Swim bladder gas gland cells produce surfactant: in vivo and in culture

C. PREM, W. SALVENMOSER, J. WÜRTZ, AND B. PELSTER
Institut für Zoologie und Limnologie, Universität Innsbruck, A-6020 Innsbruck, Austria
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Prem, C., W. Salvenmoser, J. Würtz, and B. Pelster. Swim bladder gas gland cells produce surfactant: in vivo and in culture. Am J Physiol Regulatory Integrative Comp Physiol 279: R2336–R2343, 2000.—Electron microscopical examination of gas gland cells of the physostome European eel (Anguilla anguilla) and of the physoclist perch (Perca fluviatilis) revealed the presence of significant numbers of lamellar bodies, which are known to be involved in surfactant secretion. In the perch, in which the gas gland is a compact structure and gas gland cells are connected to the swim bladder lumen via small canals, lamellar bodies were also found in flattened cells forming the swim bladder epithelium. Flat epithelial cells are absent in the eel swim bladder, in which the whole epithelium consists of cuboidal gas gland cells. In both species, Western blot analysis using specific antibodies to human surfactant protein A (SP-A) showed a cross-reaction with swim bladder tissue homogenate proteins of ~65 kDa and in the eel occasionally of ~120 kDa, probably representing SP-A-like proteins in a dimeric and a tetrameric state. An additional band was observed at ~45 kDa. Western blots using antibodies to rat SP-D again resulted in a single band at ~45 kDa in both species, suggesting that there might be a cross-reaction of the antibody to human SP-A with an SP-D-like protein of the swim bladder tissue. To localize the surfactant protein, eel gas gland cells were cultured on permeable supports. Under these conditions, the gas gland cells regain their characteristic polarity. Electron microscopy confirmed the presence of lamellar bodies in cultured cells, and occasionally, exocytotic events were observed. Immunohistochemical staining using an antibody to human SP-A demonstrated the presence of surfactant protein only in luminal membranes and in adjacent lateral membranes. Only occasionally, evidence was found for the presence of surfactant protein in lamellar bodies.

Anguilla anguilla; Perca fluviatilis

EPITHELIAL CELLS OF AIR-BREATHING organs of vertebrates are covered with a thin fluid layer, the hypophase. This hypophase is characterized by the presence of surface active agents (surfactant), which reduce and modify surface tension at the air-liquid interphase (3, 10, 21). Surfactant consists of various lipids and of surfactant proteins. Surfactant proteins may interact with lipid components and may be involved in the formation of tubular myelin, the adsorption of surfactant, and perhaps contribute to immunodefence (10, 28). Lungs and also the swim bladder originate as an outgrowth of the foregut, and the presence of surfactant in the swim bladder therefore is not surprising (3, 4). The amount of surfactant found in the swim bladder is even larger than the amount found in a mammalian lung (3). In swim bladder surfactant, however, the fraction of cholesterol and of unsaturated dipalmitoylphospholipids is much higher than in lung surfactant of mammals. This may be due, in part, to the lower temperatures encountered in the swim bladder. It may also contribute to the observation that, in contrast to lung surfactant, swim bladder surfactant mainly acts as an antigen to facilitate reopening of the bladder after a collapse or partial collapse, and it may prevent edema.

Lung surfactant is produced and secreted by specialized alveolar cells, the type II pneumocytes (12, 14, 21), and in the airways probably by so-called Clara cells (30). It is not quite clear, however, where swim bladder surfactant is produced. In the swim bladder epithelium of rainbow trout, lamellar bodies resembling the ones found in mammalian lung type II cells were found only in nonciliated cells, which apparently are not involved in gas secretion (1). In cod, for example, a physoclistous fish in which the connection between swim bladder and foregut (ductus pneumaticus) is lost during early development, bodies filled with a dark amorphous matrix are present in so-called gas-secreting cells (2), whereas ciliated cells, which, in the trout swim bladder, supposedly are necessary to move the mucouslike material secreted from the lamellar bodies to the pneumatic duct (1), are absent. For larval vallelye (Stizostedion vitreum), it has been proposed that the surfactant enters the swim bladder via the common bile duct (13), which means that surfactant may also be produced from other structures and transported into the swim bladder. Furthermore, in compact gas glands, intercellular lumina called “ducts” or “secretory canals” have been described (5, 27). It is not known whether these canals contain surfactant as well, and if so, from where this surfactant originates.

The present study therefore was set out to test the hypothesis that various cell types are able to produce swim bladder surfactant by comparing two species with a completely different swim bladder morphology.

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Address for reprint requests and other correspondence: B. Pelster, Institut für Zoologie und Limnologie, Universität Innsbruck, A-6020 Innsbruck, Austria (E-mail: Bernd.Pelster@uibk.ac.at).
In the physoclistous perch, like in many other fish, the lining of the swim bladder consists of flattened epithelial cells. Special gas gland cells, which acidify the blood to reduce the effective gas-carrying capacity and to increase gas partial pressure, are lumped together and form several small, compact structures (gas glands) that are located beneath the flat swim bladder epithelium (18, 24). In eels, the ductus pneumaticus persists throughout development (physostomous fish). In this species, the whole swim bladder epithelium is made up of cuboidal gas gland cells, and the covering epithelial cells are missing. If surfactant is produced in the swim bladder tissue, in perch the flattened epithelial cells might be responsible. Because these cells are not present in the eel swim bladder, in this species the gas gland cells may serve a dual function and secret acidic metabolites at the basolateral membranes and surfactant at the luminal membranes. A previous study, in which carbonic anhydrase was localized histochemically (using Hansson’s method) in basolateral membranes of gas gland cells of both species, demonstrated that the comparison of these two species appears to be especially useful to understand swim bladder function (29). Unfortunately, the resolution of the histological sections of this study do not allow for the identification of lamellar bodies, for example, so that a more detailed ultrastructural analysis was performed to identify and localize cell components involved in surfactant storage or release. Furthermore, immunologic studies were performed to identify surfactant proteins and to localize these proteins in cell membranes.

MATERIALS AND METHODS

Electron microscopy. Individuals of the European eel Anguilla anguilla (n = 45, body mass 150–450 g) and of perch Perca fluviatilis (n = 8, body mass 10–50 g) were obtained from local fishermen and kept in a freshwater aquarium at 12–15°C for up to 2 mo. Eels were not fed and kept mostly in the dark. Perch were kept at local light regime and fed with tubifex worms every second or third day. The animals were killed by decapitation and subsequently dissected. Samples of the swim bladder epithelium were cut into small pieces and immediately placed into 2.5% glutaraldehyde in 10-mM phosphate buffer (Dulbecco’s formula, pH 7.4) for 30 min, washed, and postfixed in cold 2% osmium tetroxide containing 2.5% potassium ferrocyanide for 90 min. The samples were dehydrated after fixation with the use of graded methanol.
series, critical-point dried with liquid CO\textsubscript{2}, sputtered with gold, and examined with a Zeiss DSM 150 microscope (Zeiss).

**Cell culture.** The preparation of the gas gland cells followed the procedure described previously (17, 19). Gas gland cells were seeded onto Anodisc 13 membranes (Whatman), cultured until they reached confluence, and then transferred into chambers that allowed us to perfuse the apical and basal side of the permeable support with different media. The basal side was perfused with culture medium, DMEM F12 (Gibco), supplemented with 10 µmol/ml alanine-glutamine, 0.5% eel serum, 0.1% BSA, 1 µg/ml gentamicin, 1 µg/ml kanamycin, whereas the apical side was perfused with pure buffer solution consisting of (in mM) 140 NaCl; 5.4 KCl; 1.0 MgCl\textsubscript{2}; 10 HEPES; and 0.5 EGTA.

**SDS-PAGE and Western blot analysis.** Protein of a swim bladder homogenate was separated by SDS-PAGE with the use of the NuPage buffer system. Electrophoresis was performed with Power Ease 500, X-Cell II using NuPage 10% Bis Tris gels (all from Novex). SDS-PAGE was done under reducing conditions (DTT, 125 µM).

The electrophoretic transfer of proteins to a nitrocellulose membrane was performed with the use of Power Ease 500 (Novex). The transfer was conducted for 1 h at a constant voltage of 25 V (160 mA). The nitrocellulose membranes were placed into a sealed bag containing 0.2% I-Block (casein-based blocking agent; Tropix) or 5% milk powder and 0.1% Tween 20 (Sigma) in 10-mM phosphate buffer and gently agitated for 1 h at room temperature. After the washing, the membranes were incubated overnight at 4°C with the rabbit anti-human surfactant protein A (SP-A) antibody 1:5,000 in buffer containing 3% milk powder. The membranes were then washed and incubated for 1 h with Sigma A 1949 monoclonal rabbit IgG 1:10,000 (conjugated with peroxidase) in 3% milk powder at room temperature. Visualization was done by enhanced chemiluminescence (Amersham Life Science).

Western blot analysis to test for the presence of SP-D-like proteins in swim bladder homogenate was performed basically according to the procedure described for SP-A. SDS-PAGE was again done under reducing conditions, with the use of MOPS buffers and a 10% Bis Tris gel. After blocking, the membrane was incubated with rabbit anti-rat SP-D antibody (1:250).

**Immunohistochemistry.** Cells were fixed in 4% paraformaldehyde in 10-mM phosphate buffer (pH 7.4) for 1 h, washed, blocked for 1 h with 0.2% I-Block (Tropix) and 0.2% Triton X-100 (Sigma) in 10-mM phosphate buffer, and incubated 1:100 with the rabbit anti-human SP-A antibody in blocking buffer overnight at 4°C. After the washing, the samples were incubated with the swine anti-rabbit IgG TRITC-conjugated antibody (Dako), diluted 1:100 in blocking buffer, and embedded in Vectashield (Vector Laboratories).

Live-cell staining was performed with 50 nM LysoTracker Green (Molecular Probes) in 10-mM phosphate buffer containing 10 mM glucose for 1 h. Fixation of the cells after live-cell staining was performed with 4% paraformaldehyde and 0.5% glutaraldehyde. Acetone was used for permeabilization instead of Triton X-100. Analysis was performed with the use of a laser-scanning microscope (Zeiss, LSM 510).

**Data analysis.** Data obtained with the laser-scanning microscope were processed on a O2 work station (Silicon graphics) with the use of appropriate software packages (Imaris, 2.6.8) from Bitplane (Bitplane AG). For contrast enhancement and deconvolution, the software package “Huygens, 2.0” (Scientific Volume Imaging BV) was used before three-dimensional visualization with the Isosurface module of Imaris 2.6.8.

**RESULTS**

Gas gland cells of the European eel form a monolayer spread over the secretory part of the swim bladder epithelium (Fig. 1A). Spread over the whole cell we found small lamellar bodies, but most of them are localized in the apical region. In contrast to the eel, the inner layer of the swim bladder of the physoclist perch includes two types of cells, the gas gland cells, lumped together to several small but multilayered glands, and flattened squamous epithelial cells (Fig. 1B). Sometimes a canal can be seen within the gas gland cells. Near these canals small lamellar bodies are present within the cytoplasm of gas gland cells. A close-up of such a canal revealed the presence of tubular myelin in the lumen of the canal (Fig. 1C). The second cell type forms the lining of the swim bladder lumen. These cells

![Fig. 2. Scanning electron micrograph of the epithelium of the perch swim bladder. A: overview of intact swim bladder epithelium in the nongas gland region. B: swim bladder epithelium in the gas gland region with canal openings. The apical membranes show numerous microvilli, and electron dense material (→) is present in the lumen of the canals.](image-url)
are flattened cells and form a monolayered epithelium between gas gland cells and the lumen. Small microvilli characterize the cells at their apical side. These epithelial cells are also characterized by the presence of a large number of lamellar bodies (Fig. 1B).

A scanning electron micrograph of the epithelial surface of the swim bladder wall in a region where no gas gland cells are found shows the classical picture of an epithelial lining (Fig. 2A). A scanning electron micrograph of a region where gas gland cells are located, however, reveals the presence of pores with a diameter of ~5 μm (Fig. 2B). Occasionally, electron-dense material is observed within the lumen of the pore opening. The surface of the epithelial cells is characterized by the presence of microvilli. These electron microscopical observations are summarized to a schematic drawing

Fig. 3. Schematic drawing of the swim bladder gas gland cells. A: swim bladder of the European eel where gas gland cells form a monolayer over the secretory part of the swim bladder epithelium. The gas gland cells show a clear polarity with a remarkable basal labyrinth and lamellar bodies. At the apical membrane, the cells form small microvilli. Below the gas gland cells a thin lamina propria containing collagen fibers (cf), fibroblasts (f), and blood vessels is present. B: swim bladder of the perch, where the gas gland cells form a distinct gas gland. Flattened epithelial cells are facing the lumen. These cells contain a large number of lamellar bodies. The gas gland cells are located in several layers between the lamina propria, where blood capillaries and nerves (nv) are found. The cells show a well-developed basal labyrinth when they are lying near blood vessels. In some cells, a canal is present with small microvilli and tubular myelin. Lamellar bodies are present in the cytoplasm near the canal. bm, Basement membrane; p, pore.
of eel gas gland cells (Fig. 3A) and of the perch gas gland (Fig. 3B).

The presence of surfactant in swim bladder cells was analyzed by Western blot analysis using specific antibodies directed against SP-A and -D. The anti-human SP-A antibody showed a cross-reactivity with an eel swim bladder epithelium protein of ~65 kDa (n = 6 animals), probably representing the SP-A dimer (Fig. 4A). In four experiments, the antibody also cross-reacted at ~120 kDa, which could represent the SP-A tetramer, and a band was observed at ~45 kDa. Additional Western blots using a rabbit anti-rat SP-D antibody (n = 5 animals) again resulted in a band at ~45 kDa. A typical result is shown in Fig. 4A.

Figure 4B shows the typical results of the Western blot analysis performed with swim bladder tissue of the perch (in total, 5 animals were tested). With the use of SP-A antibodies, we found a band at ~64 kDa, which could represent the dimer of the SP-A. Again, there was some cross-reactivity at 45 kDa. Additional Western blots using rabbit anti-rat SP-D antibody (n = 5) again resulted in a band at ~45 kDa. A typical result is shown in Fig. 4B.

A localization of surfactant protein was attempted with the use of cultured eel gas gland cells. Gas gland cells of the European eel cultured on permeable supports in a superfusion chamber show the typical polarity as found in vivo. The cells are of cubical shape and show lateral foldings of the membrane and a basal labyrinth. Cultured gas gland cells show a Golgi apparatus, long and slender mitochondria, and a large number of lamellar bodies (Fig. 5A). In some sections, we observed that these lamellar bodies undergo exocytosis (Fig. 5B).

Subsequent immunohistochemical studies (using primary cultures of 8 individual eels) also demonstrated the presence of SP-A in cultured gas gland cells of the eel, and the distribution of SP-A was highly polar. Fluorescence was only observed near the apical and lateral membranes close to the apical side, but not in basal membranes (Fig. 6). In addition, we also incubated the cells with LysoTracker Green to stain acidic vesicles, for instance lamellar bodies. Live-cell staining with LysoTracker Green showed a large number of acidic vesicles spread over the whole cell. Colocalization of LysoTracker Green and SP-A only occasionally revealed the presence of SP-A in lamellar bodies located near the cell membrane.

Figure 7 shows a three-dimensional reconstruction of a cell obtained after deconvolution of the data. SP-A (red) is only present on the apical side of the cultured
cell; there is no SP-A on the basal side. Acidic vesicles (green) are more concentrated on the apical side, but occasionally they are also found in the basal part of the cell.

DISCUSSION

Although the presence of surfactant in the swim bladder lumen is well established, the present study demonstrates that surfactant is also present in the secretory canals of compact gas glands and is produced in two different cell types in swim bladder tissue, namely in flattened epithelial cells, if present, and in gas gland cells. Furthermore, eel gas gland cells cultured under appropriate conditions retain the capacity to produce and secrete surfactant at their luminal membranes. In mammals, type II pneumocytes are responsible for the production of surfactant, and mammalian lung type II cells in appropriate culture conditions also produce surfactant similar to the in vivo situation (6, 7, 12).

Within recent years, surfactant has been found in all air-breathing structures of the vertebrates, and an analysis of surfactant proteins revealed that the molecular weight of these proteins varies among species and even within a species, but nevertheless, these proteins are highly conserved (15, 25). With the use of antibodies to human SP-A and -B, the presence of SP-A and SP-B-like proteins in lung epithelial cells of the Australian lungfish (Neoceratodus forsteri) was demonstrated (20), and Neoceratodus is the most primitive member of the Dipnoi, which evolved more than 300 million years ago. Similarly, presence of SP-A-like proteins in air-breathing organs of the African lungfish (Protopterus senegalensis), another species of Dipnoan fish, and in air-breathing organs of other Acanthopterygian fishes (which include the teleosts) has been confirmed with the use of antibodies to mouse SP-A (25). Teleosts evolved much later than the Dipnoan fishes in the Triassic period (9), and the teleost swim bladder may be regarded as a specialization of a primitive air-breathing organ of the Acanthopterygian fishes (8, 9), or it may originally have been an organ with hydrostatic function (11). The teleost swim bladder primarily functions as a buoyancy organ (18), nevertheless, with a probe designed on the basis of a mouse cDNA sequence, mRNA of SP-A-like proteins has been identified in goldfish swim bladder (25). With the use of antibodies to mammalian SP-A, SP-A-like proteins have also been detected in the intestine and swim bladder of carp (22) and in the swim bladder of perch and eel (present study). This clearly confirms the conservative nature of the surfactant system and underlines the importance of surfactant for swim bladder function.

Although in mammalian alveoli, surfactant is produced by specialized epithelial cells, in the perch swim bladder, epithelial cells as well as gas gland cells produce and secrete surfactant. In terms of evolution, this could mean that, initially, epithelial cells were capable of producing surfactant, and gas gland cells are specialized epithelial cells that retained this capability and, in addition, specialized for the production of acidic metabolites. In the eel, the whole epithelium differen-

Fig. 6. Immunohistochemical localization of SP-A (red fluorescence) and of acidic vesicles (green/yellow fluorescence) in primary cultured eel gas gland cells. The confocal image shows 3 optical sections of a gas gland cell in the xy, yz, and xz planes. The white bars in the xy section indicate the location of the other 2 sections; the white bar in the xz section indicates the location of the xy section. Note that SP-A immunoreactivity is located at apical and lateral cell membranes, but there is no immunoreactivity at basal membranes. Most of the acidic vesicles are located in the cytoplasm near the apical membrane, but occasionally they are also present in the basal part of the cell (white arrow in the xz section).

Fig. 7. Three-dimensional reconstruction of SP-A distribution (red) and localization of acidic vesicles (green) in cultured gas gland cells calculated on the basis of confocal images as shown in Fig. 6. See text for further explanation.
ties into gas gland cells, which now serve a dual function. They produce acidic metabolites, released at the basolateral membranes, as well as surfactant, released at apical membranes.

In species with a compact gas gland, located below a layer of flattened epithelial cells, the gas gland cells do not project into the swim bladder lumen. Compared with the eel swim bladder, this arrangement increases the distance between blood vessels and swim bladder lumen, which is crucial for the diffusion of gas into the swim bladder. Nevertheless, the canals in the perch gas gland cells apparently establish direct contact to the lumen and thus significantly reduce the diffusion distance between blood vessels and gas phase. This argument of course is only valuable if these canals remain gas filled and do not collect fluid. Fluid-filled structures typically can be identified by the presence of some kind of precipitation in electron microscopical studies, but none of our pictures provided any evidence for the existence of fluid-filled canals. Scanning electron micrographs demonstrate that these canals open into the swim bladder and that they contain lamellar or tubular material, which most likely is surfactant. The lamellar bodies found near the membranes lining these canals indicate that this surfactant is produced from gas gland cells. The surfactant probably is necessary to prevent a collapse of the canals during volume changes of the swim bladder.

Although the surfactant lipids are released into the swim bladder by exocytosis of lamellar bodies, the situation is less clear for the surfactant proteins. Surfactant proteins have been detected in lamellar bodies, but the secretion of hydrophilic SP-A and of hydrophobic SP-B and SP-C apparently can be regulated in a different way (16, 26). In the air-breathing organ of Neoceratodus, immunogold staining revealed the presence of SP-A and of SP-B-like protein in lamellar bodies and in the cytoplasma, but in lamellar bodies the signal was not very pronounced (20). In our study, SP-A occasionally was detected in acidic vesicles of eel gas gland cells. For rat type II cells, it was estimated 7–8% of total SP-A appears to be associated to lamellar bodies (16). Thus at least part of the SP-A is secreted with lamellar bodies.

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