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Ultrastructural and immunocytochemical evidence for the presence of polarised plasma membrane H^+-ATPase in two specialised cell types in the chick embryo chorioallantoic membrane

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ABSTRACT

The chick embryo, confined in the eggshell, has to dispose/buffer the acid generated by its metabolism, as well as to release calcium from the shell which is used for growth. To localise H^+-ATPase, electron microscope and immunocytochemical studies were conducted on chorioallantoic membranes of 15–17 d chick embryos. Ultrastructural studies of the villus cavity (VC) cells in the chorionic epithelium demonstrated that their apical plasma membrane, juxtaposed with the shell membranes, contains microvilli as well as microplicae which possess 9–10 nm studs at a density of 16700 particles/μm^2, a characteristic feature of the polarised H^+-ATPase pump. Immunocytochemical staining, using a monoclonal antibody to the 31 kDa subunit of H^+-ATPase, confirmed the presence of large amounts of the vacuolar H^+-ATPase in the VC shells with a distribution highly polarised towards the eggshell membranes. Immunoelectron-microscopic localisation studies using a rabbit antiserum to whole bovine H^+-ATPase and immunogold technique, confirmed the localisation of H^+-ATPase at the apical microvilli/microplicae as well as in the subapical vesicles. In the allantoic epithelium, the presence of mitochondria-rich (MR) cells was confirmed; it was shown that these cells extend through the full thickness of this epithelium. The MR cells also contained large numbers of 9–10 nm studs, typical of proton secreting cells, in their apical plasma membrane. This was confirmed by immunocytochemical staining which showed abundant localisation of H^+-ATPase in these cells; this localisation was, however, diffuse rather than apical. We conclude that VC cells in the chorionic epithelium, and most (if not all) MR cells in the allantoic epithelium are able to secrete acid, and hence contribute to the acid-base homeostasis in the chick embryo. Our localisation of H^+-ATPase suggests that VC cells secrete H^+ towards the eggshell which is buffered by calcium carbonate, resulting in release of bicarbonate and calcium, which in turn will be used by the chick embryo for further buffering of the generated acid and for bone mineralisation, respectively. The MR cells secrete H^+, analogous to kidney intercalated cells, and may be responsible for the progressive acidification of the embryonic urine.

Key words: Acid-base balance; acid secretion; proton secreting cells; proton ATPase.

INTRODUCTION

The chick embryo interchanges gases and water vapour with the environment through the pores in the eggshell. While the adult animal can regulate the concentration of gases in the blood by decreasing or increasing lung respiratory exchange, the embryo is unable to do so because the fixed pore size of the shell limits the magnitude of the exchange.

As the chick embryo grows, the production of CO_2 gradually increases, as does its concentration, both inside the egg, as measured in the air chamber (Wangensteen & Rahn, 1970) and in the blood of the embryo (Dawes, 1975; Dawes & Simkiss, 1969, 1971).

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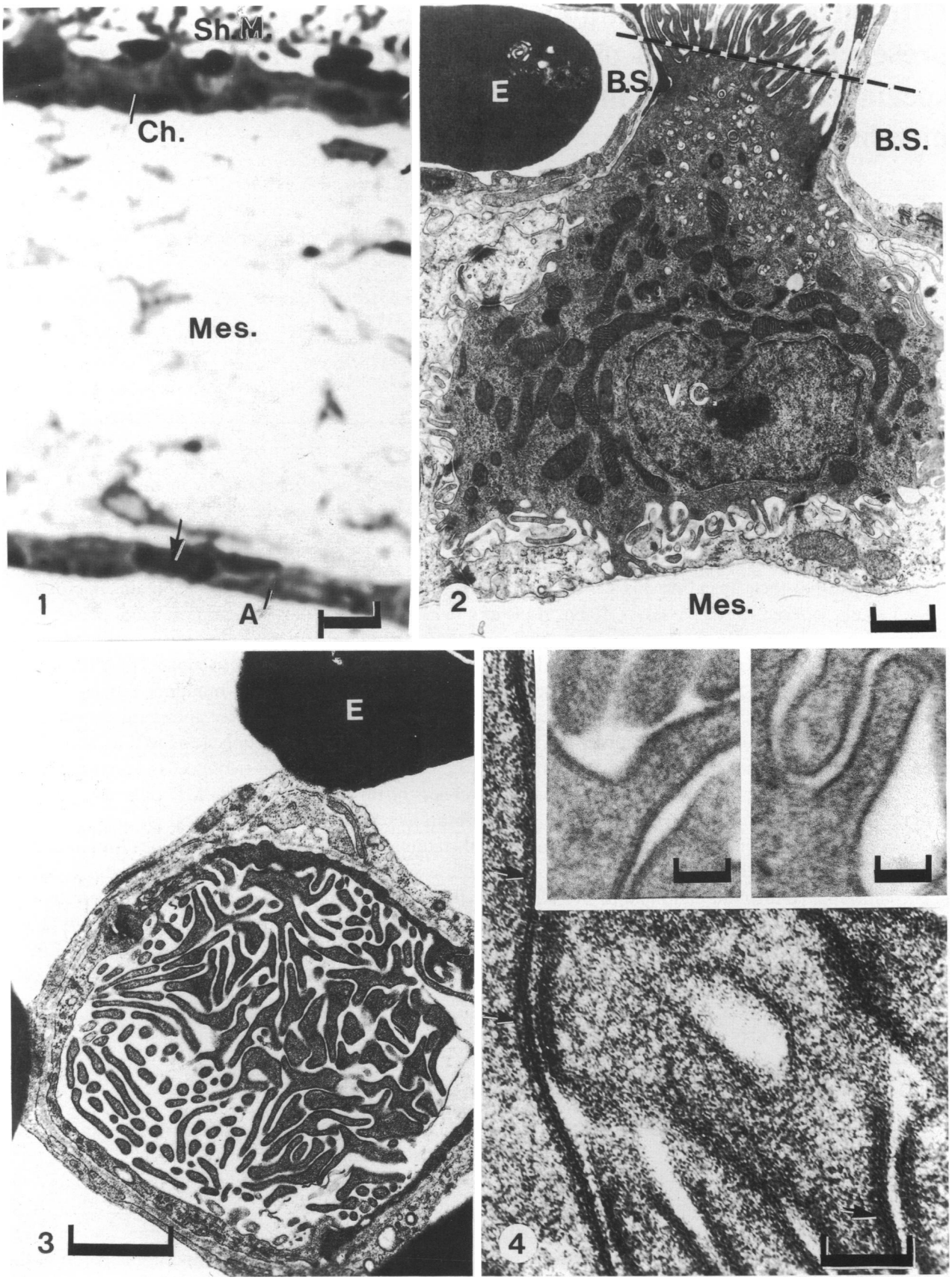

Fig. 1. Light micrograph of a cross section of the chorioallantoic membrane from a 17 d chick embryo stained with toluidine blue, A, allantoic epithelium; Ch, chorionic epithelium; Mes., mesenchyme; Sh.M, shell membrane. Arrow, dark cell in allantoic epithelium (MR cell). Bar, 10 μm.

Despite this increase in blood P_{CO_2}, the pH of the plasma remains constant (Abramovici, 1967; Dawes & Simkiss, 1969), because the increase in P_{CO_2} is accompanied by an increase in the concentration of bicarbonate (Dawes & Simkiss, 1969, 1971). The mechanism(s) for the increase in plasma bicarbonate concentration have not been established, but the embryonic kidney and the shell have both been suggested to contribute (Crooks & Simkiss, 1974; Dawes & Simkiss, 1969).

During the second half of its development, the chick embryo obtains most of the required calcium from the shell, which is mainly composed of insoluble $CaCO_3$ (Johnston & Comar, 1955; Simkiss, 1961). The chorioallantoic membrane is in direct contact with the shell membrane, and its outer layer—the chorionic epithelium—is probably not only responsible for the limited respiratory exchange, as described above, but also for the solubilisation and absorption of the shell mineral. It has been generally assumed that the solubilisation of the shell mineral is mediated by secretion of H^+ by the chorionic epithelium (Owczarzak, 1971; Coleman & Terepka, 1972; Narbaitz et al. 1981; Narbaitz, 1987). However, there is only indirect evidence for such secretion. A specialised cell in the chorionic epithelium, the 'villus cavity' (VC) cell, has been suggested as being responsible for the secretion of an acid substance or H^+ on the basis of its ultrastructural characteristics and high carbonic anhydrase activity (Skalinsky & Kondalenko, 1963; Leeson & Leeson, 1963; Owczarzak, 1971; Coleman & Terepka, 1972; Rieder et al. 1980; Anderson et al. 1981).

During the last few years intensive ultrastructural, morphometric and immunocytochemical studies have been carried out on cells known to secrete H^+ in the mammalian kidney, and in the amphibian and reptilian urinary bladders (see reviews by Madsen & Tisher, 1985 and Madsen et al. 1988). These studies have shown that H^+ secreting cells have several common characteristics: they are rich in carbonic anhydrase activity (this enzyme hydrates CO_2 to H_2CO_3 which subsequently dissociates into H^+ and HCO_3^-), they have a vacuolar-type H^+-ATPase in their apical plasma membrane, and a Cl^-/HCO_3^- anion exchanger in their basolateral membrane, implementing H^+ secretion into the lumen and HCO_3^- transport to the blood, respectively. Proton-secreting cells are also rich in mitochondria (proton secretion has high energy requirements) and display apical microvilli and microplicae (representing an amplification of the plasma membrane required for H^+ secretion) as well as subapical tubulovesicular structures. The latter represent active endocytosis-exocytosis, a process required for shuttling of the H^+-ATPase-containing membrane vesicles between the apical cytoplasm and the plasma membrane (Bastani et al. 1991).

The vacuolar H^+-ATPase responsible for proton secretion in the mammalian kidney has been identified (Gluck & Al-Awqati, 1984) and localised immunocytochemically in the plasma membrane of proton-secreting cells (Brown et al. 1987, 1988*a*, *b*; Brown, 1989; Bastani et al. 1991). In addition, Brown et al. (1987, 1988*a*) demonstrated, with high-resolution electron microscopy, that the H^+-ATPase can be identified as rows of 9–10 nm studs located at the cytoplasmic face of the plasma membrane. These studs are arranged in regular groups which consistently show a density of 16700 studs per μm^2. The pump has also been identified with freeze-fracture techniques (Brown et al. 1987). This arrangement has been observed consistently in proton-secreting cells, not only of mammalian kidney, but also in the urinary bladder of turtles (Madsen & Tisher, 1985).

With the foregoing in mind, we re-examined the ultrastructure of VC cells in the chorionic epithelium and the MR cells in the allantoic epithelium of chick embryos, using electron microscopical (EM), immunogold EM, and immunocytochemical localisation techniques with antibodies to the vacuolar H^+-ATPase. Preliminary results of this work have been reported (Narbaitz et al. 1992).

MATERIAL AND METHODS

Eggs from White Leghorn hens were obtained from a commercial source and incubated at 37.5 °C with 60% relative humidity. 10 eggs were opened between the 15th and 17th days of incubation and several

Fig. 2. Electron micrograph showing part of the chorionic epithelium. This and all the following figures correspond to membranes from 17 d embryos. B.S., blood sinus; E, erythrocyte; Mes, mesenchyme; VC., villous cavity cell. Interrupted line indicates direction of section in Figure 3. Bar, 1 μm.

Fig. 3. Cross section of upper portion of VC cell as indicated in Figure 2. Note that most apical cell processes are folds (microplicae) rather than villi, E, erythrocyte. Bar, 1 μm.

Fig. 4. Higher magnification of the microplicae of a VC cell. Note the typical rows of 10 nm studs attached to the inner side of the plasma membrane (arrows). Insets. Apical (left) and basal (right) membranes from the same VC cell. Notice the studs which are found only at the apical membrane. Bar, 0.1 μm.

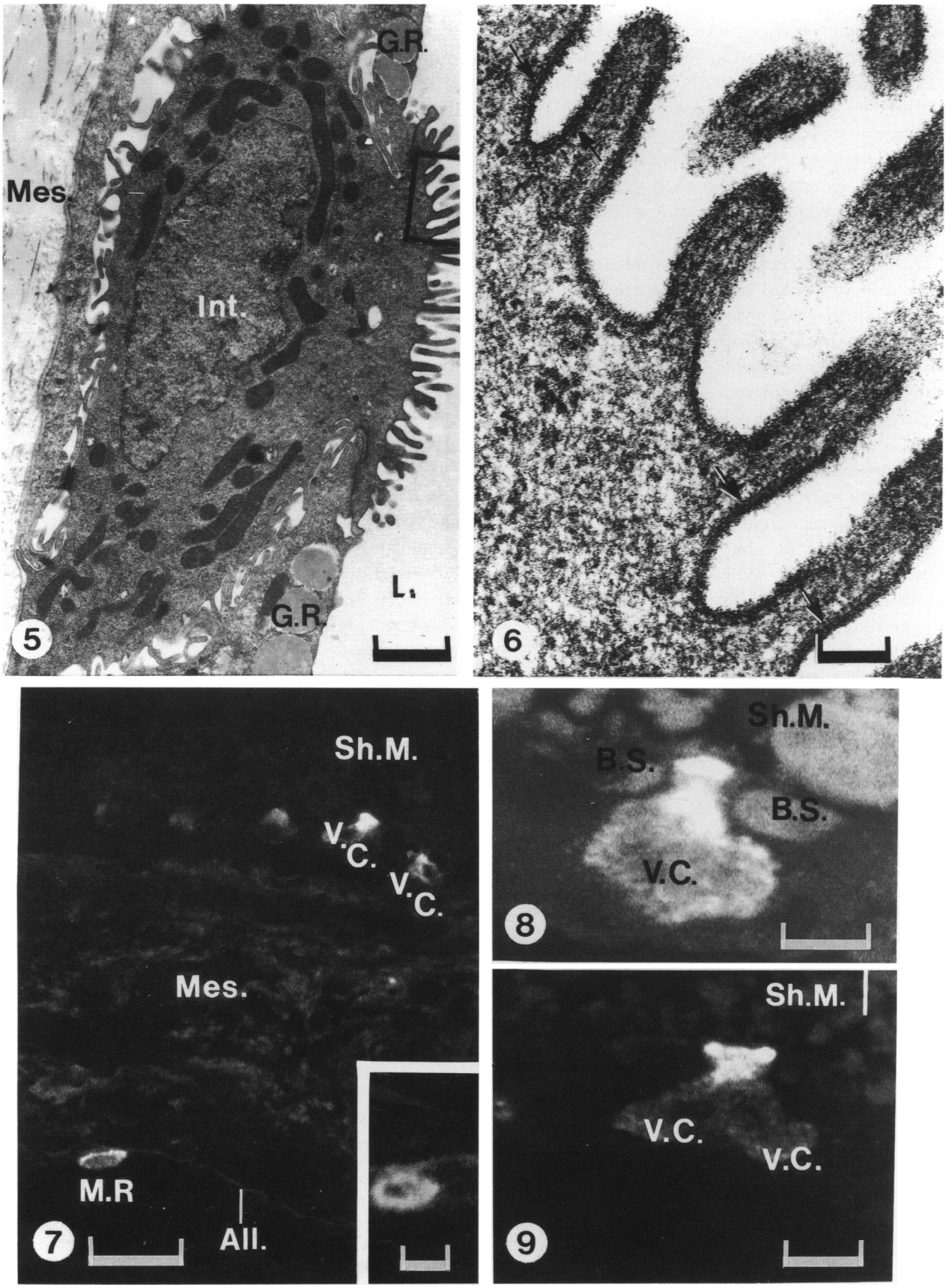

Fig. 5. Portion of the allantoic epithelium. G.R., granule-rich cell; Int., intercalated or mitochondria-rich cell. Note that it contacts both the lumen (L) and the basal lamina. The part of the cell enclosed by an incomplete rectangle is magnified in Figure 6. Mes., mesenchyme. Bar, 1 μm.

portions of the chorioallantoic membrane from each egg were fixed overnight in half-strength Karnovsky's fixative (Karnovsky, 1965). Only portions of the chorioallantoic membrane which had direct contact with the shell membrane were used for this study. The tissues were then washed with 0.1 M cacodylate buffer containing 0.2 M sucrose, postfixed in osmium tetroxide, dehydrated in ethyl alcohol and embedded in Araldite. Sections (1 μm) were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate according to Reynolds (1963). Sections were studied with a Philips 300 transmission electron microscope.

For immunocytochemistry, small portions of the chorioallantoic membranes from embryos of the same ages were fixed in B5 fixative (0.22 M $HgCl_2$, 90 mM Na acetate, and 3.7% formaldehyde) for 3 h and subsequently embedded in paraffin. Sections (4 μm) were immunostained using a monoclonal antibody against a synthetic peptide from the C-terminus of the 31 kDa subunit of the renal vacuolar H^+-ATPase (a generous gift of Dr Stephen Gluck, Renal Division, Jewish Hospital, Washington University, St Louis, MO) as described before (Bastani et al. 1991). Binding of the antibody was followed by fluorescein-labelled (FITC) goat antimouse IgG (1:50, v/v dilution; Fisher Scientific, St Louis, MO). Immunofluorescence was detected and photographed by both epifluorescence (Nikon Optiphot-2 epifluorescence microscope; Nikon Incorporation, Instrumental Group, NY, USA) and confocal (Biorad MRC 500 confocal microscope equipped with an argon laser; 1 μm optical section thickness) microscopy.

For immunogold labelling, chorioallantoic membranes from 16 d chicken embryos were fixed in 4% paraformaldehyde, rinsed in buffer, dehydrated in a graded series of ethanol, embedded in LR White medium, and polymerised at 50 °C overnight. Ultrathin sections were mounted on formvar-coated nickel grids and immunolabelled using an indirect colloidal gold labelling technique. Nonspecific binding was blocked by a 20 min incubation at room temperature in phosphate buffered saline (PBS, 58 mM Na_2HPO_4, 18 mM KH_2PO_4, 75 mM NaCl) containing 0.8% bovine serum albumin, 0.1% gelatin and 10 μg/ml normal goat IgG. Sections were rinsed in PBS, incubated with rabbit antiserum (R3 antibody, a generous gift from Dr S. Gluck) to whole bovine H^+-ATPase (Brown et al. 1987), diluted 1:20 in the blocking solution for 2 h at room temperature, rinsed several times in PBS, and incubated with 10 nm colloidal gold labelled goat antirabbit in blocking buffer for 1–2 h at room temperature. The final rinse was in 2 changes of PBS, followed by 5 min in PBS with 0.4 M NaCl and 0.05% Tween 20, and 2 more changes of PBS. Sections were then fixed for 5 min in 2% glutaraldehyde, rinsed in distilled water, and stained with aqueous uranyl acetate for viewing in a JEOL 100CX transmission electron microscope.

RESULTS

The histological features of the chorioallantoic membrane of a 17 d chick embryo are shown in Figure 1. Note that the membrane is formed by mesenchyme interposed between 2 epithelia: the chorionic epithelium, which is in direct contact with the shell membrane, and the allantoic epithelium which faces the allantoic cavity containing the allantoic fluid (urine).

The chorionic epithelium

This epithelium contains a superficial vascular network in close contact with the shell membrane (Figs 1, 2). In confirmation of previous reports, we observed that the space between blood vessels is occupied by cells with numerous long apical cellular processes, the VC cells (Fig. 2). Cross-sections of these cellular processes showed that a large proportion of them are microplicae rather than microvilli (Fig. 3).

As shown in Figure 2, VC cells have an electron-

Fig. 6. Higher magnification of part of the same cell (as indicated by rectangle in Figure 5). Note the rows of typical 10 nm studs at the cytoplasmic side of the apical membrane (arrows). Bar, 0.1 μm.

Fig. 7. Immunocytochemical localisation of H^+-ATPase in a cross section of the chorioallantoic membrane using an epifluorescence microscope. The VC cells show accentuated staining in the apical pole facing the shell membrane. The MR cell shows accentuated staining at the basal membrane. Al., allantoic epithelium; Mes., mesenchyme, M.R., MR cell; V.C., VC cells; Sh.M., shell membranes. Epifluorescence microscopy ×400. Inset: In a similar section, an MR cell is shown using the same technique. Fluorescence in this cell is diffuse throughout the whole cytoplasm. Bar, 1 μm.

Fig. 8. Similar preparation as in Figure 7 but viewed by confocal microscopy. Fluorescence is polarised at the apex of a VC cell. B.S., blood sinus; Sh.M., shell membranes; V.C., VC cell. The weak fluorescence in the shell membrane and blood sinuses was due to nonspecific autofluorescence. Bar, 10 μm.

Fig. 9. Similar preparation as in Figure 7 but viewed with confocal microscopy. Two VC cells (V.C.) share a common intervascular space. H^+-ATPase is highly polarised towards the apex of both cells. The apparent staining seen in the shell membrane is nonspecific autofluorescence. Bar, 10 μm.

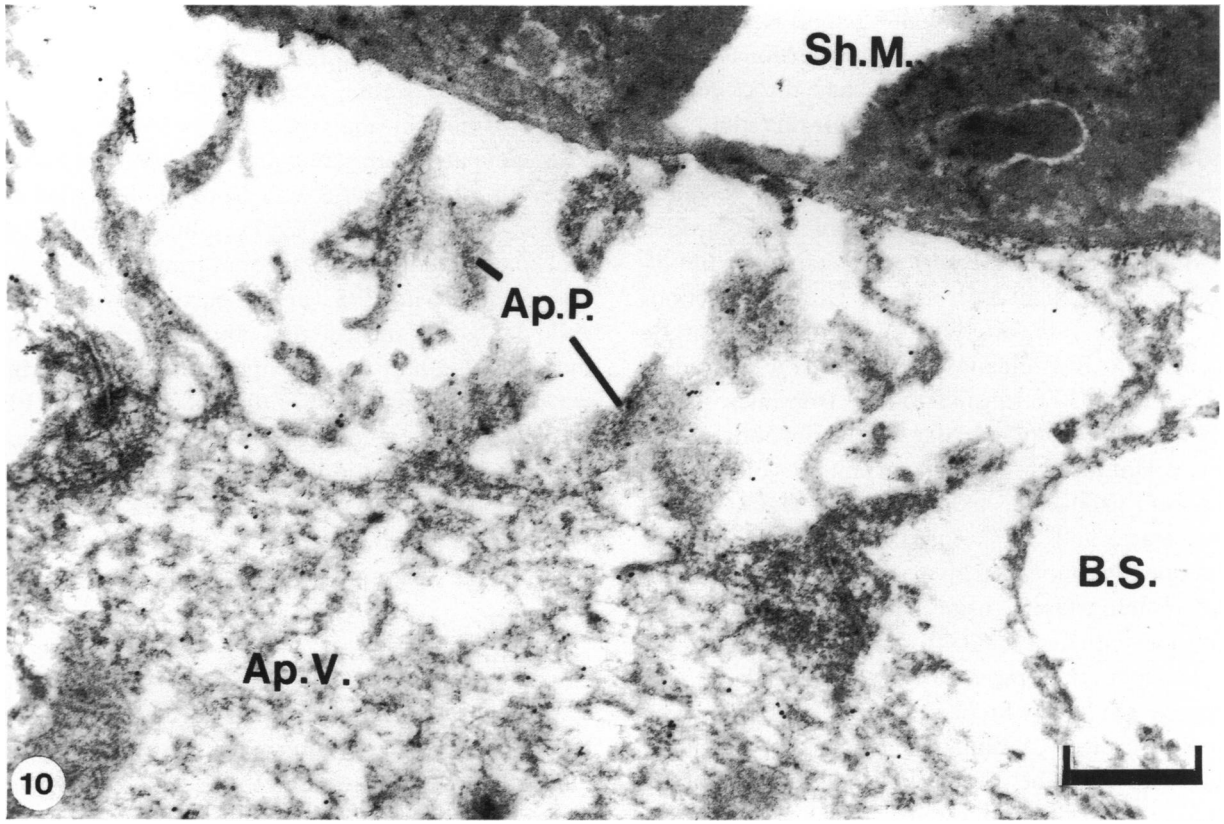

Fig. 10. Immunogold electron micrograph of the apex of a VC shell from a 16 d embryo. Gold particles are found close to the apical plicae (Ap.P.) and vesicles (Ap.V). Very few or none are seen in the cavity which surrounds the plica and in the shell membrane (Sh.M.). B.S., blood sinus. Bar, 0.5 μm.

dense cytoplasm which contains numerous mitochondria and subapical vesicles. At high magnification, the plasma membrane of microvilli and microplicae has numerous studs at its cytoplasmic side (Fig. 4). These studs are arranged at regular intervals forming rows. Each of these studs measured 9–10 nm and the density of their distribution was approximately 13 particles per 0.1 μm which is equivalent to 16700 particles per μm^2 shown to be characteristic of H^+-secreting cells in mammalian kidney and turtle urinary bladders (see Introduction). These studs were only found in the apical membrane (Fig. 4, left inset) and not in the basolateral membrane (Fig 4, right inset). All VC cells examined had the same characteristic features.

The allantoic epithelium

Our observations confirmed that the allantoic epithelium consists of 3 cell types: basal, granular and 'mitochondria-rich' cells (Coleman & Terepka, 1972). The mitochondria-rich (MR) cells stain dark with toluidine blue (Fig. 1) and are highly electrondense (Fig. 5). They have numerous large apical microvilli facing the allantoic cavity. We observed that while basal cells contact only the basal lamina, and granular cells contact only the allantoic lumen, MR cells occupy the whole thickness of this epithelium from the basal lamina to the allantoic lumen (Fig. 5).

Examination with high magnification revealed the presence of studs in the cytoplasmic side of the plasma membrane of the microvilli of MR cells with the same characteristics as those in the VC cells in the chorionic epithelium (Fig. 6). Here again the studs were limited to the apical plasma membrane and were not found in basolateral membranes. Studs were not found in granular or basal cells.

Immunocytochemistry

Immunocytochemical staining confirmed the presence of H^+-ATPase both in VC cells of the chorion and the MR cells of the allantois (Fig. 7).

The VC cells were the more numerous and up to 25% of the surface of the chorion was occupied by the fluorescent apical region of these cells. While in some of the VC cells fluorescence was either diffuse or concentrated superficially all around the cell, in the

majority fluorescence was clearly polarised towards the apex, where the apical microplicae and vesicles are localised (Figs 8, 9; cf. Fig. 2). This was confirmed by immunogold deposition, which demonstrated the presence of H^+-ATPase in the apical microvilli and microplicae and in subapical tubulovesicles (Fig. 10). In the less numerous MR cells of the allantois, fluorescence was usually diffuse throughout the cell (Fig. 7, inset), although in few cases it appeared to be located at the basolateral pole (Fig. 7).

DISCUSSION

Villus cavity cells

Previous studies on VC cells (Skalinsky & Kondalenko, 1963; Leeson & Leeson, 1963; Owczarzak, 1971; Coleman & Terepka, 1972; Narbaitz, 1972) have shown some of the ultrastructural features which were later found to be characteristic of proton secreting cells (intercalated cells) in urinary epithelia (Madsen & Tisher, 1985). These features include cytoplasmic electrondensity, abundance of mitochondria, apical microvilli and subapical vesicles. Also, similar to the intercalated cells, the VC cells have been shown to be rich in carbonic anhydrase activity (Rieder et al. 1980; Anderson et al. 1981) and to be capable of apical endocytosis (Dunn & Fitzharris, 1987). Our present electron microscopical observations show further that, similar to the intercalated cells, the apical cell processes of the VC cells are mostly in the form of microplicae rather than microvilli.

The identification of polarised H^+-ATPase (proton pump) has played an important role in confirming that intercalated cells are involved in acid-base regulation by the kidney. These cells have been found to be of 2 types, one secreting protons through the apical membrane, and one secreting them through the basolateral membrane (Madsen & Tisher, 1985; Madsen et al. 1987). The former (type A intercalated cell) has H^+-ATPase at the apical plasma membrane and the latter (type B intercalated cell) has the pump at the basolateral membrane. This conclusion is based on the electron microscopical observation of the typical 10 nm studs characteristic of polarised H^+-ATPase (Brown et al. 1987, 1988*a*; Narbaitz et al. 1991) and on immunocytochemical studies using antibodies against the H^+-ATPase (Brown et al. 1988*a*, *b*; Bastani et al. 1991; Verlander et al. 1992). Our present demonstration of studs, identical in size and distribution to those found in intercalated cells, in the apical membrane of VC cells, together with our immunocytochemical confirmation of the presence of polarised vacuolar H^+-ATPase in these same cells, strongly support the view that VC shells are indeed engaged in the secretion of protons towards the shell.

The secretion of protons by VC cells is required for the solubilisation of the eggshell calcium carbonate and release of calcium as well as bicarbonate ions. This process has been shown to be regulated by vitamin D_3 (Narbaitz et al. 1980, 1981; Narbaitz, 1987). Thus activation of VC cells may represent one of the mechanisms by which the embryo regulates both calcium balance and acid-base homeostasis. Furthermore, because cells which secrete protons through one pole are thought to secrete bicarbonate through the opposite pole, in order to prevent progressive intracellular alkalinisation, VC cells probably contribute to the gradual increase in plasma bicarbonate concentration known to occur during the second half of development (Dawes & Simkiss, 1969, 1971) by both mechanisms (apical secretion of H^+ with subsequent release of bicarbonate from the eggshell, and basolateral secretion of bicarbonate).

Mitochondria-rich (MR) cells

Coleman & Terepka (1972) have briefly described the ultrastructural characteristics of the allantoic epithelium in the chick embryo. They noted that, like the toad urinary bladder, the allantoic epithelium has 3 cell types: basal cells, granule-rich cells, and MR cells. They did not speculate on the possible function of MR cells. Similar cells have also been described in the allantoic epithelium of pig embryos (Tiedemann, 1979). Our ultrastructural and immunocytochemical studies demonstrate the presence of a H^+-ATPase in MR cells, and this appears to suggest that they are involved in proton secretion in a manner similar to the specialised cells in the turtle bladder which possess similar features (Steinmetz, 1986).

Our electron microscopical observations show that in MR cells the proton pump is located at the apical membrane and this would appear to indicate that they secrete H^+ towards the allantoic fluid (urine). This would be consistent with the fact that this fluid undergoes progressive acidification during development (Abramovici, 1967; Freeman & Vince, 1974). While our immunocytochemical studies confirm the presence of the H^+-ATPase in these cells, they show in most cases a diffuse, rather than an apical localisation. For kidney proton-secreting cells diffuse cytoplasmic localisation is usually interpreted as indicating a low degree of proton secretory activity (Bastani et al. 1991).

Since H^+-secreting cells are known to secrete HCO_3^+ through the opposite pole, MR cells of the allantois would probably contribute to the increase in plasma bicarbonate observed during the second half of development.

A few MR cells showed a basolateral location of the proton pump, although this was not confirmed by the electron microscopical observations. If confirmed, this would suggest that similar to B-intercalated cells of mammalian collecting ducts and reptilian bladders, the allantois would contain a second type of mitochondria rich cell which secretes H^+ through the base and HCO_3^- through the apex.

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