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Swimbladder gas gland cells of the European eel cultured in a superfusion system

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Abstract. Swimbladder gas gland cells are polar epithelial cells which release acidic metabolites through the membranes of an extensive basolateral labyrinth, and secret surfactant via exocytosis at apical membranes. We have developed a method to establish primary cell cultures of gas gland cells in order to establish a model system for physiological analysis of gas gland cell function *in vitro*. Isolated gas gland cells attach to collagen S coated surfaces. Cells cultured in collagen S coated petri dishes were flat and showed no histological polarity. Cells cultured on Anodisc membranes in a superfusion system, in which the apical and basal side of the cells was supplied with a saline solution and with glucose containing DMEM cell culture medium, respectively, showed a clear polarity similar to the *in vivo* situation. Measurement of lactate release at the apical side and at the basal side revealed that these cells were functionally polar and secreted at least 70% of the lactate at their basal membranes. Gas gland cells could also be cultured in an air/liquid system, in which the apical membrane was exposed to humidified air. Cells cultured under these conditions released lactate only on the basal side and histologically were similar to cells cultured in the superfusion system.

Key words: Air/liquid cell culture, *Anguilla anguilla*, Gas gland cells, Superfusion system, Swimbladder

1. Introduction

Swimbladder gas gland cells are epithelial cells specialized for the production of acidic metabolites. In spite of the fact that they usually are exposed to high oxygen tensions, they mainly produce lactic acid from extracellular glucose stores [9, 10]. As a second acidic metabolite CO_2 is formed in the pentose phosphate shunt, only traces of glucose are oxidized in the aerobic metabolism [11, 17]. This acid production and the subsequent acid release into the blood stream are essential for the reduction of the gas carrying capacity of the blood during passage of the swimbladder tissue, which results in an initial increase in blood gas partial pressures [4, 13, 15]. In a subsequent step this initial increase in gas partial pressures is then multiplied by countercurrent multiplication, and the swimbladder is filled with gas by diffusion of gas molecules from the blood into the swimbladder lumen [4, 5, 13].

At the luminal surface the swimbladder epithelium is covered with surfactant [2], and recent studies revealed, that this surfactant is also produced by swimbladder gas gland cells [16]. Gas gland cells therefore apparently serve a dual function, they are responsible for the production of acidic metabolites, which are released at the basolateral membranes, and they produce surfactant, which is secreted via exocytosis at luminal membranes.

Being able to culture gas gland cells would be extremely useful for studying cellular aspects of acid release or exocytosis of surfactant. A recent study [12] demonstrated that gas gland cells can be put into primary culture. Photomicrographs of these cells cultured on collagen gel showed typical polygonal epithelial cells, but the histology of these cells was not analyzed. Cells cultured in petri dishes typically are very flat and show no clear polarity. Gas gland cells *in vivo*, however, are highly polar with an extensive basolateral labyrinth and some microvilli in the luminal membrane [3, 18]. The present study therefore was set out to establish a superfusion culture system for gas gland cells, in which the cells can be supplied with different media from the basal and luminal side and thus should regain their characteristic polarity observed *in vivo*. Ultimately, an air-liquid system should be developed in order to mimic the *in vivo* situation, in which the luminal surface is exposed to the gas phase of the swimbladder lumen.

2. Materials

A. Computers and software.
   1. IBM compatible personal computer.
   2. Software package for LSM 510.[1]
   3. Silicon Graphics O2.[2]

4. Software package for 3D imaging Imaris, 2.6.8 and Selima 2.0 Database software.[3]
5. Software package Huygens 2.0 for deconvolution.[4]

B. Equipment

1. Erbotom T71D.[5]
2. CO_2 Water Jacketed Incubator Modell 3121.[6]
3. Laminar flow Type UVF 6.15S.[7]
4. Labo Autoclave.[8]
5. PHM 84 Research pH Meter with HA405-60-88TE-S7/12C electrode No. 104054425.[9, 10]
6. Laboport pump connected with a MF75 filter bottle with CN 0,2 µm filters.[11, 12]
7. MS 1 Minishaker.[13]
8. Refrigerated Heraeus centrifuge Labofuge 400R.[14]
9. Eppendorf Centrifuge 5417C.[15]
10. Ismatec IPC-8 Peristaltic pump.[16]
11. Pond air pump model GL 500.[17]
12. Custom designed fraction collector for collection of eight samples in parallel, programmable.

C. Microscopic equipment

1. Axiovert 25 with Varel contrast and phase contrast.[1]
2. LSM 510 with Axiovert 100 M, AttoArc HBO 100W and SNT 12V 100W, argon laser (488 nm) and helium/neon laser (543 nm), filter set for red and green fluorescence LP 560 (red) and BP 503-530 (green), HFT 488/543, NFT 545.[1]

D. Solutions and media

– Saline solution: 140.0 mM NaCl; 5.4 mM KCl; 1.0 mM $MgCl_2 \cdot 6\ H_2O$; 10.0 mM Hepes in autoclaved Millipore H_2O, adjusted to pH 7.4.
– Zelltyrode II: 0.5 mM EGTA in saline solution.
– Enzyme solution: 0.5 mg ml^{-1} serum albumin;[18] 0.22 mg ml^{-1} collagenase;[19] 0.17 mg ml^{-1} protease;[18] 0.15 mg ml^{-1} DNase;[18] 4 µl ml^{-1} elastase[20] in saline solution; sterile filtered with a 0.2 µm filter.
– Stopping solution: 10% FCS (fetal calf serum)[21] in Medium 199;[21] 0.2 µm sterile filtered.
– Washing medium: DMEM F12.[21]
– Incubation medium: 5 µg ml^{-1} ITS;[19] 16 µg ml^{-1} putrescine;[18] 6.29 ng ml^{-1} progesterone;[18] 1 µl ml^{-1} EGF (epidermal growth factor);[18] 0.5 µl ml^{-1} pituitary extract;[18] 1 mg ml^{-1} serum albumin;[18] 5 µl ml^{-1} eel serum; 2.17 mg ml^{-1} alanyl-glutamine;[18] 2 µl ml^{-1} kanamycin;[18] 1 ml ml^{-1} gentamicin[18] in DMEM F12; 0.2 µm sterile filtered.
– Incubation medium for superfusion experiments: 2.17 mg ml^{-1} alanyl-glutamine;[18] 5 µl ml^{-1} eel serum; 1 mg ml^{-1} albumin 18; 2 µl ml^{-1} kanamycin;[18] 1 µl ml^{-1} gentamycin[18] in DMEM F12;[21] 0.2 µm sterile filtered.
– Collagen coating of the filter membranes: Autoclave the filter membranes and the holder rings. Mix 1 part collagen S (Type I)[19] and 3 parts 60% ethanol (0.2 µm sterile filtered). Pipette 70 µl of this solution on filter membranes and dry them over night in the laminar flow.
– 10 mM phosphate buffer Dulbecco's formula (PBS): 0.2 M KCl;[18] 0.2 M KH_2PO_4;[18] 8 M NaCl;[18] 1.15 M Na_2HPO_4.[18]
– fluorescent dyes: JC-1;[28] MitoTracker™ [28] Green; SYTO® [28];[13] Hoechst 33342 (no. B2261).[18]
– chemicals for immunohistochemical experiments: 4% paraformaldehyde;[18] I-Block,[29] Triton X-100;[18] chicken anti Na^+/K^+-ATPase antibody no. 0126-1108;[30] FITC conjugated anti chicken IgG antibody no. F4137;[18] Vectashield no. H-1000.[30]

E. Glassware and plastics

All glassware is treated with HCl before the first use and washed with phosphate free cell culture glass cleaning solution (7X® PF, no. 76-671-21).[22] After washing the glassware for a few hours we rinse it with water and 3 times with Aqua dest. before we autoclave all our glassware for at least 30 minutes.

– 50 and 100 ml Duran laboratory glassware and petri dishes.[23]
– 100 and 250 ml Duran laboratory bottles with ISO thread.[23]
– Anodisc 13 0.2 µm membrane disc no. 6809.7023.[24]
– Minusheets 13 mm, no. 1300.[25]
– Tissue culture plate 24-well no. 83.1836.500.[26]
– 50 ml centrifuge tubes no. 62.559.001.[26]
– 15 ml centrifuge tubes no. 62.554.002.[26]
– 20 ml inject disposable syringes no. 1942 12020.[27]
– 10 ml inject disposable syringes no. 1942 12010.[27]
– 1 ml omnifix disposable syringes no. 1942 13101.[27]
– 0.2 µm Filtropur S no. 83.1826.001.[26]
– 0.45 µm Filtropur S no. 83.1826.[26]
– 3.5 ml transfer pipettes no. 86.1171.001.[26]
– 70 µm nylon cell strainer no. 2350.[32]
– 1.5 ml micro tubes no. 72.690.[26]
– silicone tubes and fittings no. 1306.[25]
– Intramedic Polyethylene Tubing PE 20 no. 427406.[32]
– Intramedic Polyethylene Tubing PE 60 no. 7416.[32]

3. Procedures

A. Preparation of the animals

Individuals of the European eel *Anguilla anguilla* (body mass 150 g–450 g) were obtained from local fishermen and kept in a fresh water aquarium at 12 °C for up to two month. The fish were not fed and kept mostly in the dark. All experiments were performed at 20 °C.

1. The eels are quickly decerebrated, spinally pithed and fixed in a special eel-holder [8]. During the preparation the gills of the eels are ventilated with air saturated fresh water at a flow rate of about 1.5 l min^{-1} to assure a sufficient oxygen supply to the tissues.
2. Open the body wall ventrally and separate the intestine from the tissue which surrounds the swimbladder.
3. Expose the swimbladder carefully and separate it from the connective tissue.
4. The artery and vein entering and leaving the swimbladder are separated from the surrounding connective tissue.
5. Insert occlusive catheters into the swimbladder artery (PE-20) and swimbladder vein (PE-60).
6. Perfuse the swimbladder with about 2 ml of saline solution until the vessels are free of red blood cells.
7. Infuse about 3 ml of the enzyme solution to start the dissociation of tissue.
8. Remove the outer layers of swimbladder tissue including the guanine encrusted submucosa.
9. Dissect the epithelium of the secretory part of the swimbladder for further isolation of gas gland cells.

B. Isolation of cells

1. Chop the swimbladder epithelium into small pieces and incubate them in 5 ml cold enzyme solution for 10 minutes.
2. Filter the solution through a 70 µm nylon mesh and transfer the isolated single cells and cell clusters cells into 10 ml stopping solution.
3. For the remaining tissue the enzyme incubation and the filtration procedure is repeated twice.
4. Cells in the stopping solution (25 ml) are sedimented by centrifugation with 100 g at 4 °C for 10 minutes.
5. Resuspend the cells in about 20 ml washing medium (DMEM F12).
6. The centrifugation and resuspension procedure is repeated twice in order to wash the cells.
7. Resuspend the cells in about 2 ml incubation medium and check the cell density and condition by microscopical inspection (phase contrast).
8. Seed the cells on collagen coated permeable supports.
9. Incubate the cells for about 2 or 4 days at 20 °C and 0,5% CO_2 until they reach confluence.
10. Renew the incubation medium every second day.

C. Cell culture

The cells are seeded onto Anodisc 13 filter membranes. These filters are assembled between the two holder rings of the Minusheets (Figure 1A). Normally these holder rings have legs on the lower half, which are removed to get better tightness in the superfusion chambers (see below). Before seeding the cells place the assembled filter membranes in culture plates (24 wells) on small tables made of polycarbonate (autoclaveable) and fill the chamber with 2 ml of the incubation medium. Then add 0.5 ml of the cell suspension onto the filter membranes and let the culture grow for two to four days. When the cells reach confluency transfer the filter membranes into the cell chambers of the superfusion system, which essentially is a modified Ussing chamber. The cell chambers are tightened with O-rings and fixed with three thumb screws (Figure 1B).

The whole superfusion system consists of these superfusion or cell chambers, an eight channel peristaltic pump, two bottles with different media for each side of the chamber, and a custom designed fraction collector (Figure 2). The bottles, cell chambers and the fraction collector are connected with silicon tubing. The peristaltic pump is set to a constant flow of 1 ml h^{-1}, and the fraction collector separately collects the two solutions in samples of one hour interval (i.e. 1 ml samples). This fraction collector permits a cooling of the samples for a couple of hours until they are frozen at –80 °C for further analysis. Each part of this setup can be autoclaved.

In the liquid cell culture experiments the cells were superfused with the incubation medium at the basal side and saline solution at the apical side. In air/liquid experiments the luminal side of the cells is exposed to humidified air. Air is sterilized by using an aquarium pump to pass it through a 0.45 µm filter and humidified with teflon air-stones in two serial 50 ml plastic bottles filled with autoclaved destilled water. The humidified air is pumped to the cell chamber by the peristaltic pump at a flow rate of 1 ml h^{-1}.

For comparison cells were also seeded on collagen S coated petri dishes. Histological examination of these cells followed classical procedures of histological tissue examination [18].

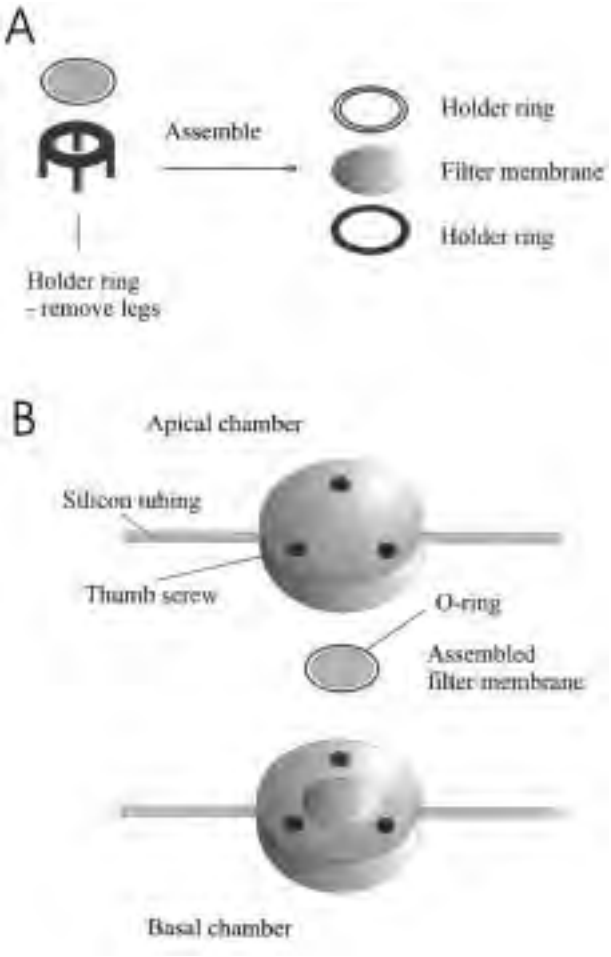

Figure 1. (A) Assembly of the filter membrane (Anodisc) and the Minusheet holder rings. The legs of the lower holder should be removed a assure a better sealing in the superfusion chamber. (B) The superfusion chamber basically is a modified Ussing chamber, in which the apical and the basal side of the filter membrane can be supplied with different incubation media. The tight sealing of the filter membrane within the experimental chamber is achieved by O-rings.

D. The Superfusion System
   1. Wash the superfusion system with 70% ethanol and Millipore H_2O and then fill the bottles with the different media.
   2. When the cell culture is confluent, insert the filter membranes with the cells into the cell chambers of the superfusion system and perfuse the apical and the basal chamber with the different media. In the first series of experiments we used cell culture medium at the basal side and saline solution at the apical side.
   3. Make sure that no air bubbles remain within the cell chambers.
   4. Adjust the flow for the media to 1 ml h^{-1}.
   5. Collect the medium and saline solution behind the cell chambers every hour for further analysis (determination of lactate concentration according to [1]).
   6. For air/liquid experiments use humidified air instead of saline solution at the apical side.

E. Live staining of cells
   1. Check the cell cultures by phase contrast microscopy.
   2. Wash the permeable supports with the cells at least two times with PBS that contains 10 mM glucose.
   3. Incubate the cells in PBS containing glucose and the JC-1 solution (1:200) for 15 minutes or in 50 nM MitoTracker™ Green in PBS containing 10 mM glucose for 45 minutes in the dark. For staining the nucleus use 10 µM Hoechst 33342 or 100 nM SYTO® 13 in PBS with 10 mM glucose.
   4. Wash the cell culture 3 times in PBS that contains 10 mM glucose.
   5. Keep the cells in the dark until fluorescent microscopic examination.

4. Results and discussion

Although swimbladder gas gland cells did not attach to uncoated plastic surface, they could be cultured on collagen S coated petri dishes. Electron microscopical examination revealed that cells cultured under these conditions were flat and had their highest elevation in the area of the nucleus, where the cell thickness was about 4 to 5 µm. The cells showed no obvious histological polarity (Figure 3). This is in stark contrast to the appearance of gas gland cells *in vivo*. Gas gland cells of the eel typically are characterized by extensive basolateral membrane foldings, sometimes termed basolateral labyrinth [3, 18]. The apical membrane includes some small microvilli, and mitochondria are present, although not numerous.

Cells cultured on a permeable support (Anodisc membranes) show a rather different histology (Figure 4). The cells are cuboidal and the nucleus does not cause any deformation of the cells. The cells are

Table 1. Fluorescent dyes and wavelengths necessary for fluorescent microscopy

Fluorescent stain	Excitation wavelength	Emission wavelenght
JC-1	488 nm	520 nm (green) and 590 nm (red)
MitoTracker™ Green	488 nm	520 nm
SYTO® 13	488 nm	520 nm
Hoechst 33342	350 nm	460 nm

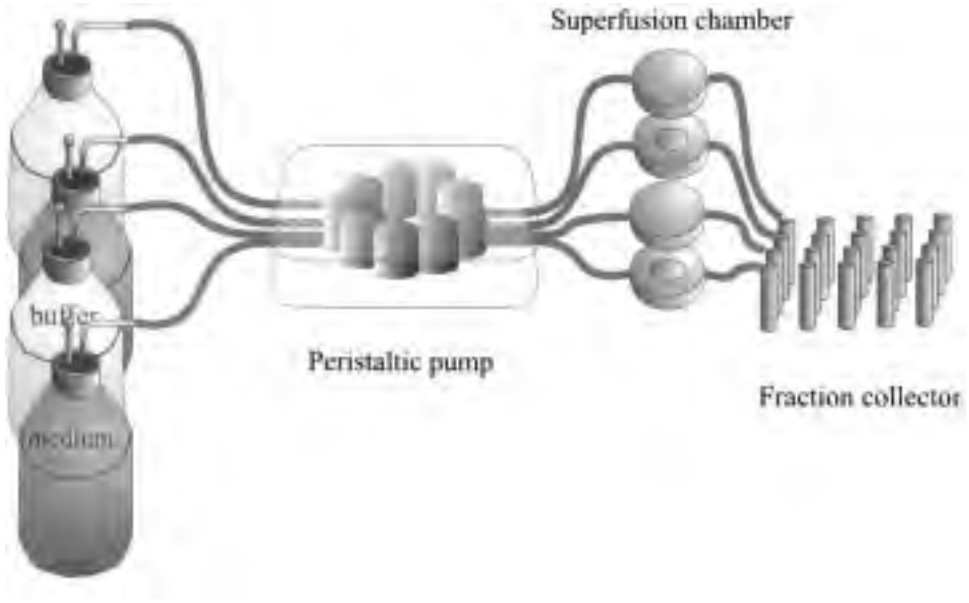

Figure 2. Set up of the superfusion system. A multi channel peristaltic pumps supplies incubation media to the superfusion chambers. The outflow is connected to a fraction collector, which collects the superfusate for further biochemical analysis.

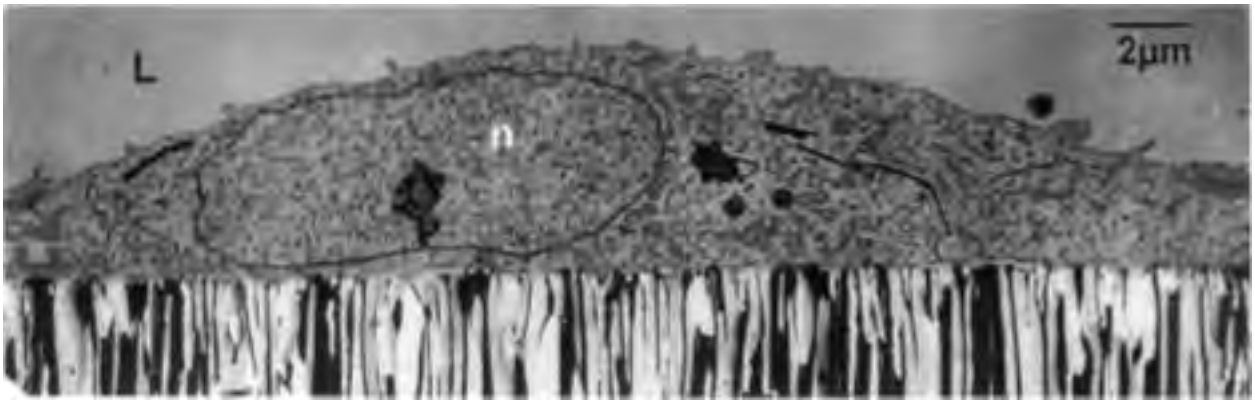

Figure 3. Electronmicrograph of a gas gland cell cultured in a collagen S coated petri dish. The cell is flat and shows no polarity. Some microvilli are present at the luminal membrane. L, lumen; n, nucleus.

about 7 to 10 µm high, which is close to the *in vivo* situation [18]. The apical membrane is characterized by small microvilli. A number of vesicles is located near the apical surface which resemble the lamellar bodies known from surfactant secreting type II cells of the mammalian lung epithelium [6, 7]. Pronounced membrane foldings were observed especially in the lateral membranes, but in some preparations also in basal membranes. Using immunohistochemistry Na^+/K^+-ATPase was localized especially in lateral membranes, but not in apical membranes (data not shown). Live cell staining using MitoTracker™ Green demonstrated the presence of filamentous mitochondria similar to the ones present in cells *in vivo* [3].

In a previous study enzyme activities of the glycolytic pathway and of the pentose phosphate shunt in gas gland cells cultured in collagen S coated petri dishes indicated that these cells retain their characteristic metabolic capacities, and these cells indeed release lactate into the incubation medium [12]. Measurement of lactate concentration in the superfusate demonstrated that cells cultured on a permeable support also produce and release lactate. Lactate concentration in the fluid of the basal side was much higher than in the fluid of the apical chamber, and a calculation of the amount of lactate released revealed, that about 65–70% of the total lactate released was released to the basal side (Figure 5). The experimental set up did not permit a measurement of the electrical resistance, but a check of cell density on the filter membrane by staining the nuclei with Hoechst 33342 and addition of phenol red to one side of the superfusion chamber revealed that the cells did not form an absolutely tight epithelium, and there was always some phenol red leaking through the membrane. In consequence, the lactate gradient from the basal side to the apical side of the chamber may

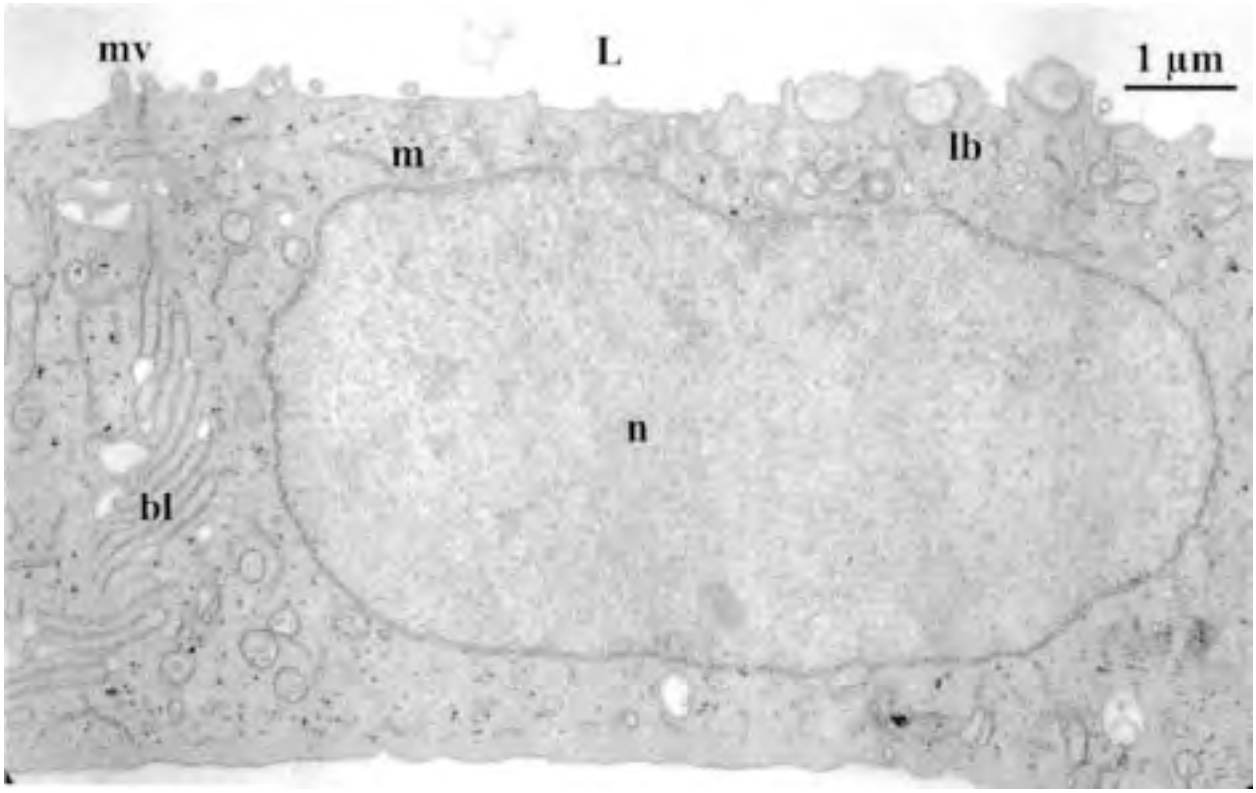

Figure 4. Electronmicrograph of a gas gland cell cultured on a permeable filter membrane. The cell is cuboidal and shows a clear polarity with some microvilli in the luminal membranes, and extensive membrane foldings in the lateral, but also in the basal membrane. The cytoplasm contains filamentous mitochondria and a Golgi apparatus. Especially near the luminal membrane lamellar bodies are observed. bl, membrane foldings; L, lumen; lb, lamellar body; m, filamentous mitochondria; mv, microvilli; n, nucleus.

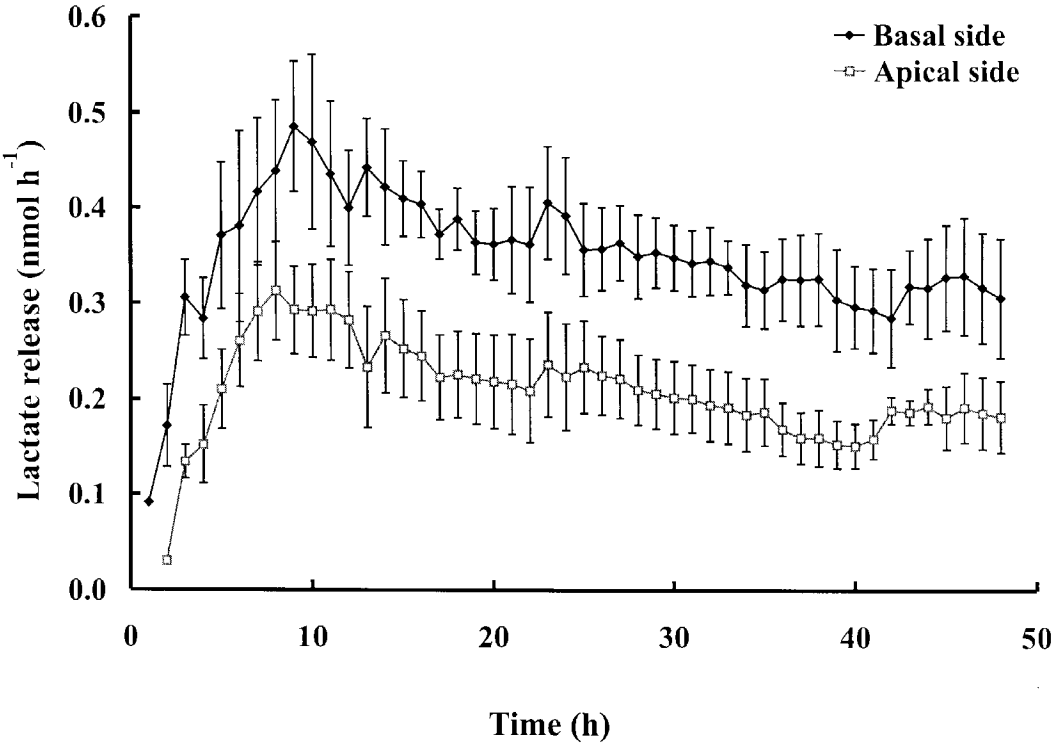

Figure 5. Average rate of lactate release by gas gland cells at the apical and the basal membrane during the first 48h of culture in the superfusion system (mean ± SE; n = 20).

have caused a paracellular diffusion of lactate from the basal to the apical side. The calculated difference in lactate release between the apical and the basal side therefore may well be an underestimation.

In an additional set of experiments the fluid of the apical side was replaced by humidified air. The histology of gas gland cells in air/liquid culture was similar to the one already described for the liquid system (data not shown). Cells cultured in the air/liquid system also produce and release lactate, and this lactate is only released through the basal membranes. Although there was no indication for a difference in cell density on the filter membrane, the total amount of lactate released from these cells was about twice as high as in the liquid system (Figure 6). Analysis of metabolic activity of eel swimbladder tissue *in vivo* suggested that hypoxia caused a decrease in the rate of lactate production [14]. Due to the higher oxygen capacity of the gas phase compared to the saline solution the oxygen supply to the cells in the air/liquid system should be much better than in the liquid system. It thus may well be that the improved oxygenation of the cells in the air/liquid system induced an increase in the metabolic activity of the cells and thus an increase in the rate of lactate release.

In summary, when cultured on permeable supports in a superfusion system by the methods of this paper, gas gland cells clearly show a histological and functional polarity. They regain the capacity to produce and release lactate to the basal side as well as surfactant to the apical side and therefore are a useful model for the analysis of gas gland cell function.

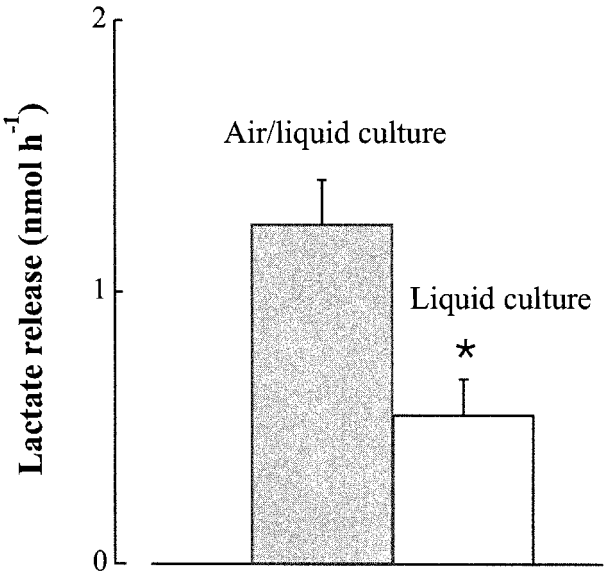

Figure 6. Average rate of total lactate release by cultured gas gland cells (in nmol·h^{-1}) during the first 48h of culture in a superfusion system with fluid supply to both sides of the superfusion system (liquid culture) and in an air/liquid system (mean ± SE; n = 20). *, significantly different with $p < 0.05$.

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Notes on suppliers

1. Carl Zeiss GmbH, Modezenterstr. 16, 1030 Wien, Austria
2. Silicon Graphics, 2011 N. Shoreline Boulevard, Mountain View, CA 94043-1389, USA
3. Bitplane AG, Technoparkstr. 1, 8055 Zürich, Switzerland
4. Scientific Volume Imaging BV, J. Geradtsweg 181, PO Box 615, 1200AP Hilversum Netherlands
5. Erbe Elektrodenmedizin, 72072 Tübingen, Germany
6. Forma Scientific Division, PO Box 699, Marietta OH 45750, USA
7. BDK, Pfullingerstr. 57, 72820 Sonnenbühl-Genkingen, Germany
8. Sanyo Scientific, 900 N. Arlington Heights Rd. Hasca, IL 60143-2844, USA
9. Radiometer A/S Empdrpvej 72,Copenhagen NV, Denmark
10. Mettler Toledo GmbH, Im Langacher, 8606 Greifensee, Switzerland
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12. Nalgene Company, 75 Panorama Creek Drive, PO Box 20365, Rochester, NY 14602-0365 USA
13. IKA®-Werke, Janke & Künkel GmbH & CoKG, Postfach 1263, 79217 Staufen, Germany
14. Heraeus GmbH Wien, Parttartgasse 34, 1230 Wien, Austria
15. Eppendorf Netheler Hinz GmbH, 22331 Hamburg, Germany
16. Ismatec Germany, Laboratoriumstechnik GmbH, Futtererstr. 16, D-97877 Wertheim-Mondfeld, Germany
17. Hoffmann Elektrogerätebau, Schißlerstr. 30, 86154 Augsburg, Germany
18. Sigma Chemicals, Hebbelplatz 7, 1100 Wien, Austria
19. Boehringer Mannheim Wien, Engelhorngasse 3, 1210 Wien, Austria
20. Serva Electrophoresis GmbH, Carl Benz Str. 7, 69115 Heidelberg, Germany
21. GIBCO-BRL Life Technologies, Hirschstettnerstr. 44/1, 1220 Wien, Austria
22. ICN Biomedicals GmbH, Thüringerstr. 15; 37269 Eschwege, Germany
23. Schott Glaswerke, Postfach 2480, 55116 Mainz 1, Germany
24. Whatman Scientific Ltd.; Whatman House, St. Leonard's Rd. 20/20 Maidstone, Kent ME16 0LS England
25. Minucells and Minutissue Vertriebs-GmbH, Starenstr. 2, 93077 Bad Abbach, Germany
26. Sarstedt, IZ Süd/Straße Nr.7/Obj. 58/A/1, A-2355 Wiener Neudorf, Austria
27. B.Braun Melsungen AG, Postfach 1120, 34209 Melsungen, Germany

28. Molecular Probes Europe BV, PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands
29. Tropix, 47 Wiggins Ave., Bedford, MA 01730, USA
30. Biogenesis Ltd, 7 New Fields, Stinsford Road, Poole BH17 0NF, England, UK
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