Scheffey, 1986; Scheffey *et al.* 1991). Our studies used, in contrast, a proton-selective electrode. Technical details concerning the use of ion-specific probes have been discussed previously (Smith and Shipley, 1990; Smith *et al.* 1994). Briefly, the proton-selective probe is composed of a glass microelectrode filled with an H^+-selective liquid ion exchanger. A bathing medium with low buffering capacity is used in order to maximize the pH gradient measured at the tissue surface. Before and after each experiment, the Nernst slope of the electrode is calibrated in solutions of different pH, and controls to detect any potential effect of additives to the medium on the specificity or selectivity of the electrode are also performed.

When the probe was moved in 50 μm lateral increments over the surface of the exposed epithelium, acidification hot-spots were detectable (Fig. 2). These 'hot-spots' most probably reflect the presence of one or more proton-secreting cell(s) immediately beneath the tip of the probe. As shown in Fig. 2, addition of the specific V-ATPase inhibitor bafilomycin (1 μmol l^{-1}) to the bath strongly and rapidly inhibited the bulk (up to 80 %) of the proton flux in this preparation from the proximal vas deferens when the probe was over one of the 'hot-spots'.

Because not all of the proton flux was bafilomycin-sensitive, we tested the effect of other inhibitors on acidification in the vas deferens to determine the origin of the remaining 20–30 % of the flux. So far we have been unable to show inhibition by amiloride (up to 1 mmol l^{-1}), implying that the Na^+/H^+ exchanger is not a major contributor, at least in the vas deferens. The carbonic anhydrase inhibitor acetazolamide (100 μmol l^{-1}) was also tested and was found to inhibit proton flux to approximately the same extent as bafilomycin in some cases, although the rate of onset of inhibition was much slower than with bafilomycin and the inhibitory effect was variable among different preparations. This different effect was expected based on the different targets of the two drugs. Bafilomycin inhibits the pump itself, whereas with acetazolamide, the transported ion (protons) would only slowly become less abundant as CAII is inhibited. Subsequent addition of bafilomycin after acetazolamide produced no further inhibitory effect on luminal acidification. In addition, it is possible that protons to supply the V-ATPase might derive from a CAII-independent source in some tissues, explaining the variability of the effect. Finally, an inhibitor of the gastric H^+/K^+ P-ATPase, Sch-28080, had no detectable effect on proton flux at concentrations of 10–50 μmol l^{-1}, when it retains specificity for the gastric ATPase (Sabolic *et al.* 1994).

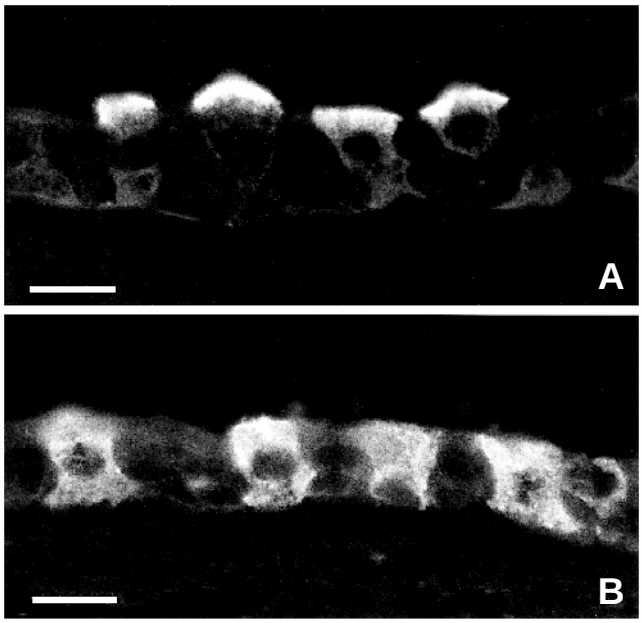

Fig. 3. V-ATPase localization in the epididymis from normal (A) and colchicine-treated (B) rats. In control rats, the V-ATPase is concentrated at the apical pole of proton-secreting cells, whereas after colchicine treatment, the V-ATPase is diffusely distributed throughout the cytoplasm. Thus, microtubules are required for the apical targeting of this enzyme. Scale bars, 10 μm.

Microtubules are required for V-ATPase targeting in the epididymis

The V-ATPase is recycled between the plasma membrane and 'stud-coated' intracellular vesicles in proton-secreting cells. In renal intercalated cells, targeting to both the apical membrane (in A-cells) and the basolateral membrane (in B-cells) is disrupted by treatment of the whole animal with colchicine, a microtubule-disrupting drug (Brown *et al.* 1991). To determine whether a similar process also occurs in the reproductive tract, we treated rats with colchicine (0.25 mg 100 g^{-1} body mass, intraperitoneally) for 6–12 h and examined V-ATPase distribution. As shown in Fig. 3, the apical polarity of V-ATPase expression was completely disrupted by the drug, suggesting that microtubules are involved in the apical trafficking and recycling process. This effect also implies that the cell biological mechanisms involved in the regulation of proton secretion by these cells are at least partially similar in the kidney and in the epididymis.

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