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Downregulation of aquaporins 1 and 5 in nasal gland by osmotic stress in ducklings, *Anas platyrhynchos*: implications for the production of hypertonic fluid

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Summary

Using primers against highly conserved regions of mammalian and bird aquaporins in RT–PCR experiments, we amplified products derived from duck (*Anas platyrhynchos*) nasal gland RNA that were identified as homologues of mammalian and chicken aquaporin 1 and aquaporin 5 cDNAs by sequencing. Using digoxigenin-labelled probes derived from these PCR products in northern blot analyses of mRNA isolated from nasal glands of untreated (naïve) or osmotically stressed ducklings (replacement of drinking water with a 1% NaCl solution), we observed a decrease in aquaporin 1 (AQP1) and aquaporin 5 (AQP5) mRNA abundance (by approximately 40%) during saline adaptation in the animals. Western blot analysis of AQP1 and AQP5 expression in the glands revealed that protein abundance decreased in a similar fashion. Immunohistochemical analysis of AQP1 distribution in cryosections of nasal gland indicated that AQP1 is mainly expressed in endothelial cells of the capillaries, but definitely not in the secretory or ductal cells of the gland. AQP5 distribution in the gland, however, seems to be different, since staining was exclusively observed in apical and basolateral plasma membranes of individual epithelial cells of the primary and central ducts, which collect fluid from the secretory tubules. The observations are consistent with the hypothesis that strongly hyperosmotic fluid is produced by the secretory cells at very low (unstimulated gland) or high (activated gland) rates. In the unstimulated gland, secretions may be diluted by aquaporin-mediated transcellular water flux while passing through the ductal system flushing the glandular ducts, thereby potentially preventing ascending infections. In the activated gland, however, downregulation of aquaporins in capillaries and duct cells may prevent dilution of the initially secreted fluid, enabling the animals to excrete large volumes of a highly concentrated salt solution.

Key words: aquaporins, osmotic stress, avian nasal gland, fluid concentration, adaptive cell differentiation.

Introduction

The avian nasal gland is unique among transporting epithelial tissues in that it is able to form a NaCl-rich fluid that is always hyperosmotic with respect to the blood (Schmidt-Nielsen, 1960). Secretion of a strongly hypertonic fluid from the glands enables marine or potentially marine birds (e.g. ducks, *Anas platyrhynchos*) to excrete excess salt while retaining free water in the body after ingestion of salty food or seawater. The concentrating ability of the glands does not involve a countercurrent exchange system as that in the mammalian kidney but seems to be an intrinsic property of the epithelium lining the secretory tubules (Butler, 2002).

Secretion from the nasal gland is controlled by the parasympathetic nervous system (Fänge et al., 1958). Acetylcholine released from nerve terminals in the secretory tissue activates muscarinic receptors on the basolateral surface of the cells which results in phospholipase C-mediated inositol phosphate production and intracellular calcium signalling (Snider et al., 1986; Shuttleworth and Thompson, 1989). The latter induces the opening of apical chloride channels, which release chloride ions from the cells to the tubulus lumen by chemical and electrical gradients. Replacement of chloride ions from the interstitium occurs through a basolateral $Na^+/2Cl^-/K^+$ cotransporter that utilizes the concentration gradient of sodium ions to energize the entry of potassium and chloride ions into the cell (Torchia et al., 1992). Accumulation of potassium ions within the cell is avoided by calcium-dependent activation of basolateral potassium channels. As in other secretory epithelia, this allows sustained secretion of

NaCl from the interstitium into the tubulus lumen *via* secondary active chloride secretion and passive transepithelial movement of sodium ions (Frizzell et al., 1979; Lowy et al., 1989), supposedly through a selective permeability of the tight junctions.

In mammalian epithelial cells (e.g. pancreatic duct cells, airway gland cells, salivary gland cells), such an active salt secretion is generally accompanied by osmotic water flux *via* water channels (aquaporins) in apical and basolateral plasma membranes (Burghardt et al., 2003; Song and Verkman, 2001) or *via* paracellular pathways (Murakami et al., 2001) so that the concentration of the secreted fluid is more or less isoosmotic when compared with interstitial fluid or blood, respectively. The avian nasal gland, however, has evolved the ability to effectively secrete salt and simultaneously restrain osmotic water flux. This may be achieved by a very limited water permeability of the tight junctions (paracellular water flux) or the apical and basolateral cell surfaces (transcellular water flux). Although little is known about the regulation of water permeability of tight junctions (Anderson, 2001; Guo et al., 2003; Schneeberger and Lynch, 2004), transcellular water flux in polarized epithelial cells is generally dependent on the presence of different isoforms of aquaporins (AQP; water channels) (Borgnia et al., 1999). Among the true water channels which transport water but no solutes, AQP1 has been identified in endothelial cells lining the capillaries and AQP5 is generally expressed in the secretory cells of glandular tissues (Burghardt et al., 2003; Gresz et al., 2004; Nielsen et al., 1997; Song and Verkman, 2001; Verkman and Mitra, 2000). In concert, these water channels provide a transcellular route for osmotic water flux from the blood space to the lumen of the secretory ducts.

In contrast to secretions from other glands in vertebrates, the secretory fluid in the avian nasal gland is always hypertonic, even shortly after the onset of secretion due to acute osmotic stress in the animals. Furthermore, during prolonged osmotic stress in ducklings (*Anas platyrhynchos*), the osmotic concentration of the secretory fluid increases from approximately 700 mmol kg^{-1} H_2O (at the onset of secretion) to 1000 mmol kg^{-1} H_2O at 48 h (Bentz et al., 1999) indicating that an increase in the concentrating capability of the gland is part of the adaptive differentiation response of the gland cells to sustained osmotic stress in the animals.

The aim of this study was to identify potential transcellular pathways for osmotic water flux following actively secreted sodium chloride in nasal gland tissue and to study the regulation of the molecular components of these pathways during physiological adaptation to osmotic stress in ducklings.

Materials and methods

Animals

One-day old ducklings (*Anas platyrhynchos* L.) were purchased from a commercial hatchery, kept in cages in a room with 12 h:12 h light:dark cycle and fed chick starter crumbs with drinking water *ad libitum*. Drinking water of experimental animals was replaced with a 1% NaCl solution (327±3 mmol kg^{-1} H_2O, mean ± s.d., N=12 preparations) for different periods of time. All experimental animals were between 12- and 15-days old.

Extraction of total RNA and mRNA

Nasal glands from decapitated ducklings were dissected out and rinsed in ice-cold sterile saline. The glands were homogenized with a mortar and pestle under liquid nitrogen and total RNA extraction from the homogenate was performed using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. RNA was quantified spectrophotometrically and stored in 50 μg aliquots in deionised water at –80°C.

Purification of mRNA was performed using the Oligotex mRNA Purification Kit (Qiagen, Hilden, Germany) following the protocol recommended by the manufacturer.

Analysis of RNA by RT–PCR

Equal amounts (1 μg) of total RNA were used for first strand cDNA synthesis in a 22 μl reaction volume [20 mmol l^{-1} Tris-acetate, pH 8.0, 50 mmol l^{-1} KCl, 2 mmol l^{-1} dithiothreitol, 5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} of each dNTP, 30 pmol oligo(dT) primer, 20 i.u. M-MuLV reverse transcriptase (MBI Fermentas, St-Leon-Rot, Germany)] according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed in a final volume of 25 μl containing 2 μl cDNA, 20 mmol l^{-1} Tris-HCl, pH 8.5, 16 mmol l^{-1} $(NH_4)_2SO_4$, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} of each dNTP, 0.2 μmol l^{-1} of each primer and 2 i.u. *Taq* polymerase (MBI Fermentas). Assuming that identical stretches of DNA sequences in mammalian and other bird species may resemble aquaporin sequences in the duck, primers were constructed according to conserved sequence stretches of human AQP1 mRNA [GenBank U41517 (Moon et al., 1993)], human AQP2 mRNA [GenBank Z29491 (Deen et al., 1994)], human and quail AQP4 mRNA [GenBank U63622 (Lu et al., 1996) and GenBank AF465730], human and chicken AQP5 mRNA [GenBank U46566 (Lee et al., 1996) and GenBank AJ829443], human AQP6 mRNA [GenBank U48408 (Ma et al., 1996)] and human AQP8 mRNA (GenBank AF067797). The consensus sequences for the forward and reverse primers were:

Primer 1 (forward): 5′-AGCGGNBSCCACNTCAACC-3′

Primer 2 (reverse): 5′-GGBCCNACCCARWARAYCC-3′.

Optimal results were obtained using the following PCR protocol. After preheating for 3 min at 94°C, samples were processed through 30 cycles at 94°C for 30 s, 48°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 10 min. Analysis of PCR products was performed by electrophoresis in 1.5% agarose gels, followed by densitometry of ethidium bromide-stained DNA bands using a size standard (100–1000 bp; Roth, Karlsruhe, Germany) as a reference. For control purposes, PCR products were sequenced (Agowa, Berlin, Germany).

Cloning of PCR products

For sequencing purposes and downstream applications, PCR products were cloned into the pBluescript KS vector (Stratagene, La Jolla, USA). After separation on agarose gels, PCR products of the appropriate size were cut out and recovered from the gel matrix using the DNA Extraction Kit (MBI Fermentas) according to the manufacturer's recommendations. Purified PCR products were ligated with blunt-ended pBluescript KS vector using T4 ligase (MBI Fermentas), and samples of the ligation mixtures were transferred into *E. coli* DH5a cells by electroporation (ECM 399; Harvard Apparatus, Holliston, USA).

Putative clones containing cDNA of duck aquaporins were identified by plasmid preparation (Birnboim and Doly, 1979) and subsequent restriction analysis, and the nucleotide sequences of these clones were determined (Agowa, Berlin, Germany). The partial nucleotide sequences of duck aquaporin 1 and aquaporin 5 cDNAs were deposited in GenBank under the accession numbers DQ123915 and DQ123916, respectively.

Generation of digoxigenin-labelled hybridization probes

Purified plasmid DNA of pBluescript KS containing duck aquaporine 1 or duck aquaporine 5 partial cDNAs, respectively, was used as template for generation of digoxigenin (DIG)-labelled hybridization probes by PCR. The reactions were performed in a final volume of 50 μl containing 0.2 μg plasmid DNA, 20 mmol l^{-1} Tris–HCl, pH 8.5, 2.5 mmol l^{-1} $MgCl_2$, 5 μl PCR-DIG labelling mix, 0.2 μmol l^{-1} of each primer (see above) and 1 i.u. *Taq* polymerase (MBI Fermentas). Optimal results were obtained using the following PCR protocol. After preheating for 3 min at 94°C, samples were processed through 30 cycles at 94°C for 30 s, 51°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 10 min. After separation on 1.5% agarose gels, PCR products were cut out and recovered. Concentrations of labelled PCR products were spectrophotometrically quantified and the efficacy of the labelling reactions was determined by dot blot analysis with serial dilutions of the labelled probes.

To check the specificity of the labelled cDNA probes, we performed cross-hybridization tests using plasmids containing cDNAs of AQP1 and AQP5 as templates. Electrophoresis of equal amounts of purified plasmid DNA was performed on 1% agarose gels in TAE buffer (40 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, adjusted to pH 8.0 with glacial acetic acid). The gel was incubated for 30 min in 0.25 mol l^{-1} HCl followed by an incubation step in denaturation buffer (0.5 mol l^{-1} NaOH, 1.0 mol l^{-1} NaCl) for 30 min and repeated incubations for 15 min each in neutralization buffer (1.5 mol l^{-1} NaCl, 0.5 mol l^{-1} Tris–HCl pH 8.0). After equlibration in 20× SSC buffer (3 mol l^{-1} NaCl, 0.5 mol l^{-1} sodium citrate, pH 7.0), DNA was blotted onto a nylon membrane (Roche Diagnostics, Mannheim, Germany) by vacuum blotting (Gel Dryer, BioRad, Hercules, CA, USA) for 6 h. Blotted DNA was crosslinked to the membrane by UV light for 3 min.

Prehybridization was performed in 50% formamide, 5× SSC, 2% blocking reagent (BioRad, München, Germany), 0.1% *N*-lauroyl sarcosine and 0.02% SDS for 1 h at 68°C. Hybridization probes were incubated with the blotting membranes at 68°C overnight. Membranes were washed twice for 5 min at room temperature in maleic acid buffer (0.1 mol l^{-1} maleic acid, 0.15 mol l^{-1} NaCl, adjusted pH 7.5 with NaOH) and twice for 15 min at 68°C in blocking buffer (1% blocking reagent in maleic acid buffer).

Detection of signals was performed with the DIG DNA Detection Kit (Roche Diagnostics) according to the manufacturer's recommendations, and signals were visualized on X-ray film. Images were digitized using a computer scanner.

Northern blotting

Electrophoresis of equal amounts of mRNA (3 μg per lane) was performed on denaturing agarose/formaldehyde gels (1%) as described previously (Hildebrandt and Shuttleworth, 1994). After equilibration of a gel in 20× SSC, RNA was blotted onto nylon membrane (Roche Diagnostics, Mannheim, Germany) by vacuum-assisted transfer for 6 h. RNA was crosslinked to the membrane by UV light for 3 min. Prehybridization, hybridization and detection were performed as described above. Images were digitized using a computer scanner and analyzed densitometrically using Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK) on a personal computer. ANOVA was used to test to means of densitometric data for significant differences (P<0.05).

Western blotting

Ducklings were killed by decapitation, the nasal glands were injected with ice-cold Hepes-buffered saline (Shuttleworth and Thompson, 1989) to remove the blood cells from the tissue and dissected out. Tissue samples were immediately frozen in liquid nitrogen. Sample preparation, SDS electrophoresis of proteins on 13% polyacrylamide gels, blotting of proteins onto nitrocellulose membrane and detection were performed as described previously (Hildebrandt et al., 1998). Protein concentrations in the homogenate were determined using the Bradford assay with bovine serum albumin as a standard (Bradford, 1976). After adding SDS sample buffer, equal amounts of total protein (20 μg) were loaded onto each lane of the electrophoresis gel. Detection of aquaporins using a 1:1000 dilution of polyclonal antibodies (AQP1: Alpha Diagnostic AQP11-A, San Antonio, TX, USA; AQP5: Sigma A4979, Taufkirchen, Germany) and HRP-coupled sheep anti-rabbit antibody (1:6000, Amersham Biosciences, Freiburg, Germany) as secondary antibody was performed using enhanced chemiluminescence (ECL) reagents and X-ray film (Amersham Biosciences). Images were digitized using a computer scanner and analyzed densitometrically using Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK) on a personal computer. ANOVA was used to test the means of densitometric data for significant differences (P<0.05).

In some experiments, proteins in tissue homogenates were deglycosylated before being analyzed by SDS electrophoresis and western blotting. Tissue was homogenized in buffer

containing protease inhibitors [0.5 mmol l^{-1} Pefablock (Roth, Karlsruhe, Germany), 20 μg ml^{-1} each of aprotinin and leupeptin (Sigma, Taufkirchen, Germany) and 0.2 μg ml^{-1} pepstatin from the same source]. Membrane proteins were solubilized by adding 1% SDS and 3% β-mercaptoethanol and heating the samples to 100°C for 10 min. Proteins in the cooled samples were stripped from SDS by addition of 2% Triton X-100 before 44 i.u. ml^{-1} of *N*-glycosidase F (Roche, Mannheim, Germany) were added to each sample followed by incubation at 37°C for 2 h. Reactions were stopped by heating the samples briefly to 100°C and SDS sample buffer was added before loading samples onto the electrophoresis gel.

Immunohistochemistry

Ducks were killed by decapitation and nasal glands were injected *in situ* with ice-cold 5% paraformaldehyde in phosphate-buffered saline (PBS), dissected out and quickly cut into four pieces. The tissue was fixed in ice-cold 5% paraformaldehyde PBS for another 5 h, subsequently transferred to PBS containing 20% sucrose and stored at 4°C overnight. Tissue was gently blotted dry and rapidly frozen on pulverized dry ice. Tissue cubes were frozen onto a cryotome sample platform in blocks of Tissue Tek (Cambridge Institute, Cambridge, UK) and cut into 5 μm sections using a cryotome (Leica Microsystems, Bensheim, Germany) with the chamber temperature set to –18°C and the sample platform temperature set to –22°C. Sections were transferred to poly-L-lysine-coated microscopic slides. Slides were washed in 96% (v/v) ethanol for 10 min at room temperature (RT), dried and incubated in Tris-buffered saline (TTBS: 0.0125 mol l^{-1} Tris, 0.15 mol l^{-1} NaCl, 0.1% Tween 20, pH 7.5) containing 0.1% (w/v) non-fat dry milk for 2 h at 4°C. In some experiments, slides were incubated in 3% (w/v) H_2O_2 solution for 30 min at RT to inactivate endogenous peroxidases in the tissue. Blocking and antibody incubations were performed in TTBS+0.1% non-fat dry milk as described previously (Hildebrandt et al., 1998) using 1% horse serum as blocking reagent, 1:1000 dilutions of AQP1- or AQP5-specific polyclonal antibodies [Alpha Diagnostic (AQP11-A or AQP51-A) San Antonio, TX, USA], a Vectastain (Vector, Burlingame, CA, USA) biotin–avidin–HRP (horseradish peroxidase) enhancer system according to the manufacturer's instructions and a combined diaminobenzidine/hydrogen peroxide reagent (Sigma, Taufkirchen, Germany) as the substrate for HRP. Controls were prepared without primary antibody or without biotinylated secondary antibody. Slides were rinsed in water, in increasing concentrations of ethanol and finally in xylene and dried before tissue sections were covered with DPX Mountant (Fluka, Buchs, Germany) and a coverslip. Tissue sections were viewed using a Nikon Eclipse TE300 microscope equipped with Plan- and Hoffmann modulation contrast objectives and a Nikon DXM1200 digital camera.

Results

To identify the subtypes of aquaporins expressed in glandular tissues of ducklings, we used a pair of PCR primers according to sequences that are highly conserved in all water-specific isoforms of mammalian and chicken aquaporins. After optimization of PCR parameters, we obtained two PCR products of the expected size (approximately 440 bp) which were identified as partial sequences of the duck homologues of aquaporin 1 and aquaporin 5 by sequencing (GenBank DQ123915 and DQ123916). No other PCR products related to aquaporins were detected. The sequenced portions of duck aquaporins 1 and 5 show high degrees of similarity with the mammalian (84–88% at the amino acid level) or other bird (chicken, quail; 96–99%) sequences, respectively (Fig. 1).

Although only rarely reported in mammalian systems, it seems possible that aquaporin expression may be regulated during cellular differentiation. To study potential transcriptional regulation of AQP1 and AQP5 expression in nasal gland under conditions of osmotic stress in the ducklings, we prepared specific DIG-labelled cDNA probes for hybridization and detection of AQP1 or AQP5 mRNA, respectively. Owing to sequence similarities between AQP1 and AQP5, cross-reactivity of these probes was checked by running three different concentrations of linearized plasmids containing the cDNA inserts of AQP1 or AQP5, on agarose gels and hybridizing the blotted cDNAs in a crosswise manner with AQP1- and AQP5-specific probes. The probes showed a moderate cross-reactivity, but bound to their specific targets with much higher efficiency (not shown) indicating that co-hybridization with both probes in the same assay was possible. Northern blot analysis using mRNA preparations from gland tissue of untreated, or naive (fw; given freshwater) and osmotically stressed (sw; given saltwater) animals showed that the size of duck AQP1 mRNA was approximately 3.1 kb, the size of duck AQP5 mRNA was approximately 1.7 kb (Fig. 2).

To compare mRNA abundances in gland tissue from fw ducklings with those in sw ducklings, we determined DIG signal densities in northern blots obtained with AQP1- or AQP5-specific probes (Fig. 2B,C), which were standardized using ethidium bromide fluorescence signals of residual 28S and 18S bands as internal references. The relative amount of AQP1 mRNA in nasal glands of sw ducklings was approximately 64% of the amount in gland tissue from fw animals (N=12, P<0.05). A similar decrease to approximately 61% of the fw level was observed in northern blot experiments with sw tissue using the probe for AQP5 mRNA (N=12, P<0.01). This indicates that AQP1 as well as AQP5 mRNAs are downregulated during adaptive differentiation of glandular tissue to osmotic stress in the animals.

Using AQP1-specific antibodies, western blot analysis of nasal gland tissue extracts resulted in labelling of a weak band at 32–35 kDa (Fig. 3A, upper arrow) that was present in samples of fw and sw animals. However, after deglycosylation, a second band at 28 kDa (Fig. 3A, lower arrow) appeared on the blots. Similar shifts in molecular mass could be observed in samples obtained from duck lung and mouse lung, where the expression level of AQP1 is much higher than that in duck nasal gland. This identified AQP1 in duck nasal gland as a glycoprotein. AQP1 bands in western blots with duck salt gland

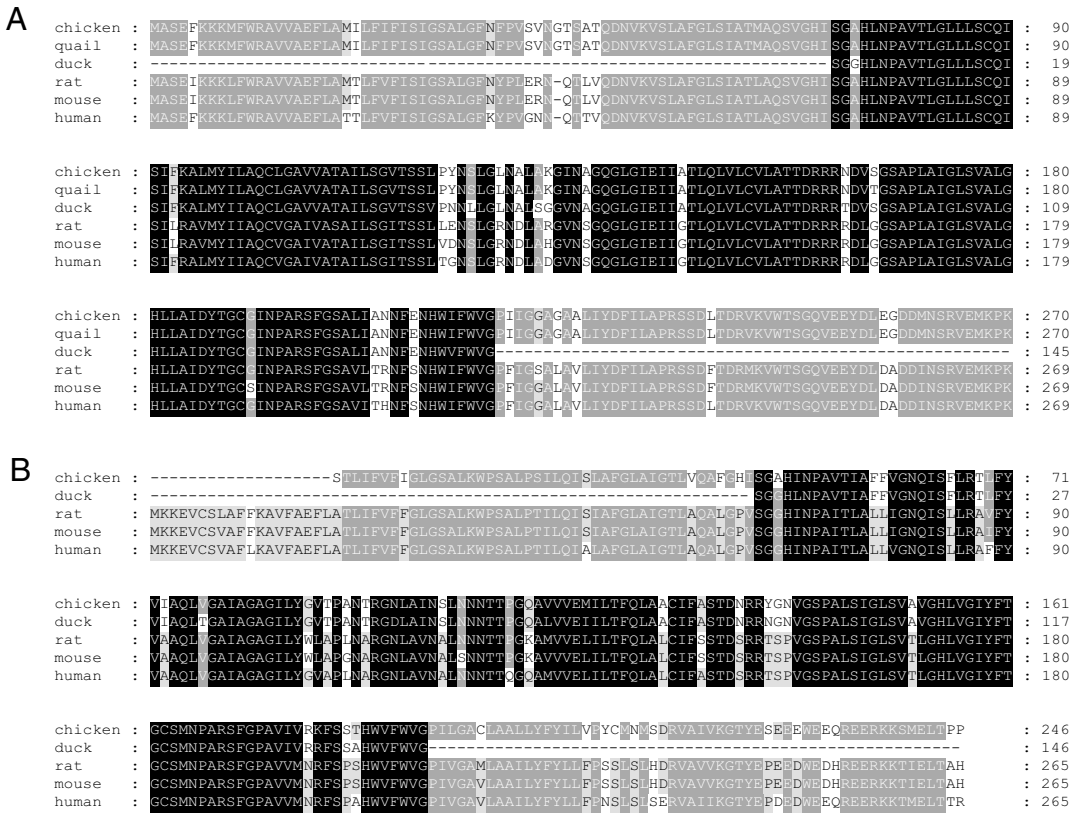

Fig. 1. Alignments of amino acid sequences of aquaporin 1 isoforms (A) and aquaporin 5 isoforms (B) in mammals and birds with the partial duck sequences obtained in this study. Amino acids with a black background are conserved in all species, black letters on a grey background indicate amino acids that are different in mammals and birds, white letters on grey background indicate N- and C-terminal portions of sequence in mammals and birds which have not been determined in ducks. GenBank accession numbers for AQP1: chicken, NM001039453; quail, AY692368; rat, NM_012778; mouse, NM_007472; human, BC022486. Accession numbers for AQP5: chicken, AJ829443; rat, U16245; mouse, NM_009701; human, NM_001651.

extracts are extremely faint, especially when compared to tissues that express this protein at high rates (lung) and seem to have a molecular mass that is somewhat lower than the proteins in duck or mouse lung. However, after deglycosylation, the protein core of AQP1 can be detected at 28 kDa exactly in line with the AQP1 isoforms of duck and mouse lung. This indicates that AQP1 is present in nasal gland at very low levels, but may have a somewhat different running pattern in SDS gels, probably due to differences in glycosylation compared with the proteins from lung.

Using AQP5-specific antibodies, western blots of duckling nasal gland proteins showed only one band at approximately 27 kDa (Fig. 3B, arrow) the molecular mass of which remained unchanged when samples were pretreated with *N*-glycosidase F, indicating that AQP5 in nasal gland is not glycosylated. Whether the additional bands of higher molecular mass in duck lung and of lower molecular mass in deglycosylated samples from mouse lung are related to AQP5 or represent non-specific cross reactions of the AQP5 antibody was not elucidated.

Comparison of expression levels of aquaporins by quantitative western blotting revealed significant differences in gland tissue isolated from fw and sw animals (Fig. 3C,D). The band intensity of deglycosylated AQP1 in the sw samples was approximately 64% of that in fw samples (N=5, P<0.01). A similar decrease in protein abundance was observed for AQP5 which, in sw samples, had only 51% of the intensity of that detected in fw samples (N=5, P<0.001). Owing to the use of full homogenates in these quantitative western blot experiments, these data indicate that AQP1 and AQP5 are downregulated during adaptive cell differentiation in the gland and not just shifted from one membrane compartment to another.

To identify the exact location of AQP1 and AQP5 expression in nasal gland tissue, we prepared cryosections of nasal gland tissue of fw and sw animals (Fig. 4). Immunohistochemical detection of AQP1 in cryosections of fw nasal gland tissue revealed an association of signals with small blood vessels and capillaries in the glandular tissue (Fig. 4C). No signal was detected in cells of the chloride-secreting epithelium or the duct epithelial cells. Tissue slices processed as usual without primary antibodies (AQP1) in the incubation medium did not show any specific staining (Fig. 4C,D, inserts). AQP5-related signals, however, were observed in apical as well as basolateral plasma membranes of individual epithelial cells of the central

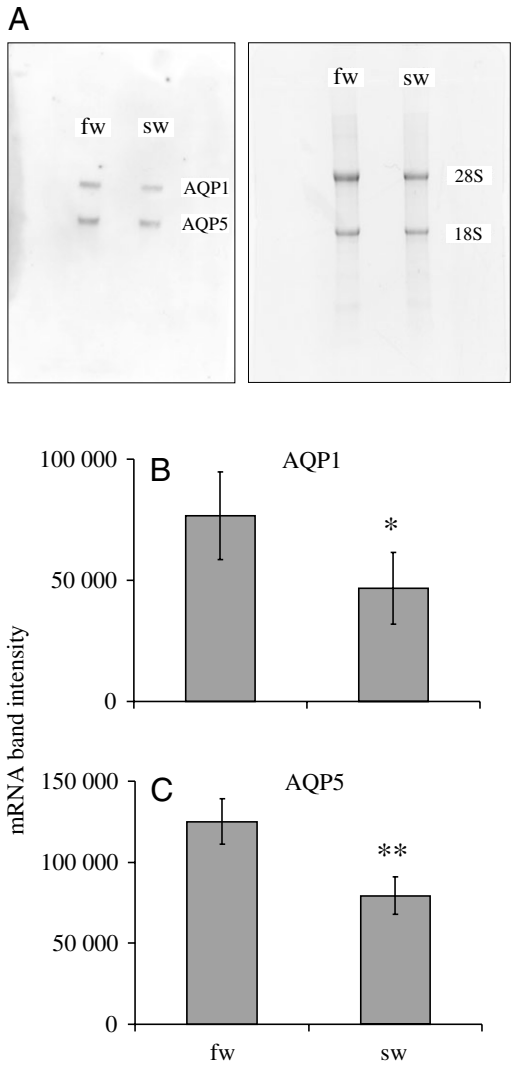

Fig. 2. Northern blot analyses of mRNA isolates from nasal glands of untreated (fw) and osmotically stressed (sw) ducklings using AQP1- and AQP5-specific DIG-labelled cDNA probes. (A) Left panel: example of a northern blot with simultaneous detection of AQP1 and AQP5 mRNAs; right panel: inverse fluorescence image of an ethidium bromide-stained agarose gel used for northern blotting. The positions of residual ribosomal RNAs (28S, 18S) in the mRNA samples loaded onto the gel lanes are indicated. These signals were used to control for even loading of the gels. (B) Relative intensities of northern blot signals in mRNA isolates from nasal glands of untreated (fw) and osmotically stressed (sw) ducklings obtained with AQP1-specific cDNA probes (means ± s.d., N=12, *P<0.05, ANOVA). (C) Relative intensities of northern blot signals in mRNA isolates from nasal glands of untreated (fw) and osmotically stressed (sw) ducklings obtained with AQP5-specific cDNA probes (means ± s.d., N=12, **P<0.01, ANOVA).

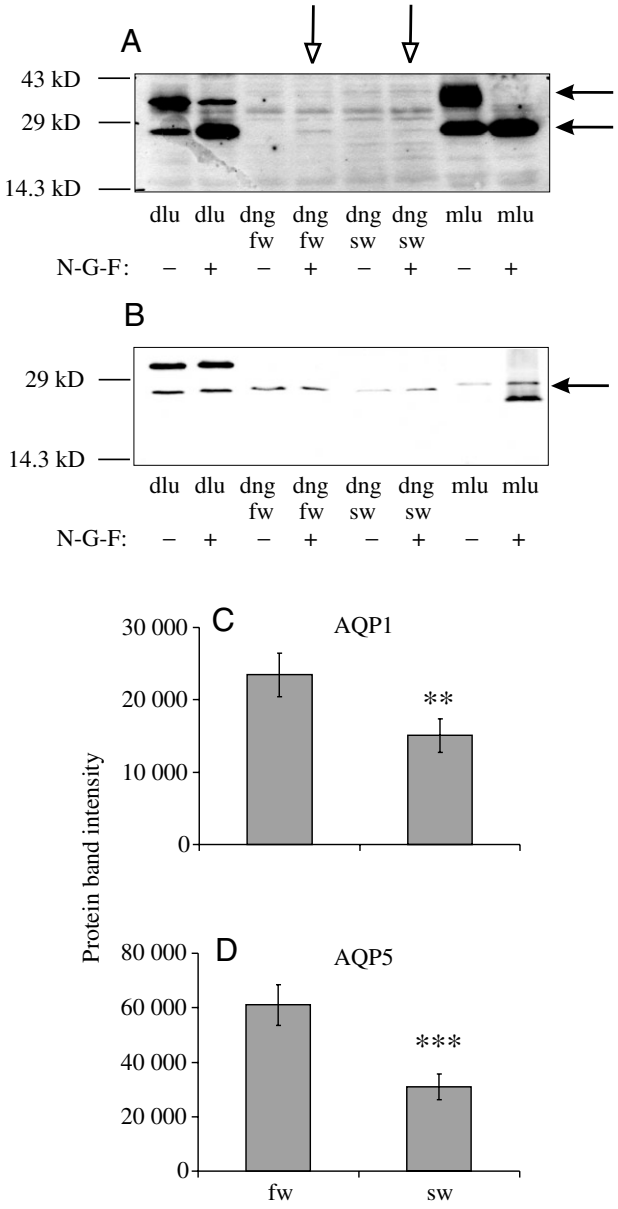

Fig. 3. Western blot analyses of aquaporins in nasal glands of untreated (fw) and osmotically stressed (sw) ducklings using AQP1- and AQP5-specific antibodies. (A) AQP1 western blot of membrane proteins from duck lung (dlu), duck nasal gland (dng) and mouse lung (mlu) with (+) or without (–) sample pretreatment with *N*-glycosidase F (N-G-F). Note the obvious difference in total AQP1 content in lung and nasal gland preparations and the appearance of bands at a molecular mass of 28 kDa in the nasal gland samples after deglycosylation (open arrowheads) which are absent in untreated nasal gland samples. (B) AQP5 western blot of membrane proteins from duck lung (dlu), duck nasal gland (dng) and mouse lung (mlu) with (+) or without (–) deglycosylation using *N*-glycosidase F (N-G-F). (C) Relative intensities of western blot signals in protein isolates from nasal glands of untreated (fw) and osmotically stressed (sw) ducklings obtained with AQP1-specific antibodies (means ± s.d., N=5, **P<0.01, ANOVA). (D) Relative intensities of western blot signals in protein isolates from nasal glands of untreated (fw) and osmotically stressed (sw) ducklings obtained with AQP5-specific antibodies (means ± s.d., N=5, ***P<0.001, ANOVA).

ductules (Fig. 4E,F), whereas many other cells lining the larger ducts were negative for AQP5 as were the secretory epithelial cells (Fig. 4E). Tissue slices processed as usual without primary antibodies (AQP5) in the incubation medium did not show any specific staining (results not shown). In nasal gland tissue isolated from osmotically stressed animals, the AQP1-related signal was much less intense compared with that in tissue from fw animals (Fig. 4C,D). A similar difference in signal intensity was observed when tissues were probed with AQP5 antibodies (Fig. 4E,G). In gland tissue isolated from sw animals, AQP5 is hardly detectable and may be limited to very low amounts in the apical plasma membrane of some of the epithelial cells lining the larger ducts (Fig. 4G,H).

There are apparent differences in the degree of AQP5 downregulation as detected by western blotting and immunohistochemistry. Although we cannot at present explain these differences, it is very likely that they result from differences in binding behaviour of the different primary antibodies used in the two procedures. We used a polyclonal antibody from Sigma for the western blot experiments and a different polyclonal antibody from Alpha Diagnostics for immunohistochemistry. These antibodies gave the best results in the respective procedures, but may have displayed different degrees of density changes due to downregulation of the protein in the differentiating gland tissue.

Discussion

Exposure of ducklings to sustained osmotic stress by replacing their drinking water with a 1% NaCl solution induces adaptive hyperplasia and cell differentiation in different subsets of cells in the nasal glands (Ernst and Ellis, 1969; Hildebrandt, 2001; Hossler, 1982; Müller and Hildebrandt, 2003). Soon after animals start drinking saline, body fluid osmolality increases (Bentz et al., 1999) triggering central osmoreceptors which, in turn, activate parasympathetic nerves supplying the nasal glands (Gerstberger and Gray, 1993). An intact nerve supply of the gland is essential for induction of osmotic stress-mediated adaptive processes (Hanwell

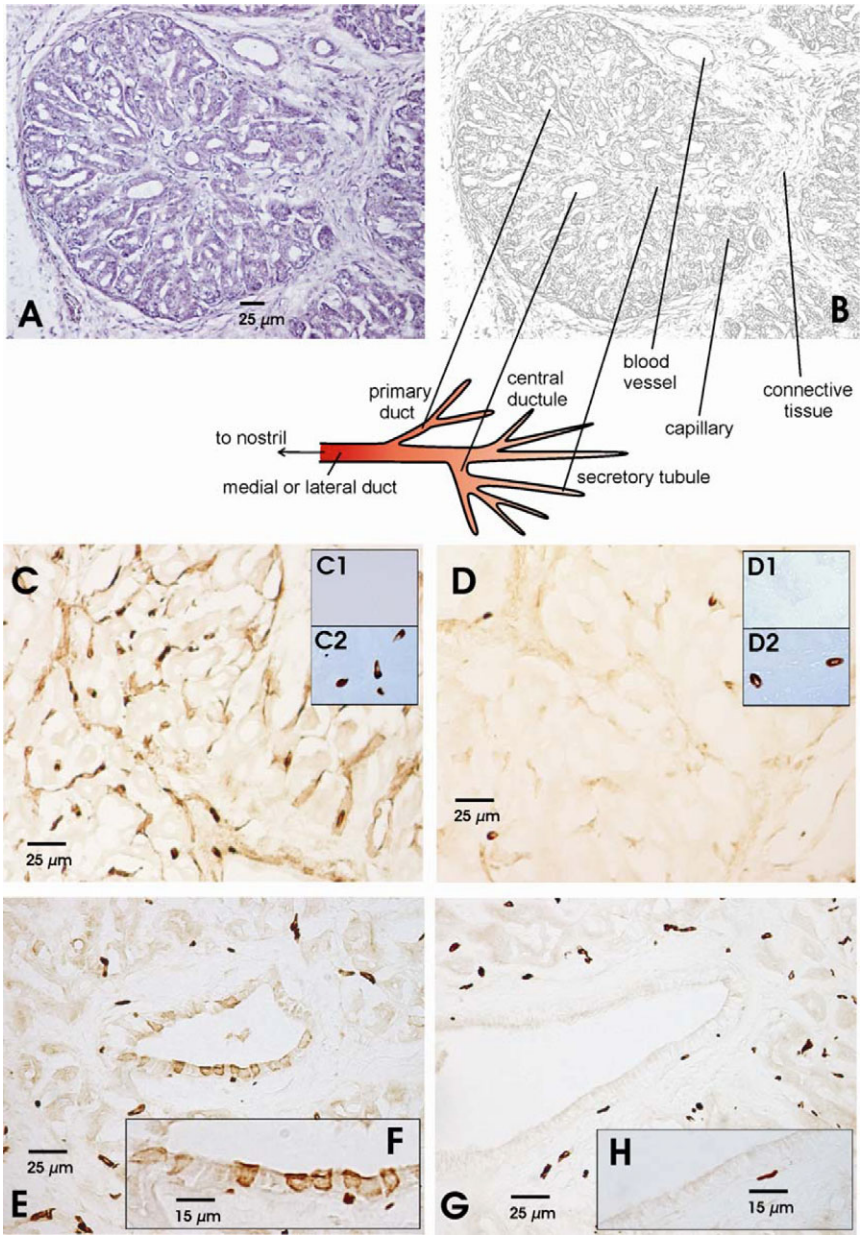

Fig. 4. Survey of a cross-cryosection (5 μm thickness) of nasal gland tissue stained with Hemalaun and Eosin (A), description of structural correlates (c.f. Butler et al., 1991) (B) and immunohistochemical detection of AQP1 (C,D) or AQP5 (E–H), respectively, in nasal gland tissue obtained from naïve (C,E) or osmotically stressed (D,G) ducklings. Specific signals at the sites of antibody binding are visible as brownish precipitates, which are products of the peroxidase-reactions with diaminobenzidine and hydrogen peroxide as substrates. The dark speckles within some of the capillaries are red blood cells (RBCs). Control experiments, in which cryosections were preincubated with (C1,D1) or without (C2,D2) 3% hydrogen peroxide-solution for 30 min at room temperature before immunostaining without primary antibody, revealed that endogenous peroxidases of RBCs reacted non-specifically with the peroxidase substrates. Note the presence of AQP1 in capillary endothelial cells in tissue of naïve animals (C). Epithelial cells of the secretory tubules or the ducts are devoid of AQP1-related signals. Note the expression of AQP5 in individual cells lining the primary and central ducts. Apical as well as basolateral plasma membrane compartments of ductal cells in glands of naïve ducklings are labelled (F). Much less immunostaining was observed in nasal gland cryosections obtained from osmotically stressed ducklings using AQP1- (D) or AQP5-specific (G,H) antibodies, respectively.

and Peaker, 1975). An important element of these adaptive changes in the gland seems to be an increase in the tightness of the secretory epithelium since the osmotic concentration of the secretory fluid increases by a factor of 1.5 between the onset of secretion at 2 h and 48 h of sustained osmotic stress in ducklings whose drinking water had been replaced with a 1% NaCl solution (Bentz et al., 1999). Although at least a portion of this effect may be a consequence of changes in molecular composition and architecture of the tight junctions between the epithelial cells lining the secretory tubules, we obtained evidence in this study that the expression of aquaporins in selected cell types of nasal gland tissue changes during the physiological adaptation of the animals to osmotic stress.

Using a RT–PCR approach, we searched for duck homologues of mammalian isoforms of aquaporins that are known to exclusively conduct water. We found two PCR products that resemble fractions of mammalian AQP1 and AQP5 isoforms at the amino acid level (Fig. 1). Detection of only two aquaporin isoforms does not exclude that other aquaporin subtypes may be expressed in the gland.

We cloned the PCR products in order to use the duck-specific cDNAs as probes for hybridization experiments. Since the sizes of AQP1 and AQP5 transcripts were clearly different and the degree of cross-reactivity of the cDNA probes was only of minor quantitative significance, it was possible to use both probes simultaneously in northern blot experiments to quantify the mRNA levels of both isoforms in RNA samples obtained from the quiescent glands of ducklings given freshwater (fw) or ducklings that had been given a 1% NaCl solution for 48 h (osmotically stressed animals; sw; Fig. 2). Specific signals for AQP1 and AQP5 transcripts were quantified by densitometry and revealed that both transcripts were significantly less abundant in sw glands than in fw tissue (Fig. 2B,C). This indicates that either the transcription rates of both genes or the stability of the transcripts are altered during adaptive differentiation in the gland.

Western blot experiments using antibodies against the mammalian AQP1 and AQP5 isoforms revealed that both proteins are expressed in nasal gland tissue of fw animals at a low level (Fig. 3), but are even less abundant in nasal glands of sw animals (Fig. 3C,D).

Using AQP1 antibodies, two bands at 32–35 kDa and a band at 28 kDa were detected in nasal gland membrane preparations. Both bands were also detected in membrane preparations of mouse lung used as a reference, since mammalian lung cells express AQP1 at high levels (King et al., 1997; King et al., 2002). Deglycosylation of solubilized membrane proteins from untreated nasal gland and from mouse lung using *N*-glycosidase F resulted in loss of signal intensity in the 35 kDa band and in a concomitant increase in signal intensity in the 28 kDa band, indicating that the higher molecular mass bands represent glycosylated forms of AQP1 (Fig. 3A). This is consistent with previously reported findings in mammalian cells that AQP1 generally occurs in the glycosylated state (Denker et al., 1988; Smith and Agre, 1991) and confirms this for the avian nasal gland.

Western blotting using AQP5 antibodies revealed weak signals at 27 kDa in untreated nasal gland tissue and mouse lung (Fig. 3B). Neither these nor higher molecular mass signals of unknown specificity changed in intensity after deglycosylation of solubilized proteins, indicating that AQP5 may not be subject to glycosylation in these tissues. Although potential glycosylation sites have been identified in the rat AQP5 protein sequence (Raina et al., 1995), direct evidence for AQP5 glycosylation in mammalian tissues is lacking (King et al., 1997). Both, AQP1 and AQP5 are obviously downregulated during adaptive differentiation of the nasal gland tissue under osmotic stress in the ducklings, since protein abundance was almost twofold higher in nasal gland of untreated compared with that of stressed animals (Fig. 3C,D). These differences in protein abundance match the differences in mRNA abundance (Fig. 2) indicating that transcription rate or mRNA stability may regulate protein abundance in this tissue rather than the rates of protein synthesis and degradation.

Immunohistochemical detection of AQP1 in cryosections of nasal gland tissue from untreated animals revealed an association of signals with small blood vessels and capillaries in the glandular tissue (Fig. 4C). No AQP1 was detected in epithelial cells in the secretory tubules, primary or central ductules. This indicates that AQP1 expression in the glands of untreated animals may facilitate the generation of osmotic equilibrium between blood and interstitium by allowing transcellular water flux through the endothelial cells. In accordance with the results of the western blot experiments, AQP1 specific signals in tissue slices were highly attenuated in samples isolated from osmotically stressed animals (Fig. 4D) compared with samples from untreated animals (Fig. 4C). Since the cryosections of glands from both treatments were prepared simultaneously and were subsequently processed on the same microscopic slide, it can be excluded that this difference is due to variations in experimental procedures, but represents true differences in protein abundance.

Using AQP5-specific antiserum, a different distribution of signals was found in cryosections of untreated nasal gland (Fig. 4E,F). In this case, individual cells in the epithelial lining of the primary and central ductules were labelled, again without any staining in the secretory cells. AQP5-positive duct cells, however, showed specific staining in both apical and basolateral membranes (Fig. 4F). By contrast, but again in accordance with results of the western blot experiments, AQP5-specific signals were very weak in nasal gland tissue isolated from osmotically stressed animals (Fig. 4G,H) indicating that AQP5 is downregulated during adaptive differentiation in nasal glands of osmotically stressed animals.

Taken together, these results indicate that nasal gland tissue in animals drinking freshwater expresses very limited amounts of AQP1 and AQP5 which, however, allow transcellular passage of water from the blood space to the lumen of primary and central ducts following the osmotic gradient across the epithelium. In the initial phase of osmotic stress, this pathway for water transport seems to be successively shut down by downregulation of aquaporins in the capillary endothelium as

well as in the glandular duct cells. The signalling cascades responsible for these effects are not known. Sustained cAMP signalling that has been implicated in downregulation of aquaporin 5 in mouse lung epithelial cells (Sidhaye et al., 2005) may actually occur in the initial phase of nasal salt gland differentiation when animals are osmotically stressed (Hildebrandt, 1997). Whether aquaporin ubiquitinylation and proteasomal degradation, as observed in cultured fibroblasts (Leitch et al., 2001), is the mechanism underlying this loss in aquaporins remains to be elucidated. However, as indicated by the almost identical rates of mRNA and protein losses during osmotic stress, it is more likely that changes in AQP1 and AQP5 gene transcription or the stability of the respective mRNAs are the relevant factors determining protein abundance.

In the final stage of the adaptive process at 48 h of osmotic stress, the paracellular route may be the only remaining transepithelial pathway for water flux through capillary endothelia and secretory as well as ductal epithelia. This reorganisation process of the gland may fully account for the observed increase in osmolality of the secreted fluid under sustained osmotic stress in ducklings (Bentz et al., 1999) and may also explain the observation (Schmidt-Nielsen, 1960) that the maximum concentration of secreted fluids is, although different in different bird species, invariant in individuals of the same species and independent of the degree of osmotic loading of the individuals. Upregulation of individual aquaporin subtypes other than AQP2 at the transcriptional (Borok et al., 2000; Hoffert et al., 2000; Martinez et al., 2005; Umenishi and Schrier, 2003) or posttranslational level (Leitch et al., 2001; Matsuzaki et al., 1999; Sidhaye et al., 2005) has already been reported, but this is, to our knowledge, the first study showing stimulus-dependent coordinated downregulation of AQP1 and AQP5 under physiological conditions.

These conclusions raise the question of why ducklings express AQP1 and AQP5 in selected nasal gland cell types under basal conditions when no visible amounts of secreted fluid is produced and excreted. Assuming that salt secretion in the secretory tubules induces a low rate of osmotic water flux through the tight junctions between the secretory cells, it is conceivable that the osmotic concentration of this primary secretion may always be the same and hyperosmotic with respect to the blood (cf. Butler, 2002). An increase in the rate of salt secretion would then result in a proportional increase in fluid volume without any change in osmotic concentration. In this way, a low constitutive rate of salt secretion in the unstimulated gland would result in a very low rate of fluid production in the secretory tubules. While running downstream through primary and central ducts, osmotic water flux would dilute the originally highly concentrated fluid and increase the fluid volume. At the opening of the medial or lateral ducts, the isotonic or mildly hypertonic fluid may get absorbed by the nasal epithelium or may eventually be swallowed so that neither salt nor water is excreted to the environment. The biological significance of such a constitutive fluid production in the unstimulated gland may lie in the constant flushing of the tubular system, thereby preventing clogging or attenuating the risk of ascending infections with pathogenic bacteria. As we have shown in a recent study (Klopfleisch et al., 2005), a low percentage of ducklings suffer from infections of their nasal glands with Gram-negative bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis* and *Aeromonas hydrophila*) which enter the duct system from the environment and can destroy large portions of glandular tissue by granulomatous inflammation.

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