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Swimbladder gas gland cells cultured on permeable supports regain their characteristic polarity

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Summary

A cell culture system has been developed in which swimbladder gas gland cells from the European eel (*Anguilla anguilla*) were cultured on a permeable support. Cells seeded on Anodisc 13 (Whatman) or Costar Transwell 13 mm membranes form a confluent cell layer within the first 2 or 3 days of culture but, on the basis of measurements of transepithelial resistance, it is a 'leaky' cell layer. In a superfusion system, the apical and basal sides of the cells were superfused asymmetrically, with saline on the apical side and a glucose-containing cell culture medium on the basal side. Under these conditions, the cells continuously produced lactic acid, and approximately 60–70 % of this lactate was released at the basal side. To mimic the *in vivo* situation, the saline solution supplied to the apical side was replaced by humidified air in an additional series of experiments. Cells cultured in an air/liquid system produced even more lactate, and this lactate was only released to the basal side; there was no leakage of fluid to the apical side. After 4 or 5 days in the superfusion system, the cells were fixed for histological examination. The cells were columnar, similar to gas gland cells *in vivo*, and showed a clear polarity, with some small microvilli at the apical membrane and extensive membrane foldings at lateral and basal membranes. Immunohistochemical localization of Na^+/K^+-ATPase revealed that this ATPase was present mainly in the lateral membranes; it was never found in the apical membranes. Cells cultured in the air/liquid system showed a similar structure and polarity.

Key words: Swimbladder, gas gland cell, epithelial cell, cell culture, *Anguilla anguilla*, air/liquid culture.

Introduction

The physiology of the swimbladder has attracted attention for a long time (Jacobs, 1930; Steen, 1970; Fänge, 1983; Pelster and Randall, 1998). While the first series of studies mainly focused on its morphology and the composition of the swimbladder gas (Hall, 1924; Saunders, 1953; Fänge, 1953; Scholander and Van Dam, 1954; Dorn, 1961), another series of experiments concentrated on the physiology of the isolated or isolated perfused swimbladder (Steen, 1963; Pelster et al., 1989; Kobayashi et al., 1990). These and many other studies provided an insight into the metabolism of swimbladder cells and into the mechanisms of gas secretion and of countercurrent concentration (Fänge, 1983; Pelster, 1997). The gas gland cells of the swimbladder epithelium were shown to be highly specialized cells that lack a Pasteur effect and produce and secrete acid over a wide range of extracellular pH values. Nevertheless, detailed information about the physiology of these gas gland cells remains obscure because these preparations always include a number of different cell types and the tissue does not allow for direct access to the gas gland cells.

In this situation, the use of cultured cells as a surrogate model has often proved advantageous in enhancing our understanding of the cellular physiology of complex organs and organ systems. Cultured branchial epithelia, for example, have been used to analyze the ion-transport characteristics of freshwater gills (Wood and Pärt, 1997), and studies on cultured kidney tubular cells have significantly enhanced our knowledge about the mechanism of renal acid–base regulation (Brown and Stow, 1995; Feifel et al., 1997; Alexander et al., 1999).

Similarly, the first insight into the mechanisms of ion transport and of metabolic control in gas gland cells was obtained using cultured cells. Working with isolated and cultured cells, it could be shown that gas gland cells secrete acid *via* the Na^+/H^+ exchanger, Na^+-dependent anion exchange and diffusion of CO_2 (Pelster, 1995; Pelster and Niederstätter, 1997). In addition, the presence of a V-ATPase was demonstrated, and this may also contribute to the secretion of protons generated in the glycolytic pathway (Niederstätter and Pelster, 2000).

Initially acid production and secretion were considered to be the most important function of these cells, but a recent study has revealed that gas gland cells are also responsible for the production and secretion of surfactant into the swimbladder lumen (Prem et al., 2000). Consequently, it is now clear that gas gland cells produce and secrete acid at their basolateral

membranes, and surfactant at their apical membranes. Thus, a cell culture system in which gas gland cells retain their polarity would be a useful model for studying the mechanisms of ion regulation and ion secretion. Several studies have shown that the polarity of fish epithelial cells can be retained in culture by using permeable supports (Dickman and Renfro, 1986; Wood and Pärt, 1997). The goal of the present study, therefore, was to establish a primary culture system for gas gland cells in which the cells retain their morphological and physiological polarity. Physiological polarity was assessed by measuring lactate release. Histological polarity was evaluated by electron microscopy and by immunohistochemical localization of Na^+/K^+-ATPase.

Materials and methods

Cell culture

The preparation of the gas gland cells from the European eel *Anguilla anguilla* followed the procedure described previously (Pelster, 1995; Prem and Pelster, 2000). Gas gland cells were seeded onto collagen-coated Anodisc 13 (Whatman) or Costar Transwell 13mm (Costar) membranes and cultured for 2 to 4 days to establish an almost confluent or confluent culture on the membrane. The Anodisc filter membranes were then transferred into chambers that allowed us to perfuse the apical and basal sides of the permeable support with different media (similar to an Ussing chamber). The basal side was perfused with culture medium [DMEM F12 (Gibco), supplemented with 10 μmol ml^{-1} alanine-glutamine, 0.5 % eel serum, 0.1 % bovine serum albumin (BSA), 1 μg ml^{-1} gentamicin, 1 μg ml^{-1} kanamycin], while the apical side was perfused with pure buffer solution, consisting of (in mmol l^{-1}): NaCl, 140; KCl, 5.4; $MgCl_2$, 1.0; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid, 10; ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5. Medium and saline solutions were exchanged every day, or at least every second day, when cultured on Costar Transwell filter membranes.

The superfusion system was custom-designed as described (Prem and Pelster, 2000). Briefly, the Perspex superfusion chambers were supplied with cell culture medium and saline (see above) by an eight-channel peristaltic pump (Ismatec IPC-8, Wertheim-Mondfeld, Germany). The flow was adjusted to a constant rate of 1 ml h^{-1}. The fluid leaving the apical and basal superfusion chambers was collected as 1 ml samples in Eppendorf tubes placed in a custom-made fraction collector. The fraction collector allowed the samples to be cooled for a few hours until they were frozen for further analysis at –80 °C. The whole arrangement was autoclaved prior to each run to avoid any bacterial contamination.

Occasionally, cell preparations showed rather poor adherence and the cell density on the filter membrane was low. To check the cell density in the superfusion system, Phenol Red was added to the fluid supplied to the basal membranes (Jovov et al., 1991), and only preparations in which the Phenol Red leakage from the basal to the apical chamber was less than 2.5 % were used for experiments. In these preparations, the cell density on the filter membrane was approximately 400,000 cells cm^{-2}. In the air/liquid culture system, the fluid supplied to the apical side of the superfusion system was replaced by humidified air. Humidification of the air was achieved by bubbling the air through a series of water bottles.

Physiological measurements

The lactate content of the medium used for the superfusion system was determined in an enzymatic test according to the principle described by Bergmeyer (1974). Measurements were performed in a plate reader (fmax, Molecular Devices, Munich, Germany) using the difference in the fluorescence signals of NAD^+ and NADH.

The transepithelial resistance of cells cultured on Costar Transwell 13 membranes was determined using an EVOM epithelial voltohmmeter with ENDOHM 12 electrodes (World Precision Instruments, Berlin, Germany). In these experiments, cells were supplied with DMEM F12 on the basal side, and with saline on the apical side. Media were exchanged daily. Control measurements were performed using exactly the same system, but no cells were seeded on its filter membranes. Measurement of the transepithelial resistance within the superfusion system proved impossible.

SDS-PAGE and western blot analysis

Protein from a swimbladder homogenate was separated by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) using the NuPage buffer system. Electrophoresis was performed with Power Ease 500, X-Cell II using NuPage 10 % Bis Tris gels (all from Novex, Germany). The SDS-PAGE was performed under reducing conditions using dithiothreitol (DTT), at 125 μmol l^{-1}.

Electrophoretic transfer of proteins to a nitrocellulose membrane was performed using Power Ease 500 (Novex, Germany). The transfer was conducted for 1 h at a constant voltage of 25 V (160 mA). The nitrocellulose membranes were placed in a sealed bag containing 10 % BSA, 10 % fetal calf serum (FCS) and 0.1 % Tween 20 (Sigma) in 100 mmol l^{-1} phosphate buffer and gently agitated for 1.5 h at room temperature (22–24 °C). After washing, the membranes were incubated overnight at 4 °C with a chicken Na^+/K^+-ATPase antibody (Biogenesis, Poole, Great Britain) diluted 1:50,000 (v/v) in phosphate buffer containing 1 % BSA, 1 % FCS and 0.1 % Tween 20. The membranes then were washed and incubated for 1 h with Sigma anti-chicken IgG (A9046) at 1:10,000 (v/v) conjugated with horseradish peroxidase, in phosphate buffer with 1 % BSA, 1 % FCS and 0.1 % Tween 20 at room temperature. Antibody binding was visualized by enhanced chemiluminescence (ECL; Amersham Life Science).

Immunohistochemistry

Cells were fixed in 4 % paraformaldehyde in 10 mmol l^{-1} phosphate buffer (pH 7.4) for 1 h, washed, blocked for 1 h with 0.2 % I-Block (Tropix, USA) and 0.2 % Triton X-100 (Sigma) in 10 mmol l^{-1} phosphate buffer and incubated with the chicken Na^+/K^+-ATPase antibody (Biogenesis, Poole, Great Britain) at

a dilution of 1:100 in blocking buffer overnight at 4 °C. After washing, the samples were incubated for 1 h with Sigma anti-chicken IgG (A9046) fluorescein isothiocyanate (FITC)-conjugated antibody (Dako), diluted 1:100 in blocking buffer, and embedded in Vectashield (Vector Laboratories). Analysis was performed with a laser-scanning microscope (Zeiss, LSM 510). Cells were sectioned with a vertical resolution of 0.3 μm.

Electron microscopy

Cells on permeable supports were placed into 2.5 % glutaraldehyde in 10 mmol l^{-1} phosphate buffer (Dulbecco's formula, pH 7.4) for 30 min, washed and postfixed in 2 % osmium tetroxide containing 2.5 % potassium ferrocyanide for 90 min at 4 °C. The samples were dehydrated through a graded acetone series and embedded in Spurr's 15 low viscosity resin (Spurr, 1969). Ultrathin sections were cut with an Ultracut E (Reichert, Austria), double-stained with uranyl acetate and lead citrate and examined in an EM902 (Zeiss, Germany).

Data analysis

Data obtained with the laser-scanning microscope were processed on an O2 workstation (Silicon graphics) using an appropriate software package (Imaris 2.6.8) from Bitplane (Bitplane AG). For contrast enhancement and deconvolution, the software package 'Huygens, 2.0' (Scientific Volume Imaging BV, Netherlands) was used. Three-dimensional reconstruction of the cells was achieved with the Isosurface module of Imaris 2.6.8.

Statistically significant differences in the observations were evaluated using a one-way analysis of variance (ANOVA) followed by a multiple-comparison procedure (Bonferoni). Significance was accepted when $P<0.05$. Data are presented as means ± standard error of the mean (S.E.M.). *N* represents the number of filters from 3–5 different fish.

Results

Electron microscopical examination of swimbladder gas gland cells cultured on a permeable support (Anodisc 13 membranes in a superfusion system) with nutrient-containing culture medium at the basal side and nutrient-free saline at the apical side revealed that the cells are columnar, with a height of

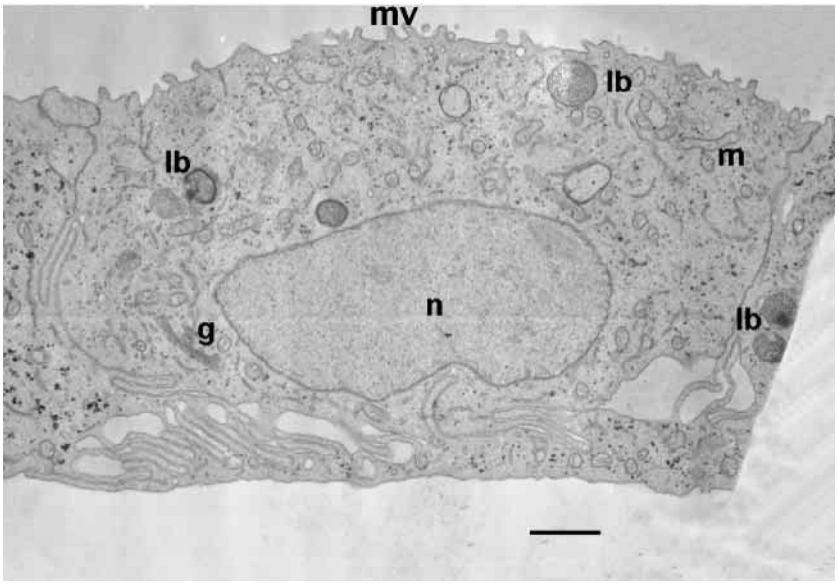

Fig. 1. Electron micrograph of a gas gland cell cultured on a permeable filter membrane. The cell is columnar and shows a clear polarity with some microvilli at the luminal membranes and extensive membrane foldings in both the lateral and the basal membranes. The cytoplasm contains filamentous mitochondria and a Golgi apparatus. Lamellar bodies are most commonly seen near the luminal membranes. g, Golgi apparatus; lb, lamellar body; m, filamentous mitochondria; mv, microvilli; n, nucleus. Scale bar, 1 μm.

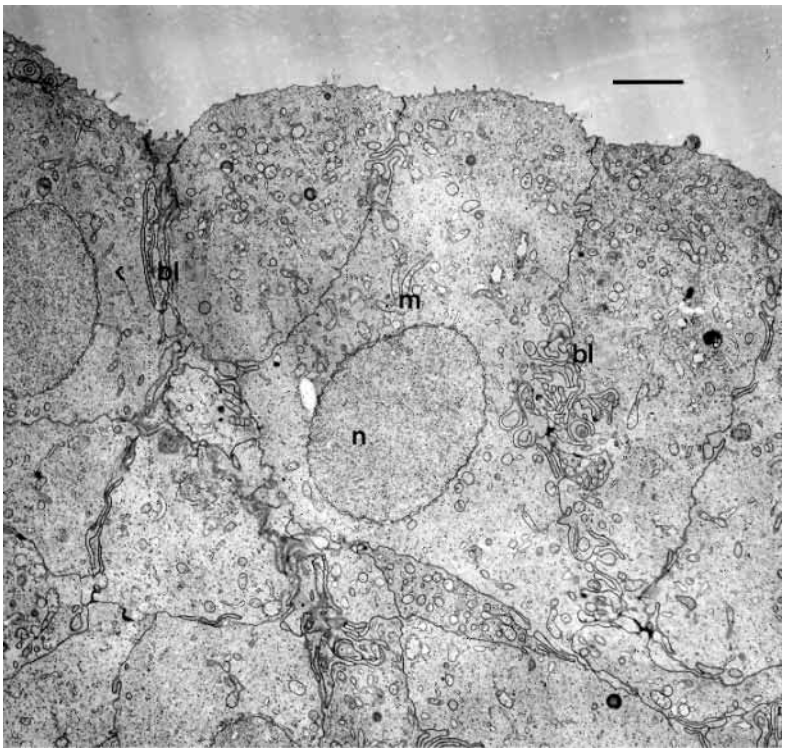

Fig. 2. Electron micrograph of gas gland cells forming a pseudostratified epithelium. In this case, only luminal cells show a clear polarity, with some microvilli at the luminal membranes and extensive membrane foldings in the basolateral membranes. bl, basolateral membrane foldings; m, filamentous mitochondria; n, nucleus. Scale bar, 1 μm.

approximately 5–10 μm (Fig. 1). The apical membrane is characterized by small microvilli. A number of vesicles, which resemble the lamellar bodies, are located near the apical surface. Pronounced membrane foldings were observed; these were usually associated with the lateral membranes, but in some preparations they were also found in basal membranes.

Occasionally, gas gland cells seeded on permeable filter membranes (Costar Transwell 13) formed a pseudostratified epithelium (Fig. 2). In this case, only the most apical cells showed some polarity, with small microvilli at the luminal membrane and membrane foldings at the lateral and basal membranes. These cells were not cultured in the superfusion system. Histological examination of cells that have been cultured in the superfusion system for 2 days did not show pseudostratification.

Cells cultured in an air/liquid system, with humidified air at the apical side and nutrient-containing culture medium at the basal side developed a polarity similar to cells cultured in a liquid system, where both sides of the cells were supplied with fluid (see Fig. 1). Histological examination of these cells showed no sign of an improved polarity (data not shown).

Using immunohistochemistry Na^+/K^+-ATPase was found to be largely confined to the lateral membranes, and was not present in apical membranes (Fig. 3). Fig. 4 shows a three-dimensional reconstruction of a cell obtained after deconvolution of the data. Fluorescence is found in the lateral membranes and in the basal parts of the cultured cell. Membrane foldings penetrate the basal part of the cells (see Fig. 1), and the fluorescence islets observed inside the cell represent these membrane foldings. The specificity of the commercially available antibody was tested using western blot analysis. Bands with a molecular mass of approximately 40 kDa and 50 kDa were observed in all homogenates tested (Fig. 5). In addition, a band with a molecular mass of approximately 60 kDa was present.

The transepithelial resistance of cells grown on permeable supports increased gradually after 2 days on the filter membrane. The highest resistance was observed after 5 days of culture (Fig. 6). After 4 or 5 days in the superfusion system (i.e. 6 or 7 days after cell seeding), the preparation became very unstable, due to detachment of cells and a lowering of cell viability. Physiological experiments were, therefore, always terminated after 5 days of recording.

In the liquid culture system, lactate release appeared to increase for the first few hours, probably reflecting an initial equilibration of the system to a steady state. After approximately 10 h in the superfusion system a stable, high level of lactate release was reached. For the following 2 or 3 days in the superfusion system, the rate of lactate release remained constant, and lactate release to the basal side always exceeded lactate release to the apical side. Approximately 60–70 % of the total lactate released appeared at the basal side,

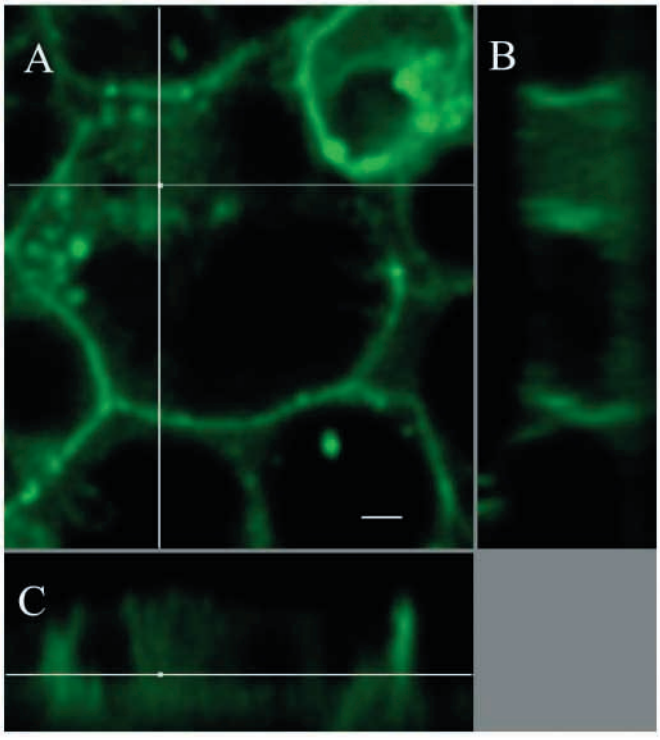

Fig. 3. Immunohistochemical localization of Na^+/K^+-ATPase in primary cultured eel gas gland cells. The confocal image shows three optical sections of a gas gland cell in the *xy* (A), the *yz* (B) and the *xz* (C) planes. The white bars in A (the *xy* section) indicate the locations of the other two sections; the white bar in C (the *xz* section) indicates the location of the *xy* section in A. Note that Na^+/K^+-ATPase immunoreactivity is located at lateral cell membranes, but there is no immunoreactivity at the apical membranes. Scale bar, 1 μm.

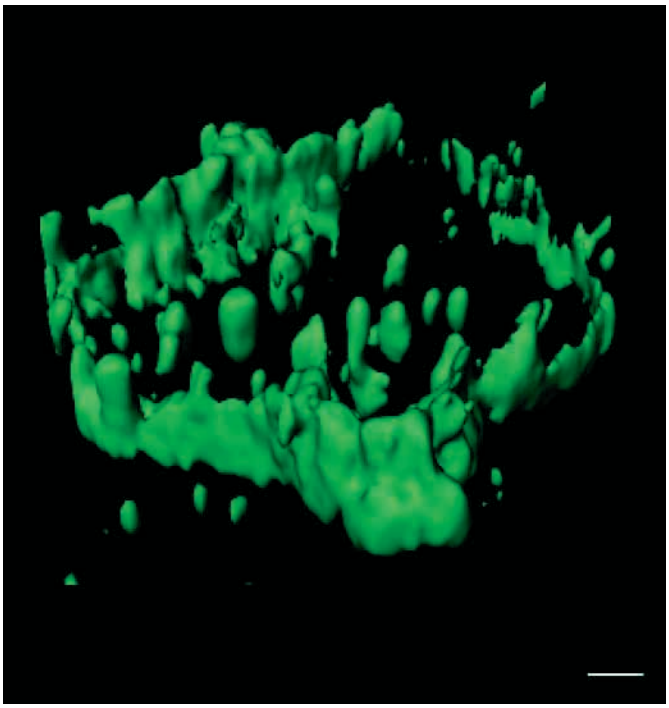

Fig. 4. Three-dimensional reconstruction of Na^+/K^+-ATPase in cultured gas gland cells, calculated on the basis of the confocal images shown in Fig. 3. Na^+/K^+-ATPase is localized to the lateral and to some basolateral membranes. Scale bar, 1 μm.

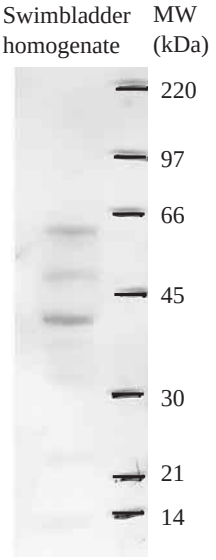

Fig. 5. A typical western blot of eel swimbladder homogenate after incubation with chicken Na^+/K^+-ATPase antibody. In negative controls performed without incubation with the primary antibody no bands were detected. Molecular mass standards (in kDa) (Amersham rainbow marker RPN756 was used) have been labelled by hand.

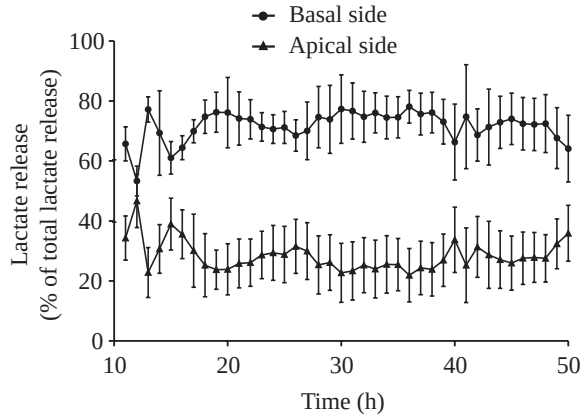

Fig. 7. A comparison of the lactate released (as a percentage of total lactate released) from the basal (circles) and apical (triangles) sides of gas gland cells cultured on permeable Anodisc 13 membranes over a period of 2 days. The total rate of lactate release in these experiments was between 0.5 and 0.6 nmol h^{-1}. Values are means ± S.E.M.; *N*=20.

and only 30–40 % at the apical side (Fig. 7). The total amount of lactate released was in the range of 0.5–0.6 nmol h^{-1}. Cells cultured in the air/liquid system released lactate only to the basal side, and no fluid leaked through to the apical side. The rate of lactate production of these cells was significantly higher than the lactate production of cells cultured in the liquid system (Fig. 8).

Discussion

The results of our study demonstrate that swimbladder gas gland cells can be cultured on permeable supports, and under these conditions, retain functional and histological polarity. While cells cultured in a Petri dish are flat and show no signs of a basolateral labyrinth (Prem and Pelster, 2000), cells cultured on permeable supports are columnar and show basolateral membrane foldings, which are clearly reminiscent of gas gland cells *in vivo*. Cells cultured on permeable supports contain vesicles that resemble the lamellar bodies found in surfactant secreting type II cells of the mammalian lung epithelium (Mason and Shannon, 1997; Mair et al., 1999). Furthermore, immunohistochemical localization of Na^+/K^+-ATPase using commercially available antibodies showed that immunoreactivity was associated with the lateral membranes and the basolateral membrane foldings of the cells, but was absent from the apical membranes. In western blots of swimbladder proteins, bands with a molecular mass of approximately 40, 50 and 60 kDa were identified by the antibody. The molecular mass of the β-subunit of Na^+/K^+-ATPase has been reported to vary between 35 kDa and 55 kDa,

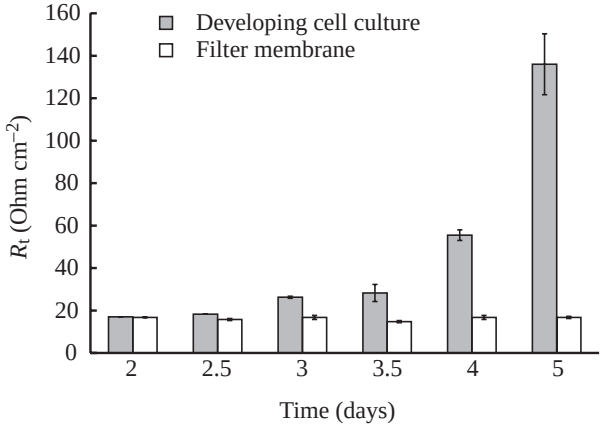

Fig. 6. Changes in transepithelial resistance R_t of cultured swimbladder gas gland cells over time with DMEM medium supplied to the basal membrane and buffer solution to the apical membrane. Cells were seeded onto Costar Transwell 13 membranes at day 0. Values are means ± S.E.M., *N*=6

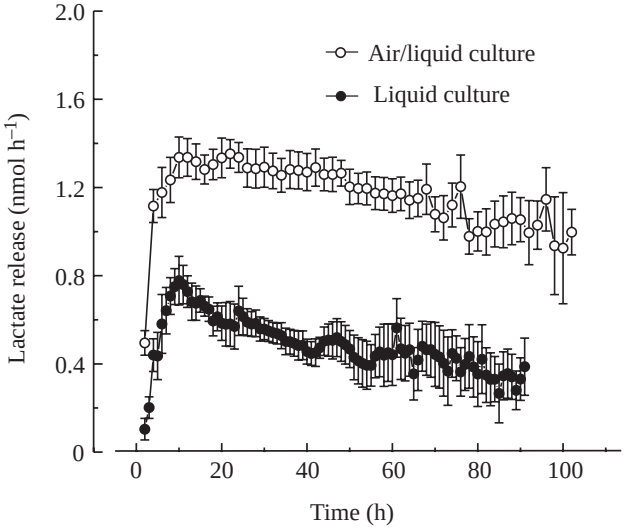

Fig. 8. Average rate of total lactate release of gas gland cells cultured on permeable supports in a superfusion system with fluid supplied to both sides of the superfusion system (liquid culture) and in an air/liquid system, in which humidified air was supplied to the apical side of the cells. Values are mean ± S.E.M.; *N*=20.

depending on the size of the *N*-linked sugars attached to the protein (Mobasheri et al., 2000). We conclude, therefore, that the bands at 40 and 50 kDa are related to the presence of β-subunit. The band at 40 kDa is the dominant band but, depending on the preparation, the size of attached sugar molecules may vary, giving rise to an additional fainter band at 50 kDa. The band at 60 kDa may represent a highly glycosylated isoform of this subunit in eel or it may be caused by unspecific binding of the antibody to a different protein. The complete absence of any reaction in the control blots, however, led us to conclude that the immunoreactivity observed in cultured gas gland cells reflects the presence of Na^+/K^+-ATPase in the lateral and basolateral membranes. While the absence of Na^+/K^+-ATPase from the apical membranes can be predicted from the structural polarity of gas gland cells, the three-dimensional reconstruction of the cells by confocal microscopy also showed an absence of the ATPase from the most basal parts of the cell membranes. It is these basal sections of the membrane, however, that are the site of attachment to the permeable supports. Attachment to the filter membranes requires the presence of special membrane proteins such as cadherins, and this might explain the absence of Na^+/K^+-ATPase from the most basal membrane sections. Our histological studies thus clearly demonstrate that gas gland cells cultured on permeable supports and superfused with different media at their basal and apical membranes retain a polarity similar to the *in vivo* situation. Similar results have been obtained with gill epithelial cells (Wood and Pärt, 1997; Wood et al., 1998; Gilmour et al., 1998; Sandbacka et al., 1999; Kelly et al., 2000) and also with kidney epithelial cells (Dickman and Renfro, 1986; Renfro et al., 1993; Dudas and Renfro, 2001).

In vivo, eel gas gland cells are exposed to a gas phase on their apical side, and we have successfully developed an air/liquid culture system to mimic the *in vivo* situation as closely as possible. An air/liquid culturing system has been used for mammalian lung epithelial cells (Dobbs et al., 1997) and, in this situation, surfactant-secreting type II epithelial cells have been cultured successfully.

Measurement of the electrical resistance of cultured gas gland cells revealed that the epithelia have only a low resistance. Gill cells in culture form epithelial layers with an electrical resistance of several thousand $\Omega\,cm^{-2}$, which is 30–50 times higher than the resistance measured for gas gland cells in the present study. In cultured freshwater gill cells, the magnitude of the transepithelial resistance increases with decreasing salinity of the media on the apical side (Wood and Pärt, 1997; Avella and Ehrenfeld, 1997; Gilmour et al., 1998). Gill cell epithelia from seawater fish in turn have a high resistance (Avella and Ehrenfeld, 1997), which clearly demonstrates that the resistance of these preparations is significantly modified by the composition of the medium. In contrast to gill cells, which form a barrier to the low-osmolarity external medium in freshwater trout, the apical side of gas gland epithelial cells is facing a gas phase. The apical surface of gas gland cells is covered with hydrophobic surfactant (Prem et al., 2000), so that a very tight epithelium is not necessary. This may explain the low electrical resistance. For native trachea sheets studied in an Ussing chamber, resistance values of approximately 125–150 $\Omega\,cm^{-2}$ have been reported (Yamaya et al., 1992), which is within the range of values measured for our cultured gas gland cells after 5 days in culture.

The physiological function of gas gland cells is to produce lactic acid and CO_2, which are secreted at their basolateral membranes (Pelster et al., 1989), and to produce surfactant, which is released at their apical membranes (Prem et al., 2000). Cultured gas gland cells produce and release lactic acid, and a comparison of the lactate contents in the superfusate of the apical side with that of the basal side revealed that approximately 60–70 % of the lactate is released at the basal membranes. This generates a gradient in lactate concentration from the basal chamber of the perfusion system to the apical side, which favours lactate diffusion from the basal to the apical chamber. Measurements of electrical resistance demonstrated that gas gland cells form a leaky epithelium. It may well be, therefore, that the measured ratio of lactate release to the apical and the basal sides is an underestimate because of paracellular diffusion of lactate from the basal chamber of the superfusion system to the apical side.

In the air/liquid culture, all the lactate was released to the basal side, and there was no fluid transfer to the apical side. At first glance, this may contradict the idea of lactate diffusion between the two sides of the Ussing chamber. However, assessing the tightness of the epithelial layer using Phenol Red clearly demonstrated that some leakage may occur in the liquid culture system, and we used only epithelial layers in which this leakage rate was below 2.5 %. Lactate is a much smaller molecule than Phenol Red, and if Phenol Red penetrates the cell layer, lactate should penetrate as well. In the air/liquid system, however, surfactant has to be taken into account. While in the liquid culture system, surfactant is secreted into a liquid phase and may be washed away; in the air/liquid culture system, the hydrophobic surfactant will cover the apical surface of the cells and thus block paracellular fluid transfer.

It is interesting to note that in the air/liquid culture system the amount of lactate released by the cells significantly exceeds that of cells in the liquid culture system. Previous experiments have shown that the rate of acid secretion as well as the rate of lactate production and release by the swimbladder tissue of the European eel decrease under hypoxic conditions (Pelster and Scheid, 1992, 1993). This observation could be explained, in part, by a decrease in blood supply and thus in glucose supply to the swimbladder tissue during hypoxia. The observed decrease in metabolic activity, however, far exceeded the decrease in blood supply, so these experiments provided clear evidence for a downregulation of metabolic activity of the swimbladder tissue during hypoxia (Pelster and Scheid, 1993). Given the significantly higher oxygen capacity of air compared with saline, the oxygen supply to the cells in the air/liquid system is certainly much better than in the liquid culture

system. Thus, the higher rate of lactate release in the air/liquid experiments may be explained by a better oxygenation of the cells. This would imply that the results of the present study support the hypothesis that the metabolism of gas gland cells is, in part, controlled by the level of oxygen availability. In this context, it should be mentioned that gas gland cells lack a Pasteur effect (D'Aoust, 1970). Thus, in gas gland cells, glycolytic flux does not decrease at higher oxygen tensions. This certainly makes sense given that gas gland cells are typically exposed to hyperoxic conditions (Pelster, 1997) and, to ensure continued gas secretion, must continue to produce lactic acid in the glycolytic pathway. The explanation for this unusual effect of oxygen on the metabolic activity of gas gland cells must await further experimentation.

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