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Localization of the vacuolar-type ATPase in swimbladder gas gland cells of the European eel (*Anguilla anguilla*)

S. T. Boesch, H. Niederstätter* and B. Pelster†

Institut für Zoologie und Limnologie, Universität Innsbruck, Austria

*Present address: Institut für Gerichtliche Medizin, Universität Innsbruck, Müllerstr. 44, A-6020 Innsbruck, Austria

†Author for correspondence (e-mail: bernd.pelster@uibk.ac.at)

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Summary

The vacuolar ATPase is a multifunctional enzyme that consists of several subunits. Subunit B is part of the catalytic domain of the enzyme and is present in two isoforms in fish as well as in mammals. Possibly, these two isoforms – vatB1 (kidney isoform) and vatB2 (brain isoform) – serve different functions. A localization of the two isoforms was attempted in swimbladder gas gland cells of the European eel *Anguilla anguilla* by immunohistochemistry. Two antibodies were produced by immunization of rabbits with synthetic peptides. Specificity of the antibodies, on the one hand, an isoform-specific antibody for vatB1 and, on the other hand, an antibody that recognizes both isoforms (vatB1 and vatB2), was confirmed by western blot analysis using recombinant proteins produced in a bacterial expression system. The immunohistochemical localization with the antibody directed against both isoforms of the B subunit revealed a positive staining in apical membranes of swimbladder gas gland cells as well as in the basolateral membranes. Significant staining was observed in vesicles located near the apical membrane. Staining with the vatB1-specific antibody resulted in a similar picture in the apical region of the cells. In contrast to the staining with the first antibody, only a poor signal was observed in the basal region. The nature of the vesicles in the apical region of the gas gland cells was determined by using an antibody directed against surfactant protein D.

Key words: swimbladder, gas gland cells, vatB1, vatB2, V-ATPase, European eel, *Anguilla anguilla.*

Introduction

Vacuolar-type ATPases (V-ATPases) are membrane-bound multi-subunit enzymes that drive the unidirectional flow of protons (Wieczorek et al., 1999) across plasma and organelle membranes. V-ATPases are found in nearly all intracellular membrane components such as endosomes, lysosomes, chlathrin-coated vesicles or Golgi-complexes (Forgac, 1998; Stevens and Forgac, 1997). In recent years, however, several studies have demonstrated the presence of V-ATPases also in the plasma membrane of cells such as kidney cells, osteoclasts, macrophages and tumor cells (Wieczorek et al., 1999; Gluck et al., 1998; Nishi and Forgac, 2002).

A functional V-ATPase consists of two major components, the membrane-spanning stalk, made up of five subunits, and the head, made up of eight subunits, which is located in the cytoplasm (Wilkens and Forgac, 2001). For some of the subunits that form the functional V-ATPase, structural differences have been found due to the expression of different isoforms of the same subunit. For subunit B, which is part of the regulatory/catalytic domain in the head region of the enzyme, two isoforms, the so-called 'kidney isoform' (B1) and the 'brain isoform' (B2) are known (Puopolo et al., 1992; van Hille et al., 1994). These subunit differences result in different properties of the mature enzyme (Gluck, 1992; Wang and Gluck, 1990). Considering ion transport, enzyme targeting or even physiological regulation of enzyme activity, the presence of different isoforms of an enzyme within the same tissue obviously suggests that these different isoforms serve different functions. For instance in mammals the kidney isoform (B1) is expressed in intercalated proton-translocating cells of the kidney, while the brain isoform appears to be expressed in most tissues.

While these two isoforms of the B subunit were originally described for mammals, in a recent study two isoforms corresponding to the B1 isoform and the B2 isoform have also been isolated from gas gland cells of the eel swimbladder (Niederstätter and Pelster, 2000). Swimbladder gas gland cells produce and secrete lactic acid and CO_2 in order to reduce the oxygen-carrying capacity of the hemoglobin during passage of the swimbladder (Pelster and Randall, 1998; Pelster, 2001), and the presence of V-ATPase in gas gland cells has been discussed in context with the secretion of protons (Pelster and Niederstätter, 1997). Indeed, during periods of intracellular acidification, inhibition of V-ATPase by application of bafilomycin significantly reduced the rate of acid secretion (Pelster, 1995; Sötz et al., 2002). Not only acidic metabolites, but also surfactant, which serves here as an anti-glue

facilitating a reopening of the swimbladder after collapse and preventing edema, is produced and secreted by gas gland cells (Prem et al., 2000). Prior to exocytosis, surfactant is stored in lamellar bodies. Lamellar bodies are organelles characterized by the presence of V-ATPase, and in gas gland cells V-ATPase may therefore be involved in proton secretion at the plasma membrane as well as in the acidification of organelles such as lamellar bodies.

Following the idea that the kidney and brain isoforms of the B subunit would indeed serve different functions, one might speculate that one isoform would be preferentially located in basolateral membranes and contribute to the secretion of protons into the extracellular space, while the other isoform might be preferentially located in multilamellar bodies located near apical membranes. The present study therefore set out to localize V-ATPase in swimbladder gas gland cells of the European eel *Anguilla anguilla*.

Materials and methods

Animals

European eel *Anguilla anguilla* L. (mass 350–500 g) were obtained from a local supplier and held in a freshwater aquarium at 16°C with a natural light cycle. The animals were not fed until experimental use. Swimbladder gas gland tissue from European eels was isolated following the procedure described by Pelster and Niederstätter (1997).

Preparation of histological specimens

After dissection, swimbladder tissue was washed twice with ice-cold 10 mmol l^{-1} Dulbecco's PBS (phosphate-buffered saline), pH 7.4. Fixation was carried out in 4% buffered paraformaldehyde at 4°C overnight. After three washes in PBS for 30 min each, the tissues were dehydrated in a series of ethanol baths (30 min in 70% ethanol, 30 min in 80% ethanol, 30 min in 90% ethanol, 3×1 h in 100% ethanol). Before the final embedding in paraffin, sequential incubations in methyl benzoate (1×overnight, 3×3–12 h), benzene (2×30 min), benzene/paraffin (1×2 h at 60°C) and paraffin (3 changes within 12–16 h) were performed.

Bacterial expression of recombinant vatB1 and vatB2

Specific primer pairs for each isoform of the B subunit of V-ATPase – vatB2 and vatB1 – were designed, which permitted the amplification of the open-reading frames (ORFs) including the native start codons but not the stop codons (see Table 1). PCR was performed at a volume of 25 µl consisting of 1×PCR buffer (Clontech, Palo Alto, CA, USA), 25 pmol of the corresponding primer, 25 µmol of each dNTP and 0.5 µl Advantage HF-2 mix (Clontech) in a GeneAmp PCR system 9700 thermocycler (Applied BioSystems, Foster City, CA, USA). Plasmids containing coding sequence of each vatB isoform were used as template (Niederstätter and Pelster, 2000). The resulting PCR products were purified, ligated into a pCR® T7/CT TOPO® vector using T/A cloning strategy (Invitrogen, Carlsbad, CA, USA) and cloned into TOPO 10F′ cells (Invitrogen). DNA from resulting clones was extracted using Qiaprep®8 Turbo MiniPrep Kit (Quiagen, Hilden, Germany).

The plasmids were analyzed in both directions by sequencing using sequencing primers T7 forward (5′-TAATACGACTCACTATAGGG-3′) and V5C-term reverse (5′-ACCGAGGAGAGGGTTAGGGAT-3′) (Invitrogen). For each isoform, a positive plasmid was selected and a transformation of BL21(DE3)pLysS (Invitrogen) was performed according to the instructions of the manufacturer (Invitrogen). Cells were grown overnight at 25°C or 30°C. The next day, 10 ml of Leibovitz (LB) medium was inoculated with 500 µl overnight culture and shaken for 2 h. For both isoforms, the cultures were split into two 5 ml aliquots, and IPTG (isopropylthiogalactoside) at a final concentration of 1 µmol l^{-1} induced expression. At selected time points (i.e. immediately, 2 h, 4 h, 5 h and 24 h after induction), cell pellets of induced and non-induced cultures were collected and immediately stored at –20°C. The presence of the induced protein was tested by SDS (sodium dodecyl sulfate) gel electrophoresis and Coomassie blue staining.

Immunological detection of V-ATPase

Antibodies (see Table 2) were produced by immunizing rabbits (BioGenes, Berlin, Germany) with synthetic peptides (Biosynthan, Berlin, Germany) corresponding to antigenic epitopes of both V-ATPase B subunits. Due to differences in the amino acid sequence, the 5′ end of the two isoforms would have been ideal for the generation of antibodies. Unfortunately, the antigenicity of the 5′ end of vatB2 was very low, so that it was impossible to direct an antibody against this region of the peptide. Therefore, one antibody was generated using an amino acid sequence close to the 5′ end specific for vatB1 (#1035), and a second antibody (#1034) was directed against a conserved amino acid sequence that is identical in both isoforms (vatB1 and vatB2). Prior to immunocytochemical staining of tissue sections, cross-reactivity of the antibodies was assessed by western-blot analysis of recombinant vatB1 and vatB2 and protein isolated from eel tissue.

Bacterial pellets from the expression experiment of both isoforms were dissolved directly in 1× Tris–glycine sample

Table 1. *Primer sequences used for amplification of expressed vatB1 and vatB2 cDNAs*

Primer	Sequence	Peptide
Eel vatB1 92/20	5′-ATG GCG ACG CTG GTA GAA AA-3′	V-ATPase subunit B1 (55.4 kDa)
Eel vatB1 1583/20 rc	5′-CTC CCT CGG GTA GAA CTC GG-3′	
Eel vatB2 32/20	5′-ATG GCA ATG AAG GCG ATC AG-3′	V-ATPase subunit B2 (56.6 kDa)
Eel vatB1 1564/24 rc	5′-GTG TTT GGA GTC TCG TGG GTA GAA-3′	

Table 2. *Description of used antibodies and their applications*

Antibody	Peptide sequence	Dilution WB	Dilution ICC	Specificity
Antibody #1034	[LPDGTKRSG]-MAP4-amide	1:100	1:40	vatB1 vatB2
Antibody #1035	Cys-NRNVELNGPEAAARQHAQA-amide	1:25	1:350	vatB1

WB, western blot; ICC, immunocytochemistry.

buffer (Novex, San Diego, CA, USA), boiled for 10 min and chilled to room temperature prior to loading. Total protein from eel swimbladder was isolated following the instructions of the manufacturer with Tri-Reagent (Sigma-Aldrich, Vienna, Austria). Proteins were electrophoretically separated under reducing conditions [125 μmol l^{-1} dithiothreitol (DTT)] using NuPage 10% Bis Tris gels (Novex, San Diego, CA, USA) and Mops [γ-morpholinopropanesulfonic acid (sodium salt)] buffer. Proteins were blotted onto PVDF (polyvinylidine fluoride) membranes (BioRad, Hercules, CA, USA) or nitrocellulose membranes (Amersham, Buckinghamshire, UK) using a constant voltage of 25 V (160 mA) for 1 h. Membranes were blocked for 1 h with 0.2% I-Block (casein-based blocking agent; Tropix, Bedford, MA, USA) and 0.1% Tween 20 (Sigma-Aldrich) in 0.1 mol l^{-1} PBS at room temperature. Primary antibody incubation was performed overnight at 4°C in blocking buffer. After additional washing steps, the membranes were probed for 1 h with a horseradish peroxidase (HRP)-conjugated second antibody [HRP-conjugated anti-rabbit immunoglobulin G (IgG), Sigma-Aldrich]. Finally, proteins were visualized using the enhanced chemiluminescence ECL detection reagents (Amersham, Buckinghamshire, UK).

For the immunocytochemical localization, paraffin sections (4–5 μm thick) from eel swimbladder were cut using an Autocut 2040 (Reichert, Vienna, Austria) and mounted on coated glass slides (dimethylsilane; Sigma-Aldrich-Chemie, Vienna, Austria). The sections were then dewaxed by a series of xylene and ethanol. After antigen retrieval by proteinase K digestion and acetylation with 10 min incubation in 0.5% anhydrous acetic acid in 0.1 mol l^{-1} Tris-HCl, pH 8.0, non-specific bindings were blocked with 10% FCS (foetal calf serum) in TBS (Tris-buffered saline). Incubation with the appropriate dilution of the primary antibodies in blocking buffer was undertaken at 4°C overnight. After five washes with TBS, slides were incubated with a polyclonal biotinylated anti-rabbit/mouse IgG (Duett-ABC Kit Solution C; Dako, Glostrup, Denmark) for 20 min. Additional washes were performed and the sections were probed with an anti-biotin alkaline-phosphatase antibody (dilution 1:100; Dako) for 1 h. Finally, a purple color reaction was developed at 4°C in a solution of 4-nitro blue tetrasodium chloride (Roche Molecular Biochemicals, Mannheim, Germany) and 5-bromo-4-chloro-3-indolylphosphate-4-toluisin salt (Roche). The sections were washed three times with TBS, mounted in Gel Mount (Lipshaw Immunon, Pittsburgh, PA) and cover-slipped. Sections were observed and photographed using bright-field light microscopy (Polyvar, Reichert, Vienna, Austria; Zeiss, Jena, Germany).

Immunological localization of surfactant protein D

To test whether the vesicles located in the apical region of gas gland cells contained surfactant, an immunohistochemical localization of surfactant protein D (SP-D) was attempted. Previously, studies have shown that an antibody directed against rat SP-D cross-reacted with a 45 kDa band of protein preparations of eel swimbladder tissue (Prem et al., 2000). Again, 4–5 μm thick sections of paraffin-embedded swimbladder specimens were used. The immunocytochemical staining was carried out as described above using a rabbit anti-rat SP-D antibody (1:1000) provided by Dr Günther Putz (Institute for Anesthesia, University of Innsbruck, Austria).

Results

Verification of the antibody specificities

Recombinant protein

In a first step, the specificity of the polyclonal antibodies #1034 and #1035 was determined using recombinant vatB1 (498 amino acids) and vatB2 (511 amino acids) produced in a bacterial system. Fig. 1 shows that the expression of vatB1, as well as of vatB2, in BL21(DE3)pLysS cells was significantly induced with 1 μmol l^{-1} IPTG. The highest level of protein expression for both isoforms was observed at 4–5 h after induction, but after 24 h the protein bands were severely diminished (data not shown). Low levels of basal expression occurred also in the non-induced bacterial cultures, probably due to the leakiness of the T7 expression system.

The western blot analyses were performed with samples taken 4–5 h after induction. For this purpose, bacterial pellets were directly resolved in the sample buffer, which resulted in some background staining. Bacterial lysate (with a protein content of 30 μg) containing each of the recombinant-expressed vatB isoforms was probed with antibodies #1034 and #1035. The results revealed that antibody #1035, directed against an amino acid sequence specific for vatB1, only recognized the recombinant vatB1 but not vatB2 (Fig. 2A). Antibody #1034, directed against a peptide located in the highly conserved region of the B subunit isoforms, indeed recognized both isoforms (Fig. 2B). The size of the detected bands (approximately 60 kDa) matched the expected molecular mass because in the expression system a 6×His tag was added to the carboxyl terminus of both proteins, increasing the

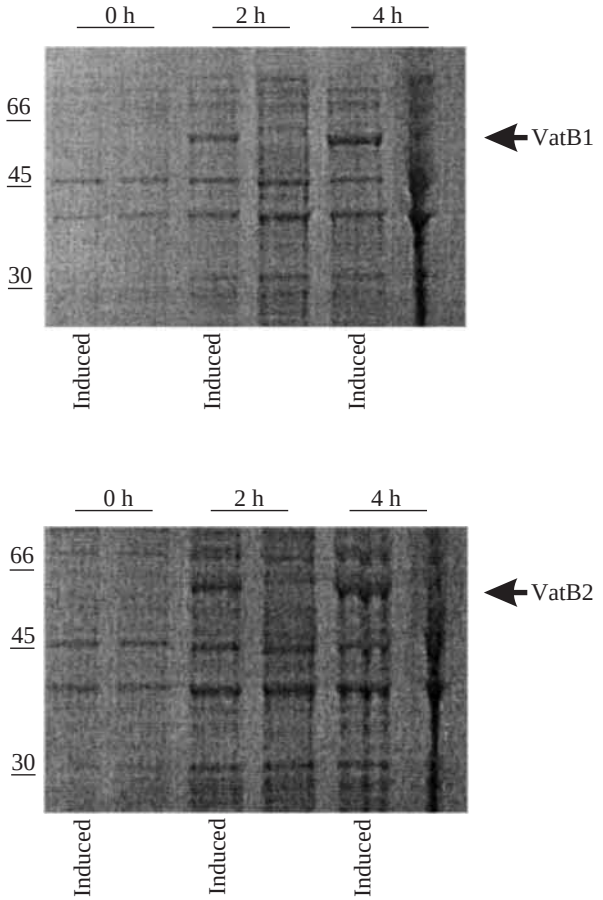

Fig. 1. Expression of vacuolar ATPase subunit B isoforms vatB1 and vatB2 in BL21(DE3)pLysS cells: bacterial lysates have been separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and stained with Coomassie blue. Expression was induced with 1 μmol l^{-1} IPTG (isopropylthiogalactoside). The expression of recombinant protein was clearly induced after 2 h, and the expression increased with time (e.g. after 4 h). Arrows indicate the position of reference vatB1 and vatB2 bands at approximately 60 kDa.

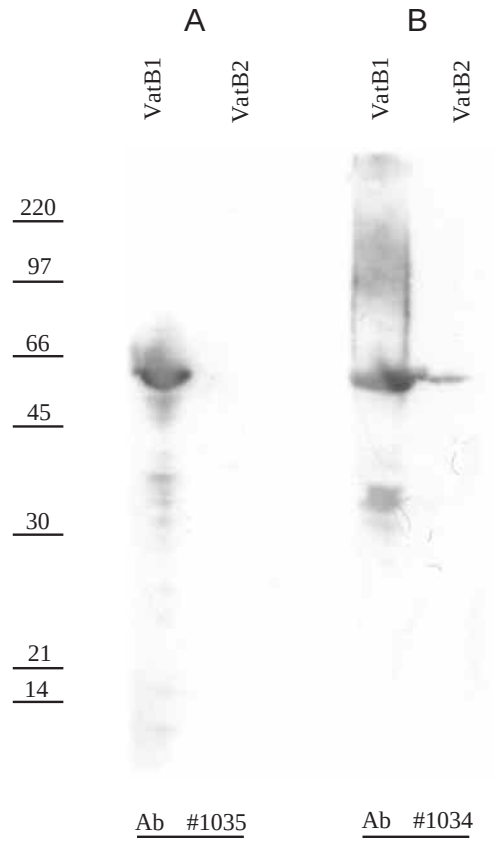

Fig. 2. Determination of specificity of the antibodies by western blot analysis with non-purified bacterially expressed eel vacuolar ATPase subunit B isoforms vatB1 and vatB2. 30 μg protein were used for each lane. (A) Western blot probed with antibody #1035, which is specific for vatB1: left lane, vacuolar ATPase B subunit isoform vatB1 (kidney isoform); right lane, vacuolar ATPase B subunit isoform vatB2 (brain isoform). (B) Western blot probed with antibody #1034, which recognizes both isoforms of the B subunit. Without primary antibody, no bands could be detected.

molecular mass by approximately 3–5 kDa. In control experiments without primary antibody, no band was detected.

Protein preparations from tissues

Antibodies were also tested in western blots with isolated total protein from the swimbladder of the European eel. We could show that the antibodies recognized a protein with a molecular mass of approximately 55 kDa (Fig. 3), which corresponds to the size of both B subunit isoforms: vatB1 (calculated molecular mass, 55.9 kDa) and vatB2 (calculated molecular mass, 56.7 kDa) (Niederstätter and Pelster, 2000). The left lane in Fig. 3 shows a western blot probed with antibody #1035, which is specific for an amino sequence of vatB1 (kidney isoform), and the right lane shows a western blot probed with antibody 1034, recognizing both isoforms. For both blots, control experiments without primary antibody were performed. In these experiments, no band could be detected.

Immunocytochemistry

Immunocytochemical staining of vatB1 and vatB2 resulted in a localization of the isoforms in gas gland epithelial cells (Fig. 4). The antibody directed against both isoforms (#1034) revealed a staining of apical as well as of basolateral membranes (Fig. 4A). A very intense signal was also observed in vesicles, mainly in vesicles located near the apical side of the cells. Staining with the antibody directed against the kidney isoform of subunit B (#1035) resulted in a weaker staining at the basolateral membrane, and many cells showed no staining at all in basolateral membranes. (Fig. 4B). The staining of the apical membrane and of vesicles in the apical region of the cells produced a similar picture as the staining with antibody #1034. With both antibodies, no staining was observed in tissue layers below the epithelial gas gland cells.

To analyze the nature of the vesicles in the apical region of gas gland cells, tissue sections were incubated with antibodies directed against surfactant protein D. The results clearly

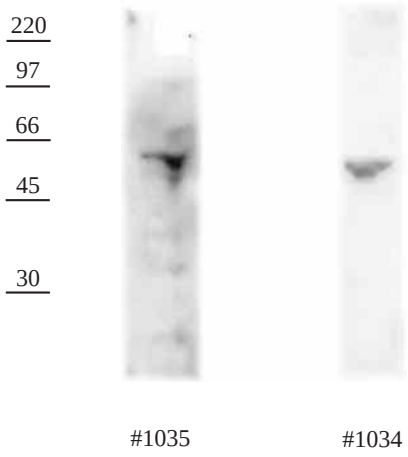

Fig. 3. Western blot analysis of eel swimbladder tissue with antibodies specific for vacuolar ATPase subunit B isoforms. 30 µg of swimbladder protein were used for each lane. Antibody #1034 recognizes both isoforms (rabbit anti-vatB1+vatB2), whereas antibody #1035 (rabbit anti-vatB1) is specific for subunit isoform vatB1. Both antibodies revealed bands of approximately 55 kDa. Without primary antibody, no bands could be detected.

showed the presence of SP-D as a marker protein, suggesting that these vesicles are multilamellar bodies (Fig. 5).

Discussion

We could demonstrate that our antibodies recognized the desired V-ATPase subunits using recombinant isoform-specific peptides. The isoform-specific antibody indeed recognized vatB1, whereas the second antibody recognized both isoforms. Furthermore, both antibodies revealed also a specific band at approximately 55 kDa in western-blot analysis of proteins from eel gas gland tissue. The results of the immunocytochemical staining clearly showed that the expression of both V-ATPase subunit B isoforms is strictly limited to the swimbladder epithelium, and no evidence was obtained for the expression of V-ATPase in cells of the submucosa or serosa. Immunocytochemical localization of the two isoforms revealed the presence of subunit B in membranes in the apical, as well as in the basolateral region, but the kidney isoform (vatB1) of this subunit was mainly concentrated in the apical region.

Preliminary experiments have shown that immunocytochemical staining of the V-ATPase subunit B with our antibodies necessarily required antigen retrieval with proteinase K. Several detection systems were tested but, due to a high level of endogenous peroxidase activity and high background staining in fluorescent methods, alkaline phosphatase was chosen as the detection system.

In swimbladder gas gland cells, two physiological functions appear to involve V-ATPase activity. Initially, it was believed that V-ATPase activity in gas gland cells was related to acid secretion at basolateral membranes (Pelster, 1995). On the other hand, gas gland cells appear to be responsible for the

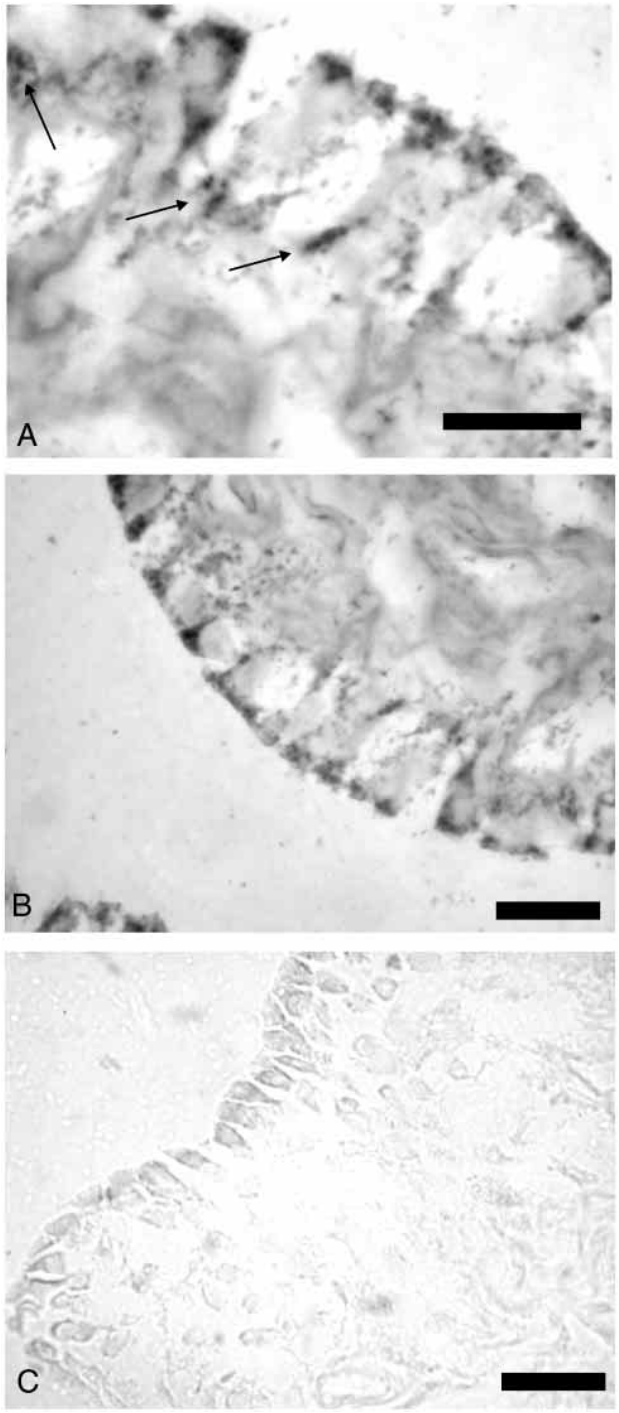

Fig. 4. Immunocytochemical staining of vacuolar ATPase B subunit isoforms vatB1 and vatB2 in swimbladder gas gland cells from *Anguilla anguilla*. Both antibodies revealed positive staining only in gas gland epithelial cells. (A) Antibody #1034 (rabbit anti-vatB1+vatB2) revealed a positive staining of apical and basolateral membranes. A very intense signal was also observed in apical vesicles. (B) Staining with antibody #1035, which is specific for the vatB1 isoform (kidney isoform), resulted in a similar picture in the apical region of the cells, but in the basolateral region very little staining was observed. No background staining was observed in the negative control (C). Scale bars, 10 µm. Arrows point to positive staining reaction in basolateral membranes.

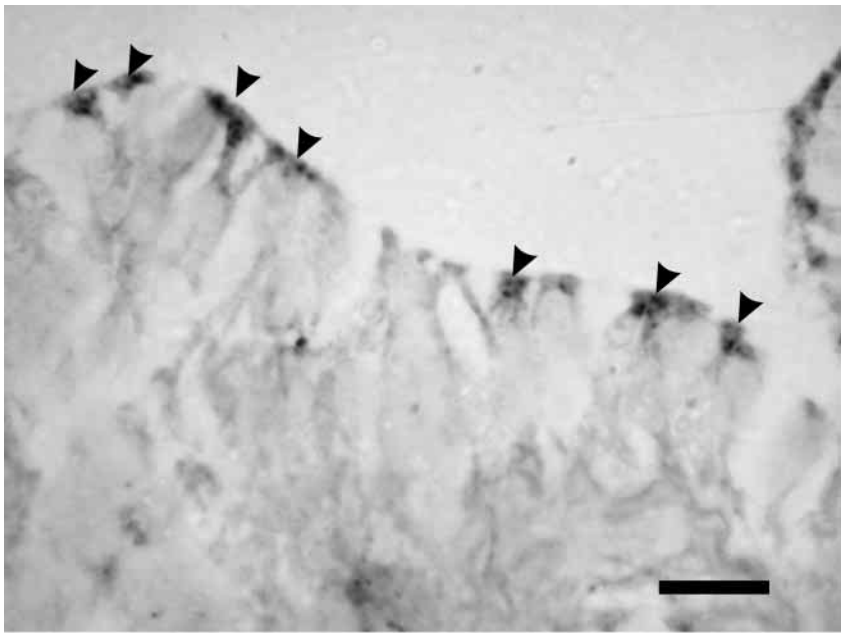

Fig. 5. Immunocytochemical localization of surfactant protein D (SP-D) in swimbladder gas gland cells of *Anguilla anguilla*. The antibody directed against human SF-D revealed staining especially of vesicles located in the apical region of the cells. Arrowheads indicate stained lamellar bodies in the gas gland cells. Scale bar, 10 μm.

secretion of surfactant at their apical membranes (Prem et al., 2000), and this includes V-ATPase activity. Surfactant is stored in multilamellar bodies prior to exocytosis, and lung lamellar bodies maintain an acidic interior by an energy-dependent process. Accordingly, it has been suggested that a V-ATPase is responsible for generating and maintaining the proton electrochemical gradient (Chander et al., 1986, 1996; Chander and Fisher, 1990). Furthermore, in isolated lung lamellar bodies, the uptake of Ca^{2+} in lamellar bodies was ATP-dependent and could almost completely be inhibited by bafilomycin A1, a specific inhibitor of V-ATPase activity (Wadsworth and Chander, 2000).

The comparison of the staining pattern using our anti-V-ATPase antibodies with earlier electron microscopical investigations led to the assumption that the vesicles in the apical region of the gas gland cells, in which the B subunit of V-ATPase is located, are multilamellar bodies (Prem et al., 2000). To prove this idea, we tested for the presence of surfactant protein D by using a specific antibody directed against SP-D. We detected a positive staining in apical membranes, where the surfactant is released into the gas gland lumen, but also in apical vesicles. The staining pattern was comparable with staining against V-ATPase in this cell region. We therefore conclude that the presence of V-ATPase in the membranes in the apical region of gas gland cells is related to the presence of lamellar bodies, in which surfactant is stored prior to secretion.

Physiological evidence suggests that, in swimbladder gas gland cells, V-ATPase is also involved in the secretion of acid. In contrast to parietal cells, in gas gland cells large amounts of lactic acid are produced even in the presence of oxygen and protons are not generated in the CO_2/HCO_3 reaction (Pelster, 2001). Furthermore, acidification of the extracellular fluid of gas gland cells was reduced in the presence of bafilomycin A1 (Pelster, 1995), and recovery of intracellular pH after an artificial acid load was significantly impaired in the presence of bafilomycin A1 (Sötz et al., 2002). In order to induce an acidification of the blood, acid must be released at basolateral membranes. A V-ATPase contributing to this acid secretion therefore must also be located in basolateral membranes. A comparison of the immunocytochemical staining pattern obtained with the antibody directed against both subunits and with the antibody binding only to the kidney isoform (vatB1) suggests that the kidney isoform is mainly located in the apical region. Previous studies have shown that vatB1 and vatB2 have slightly different biochemical properties (Breton et al., 2000; Gluck and Nelson, 1992; Wang and Gluck, 1990), suggesting that the expression of two isoforms may be connected to different functions. Following this hypothesis, we can assume that vatB2, located in the basal region of gas gland cells, is involved in acid secretion, while vatB1, located mainly in apical membranes, is involved in the acidification of multilamellar bodies, which store surfactant prior to excretion. It should be mentioned that some sections showed an inhomogeneous expression pattern, which may be related to the activity status. Because of varying data on acid secretion, it has been suspected before that the activity of V-ATPase in basolateral membranes may vary due to a shuttling of this protein, which has been nicely demonstrated in kidney cells (Brown and Breton, 2000).This would, of course, also explain an inhomogeneous expression pattern.

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