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Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.)

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Summary

The expression of a putative water channel protein, aquaporin 3 (AQP-3), has been localised within branchial and intestinal tissues from the 'silver' life stage of the European eel *Anguilla anguilla*, using a specific polyclonal antibody directed against the C-terminal of the amino acid sequence. Western blots using the AQP-3 antiserum identified the presence of a major immunoreactive protein of 24 kDa in extracts of gills from both freshwater (FW) and 3 week seawater (SW)-acclimated eels. SW acclimation induced a 65 % reduction in AQP-3 protein abundance in the gill extracts. AQP-3 immunoreactivity was apparent throughout the branchial epithelium from both FW and SW-acclimated fish, but especially so within the chloride cells, which also stained heavily with specific antisera for the β-subunit of the Na, K-ATPase. AQP-3 immunoreactivity not only colocalised with Na, K-ATPase within the basolateral tubular network but also stained the apical regions of the chloride cell where Na, K-ATPase was absent. Although there were no obvious differences in expression between the chloride cells of FW and SW-acclimated fish, considerably higher intensities of immunoreactivity were apparent near the periphery of the non-chloride cells of FW fish, especially within cells forming the base of the primary filaments and the branchial arch. AQP-3 immunoreactivity was also detected in intra-epithelial macrophage-like cells within the intestine of FW and SW-acclimated eels and in the mucous cells of the rectal epithelium of SW-acclimated fish. These results suggest that AQP-3 may play an important functional role in osmoregulation the teleostean gill but is unlikely to be responsible for the increases in intestinal water absorption that occur following SW acclimation.

Key words: aquaporin 3, teleost fish, water channel, urea, European eel, *Anguilla anguilla*, gill, intestine, immuno-histochemistry, immuno-gold.

Introduction

It is now well established that freshwater teleosts drink little and urinate large volumes to compensate for osmotic water uptake through the gills. In contrast, marine teleosts ingest relatively large amounts of seawater (SW) and absorb most of this water along with monovalent ions across the intestine. Together with active excretion of salts by the gills and other organs, this osmoregulatory strategy compensates for osmotic water loss and dehydration when in SW. The major sites of water exchange between the internal milieu and the external environment are therefore the gills and the digestive tract; the branchial epithelium is responsible for over 90 % of the total body water influx in freshwater (FW) (Motais et al., 1969; Haywood et al., 1977), with water uptake by the gut epithelium compensating for osmotic water loss through the gills in SW (Kirsch and Meister, 1982; Isaia, 1984). Although the substantial information concerning the role of ion-transporting proteins (Perry, 1997; Karnaky, 1998; Evans, 1999) has led to the development of several hypotheses concerning water flux pathways (Ando, 1983, 1985; Ogasawara and Hirano, 1984; Ando et al., 1992; Ando and Nagashima, 1996; Alves et al., 1999), the mechanisms controlling the acquisition, retention and excretion of water at the molecular level have still to be characterised (Cutler and Cramb, 2000).

We have recently cloned and sequenced a homologue of the mammalian aquaporin 3 (AQP-3) from the euryhaline eel *Anguilla anguilla* L. (Cutler and Cramb, 2002). Northern blot analyses indicated that AQP-3 was expressed in the gill, oesophagus and intestine mainly as a 2.4 kb mRNA species. Quantitative analysis revealed that the major site of mRNA expression was in the gill of FW eels and that expression was substantially reduced by up to 94 % following SW acclimation. In the intestine, the levels of mRNA expression were much lower and there was no measurable difference between FW and SW-acclimated fish (Cutler and Cramb, 2002).

Water channel aquaporins are likely to be central to the molecular and physiological mechanisms responsible for the changes in water balance that allow the successful adaptation of euryhaline teleost species to both freshwater and marine environments. Aquaporins (AQPs) are members of the ubiquitous family of channel-forming proteins also known as the major intrinsic protein (MIP) family, and function as water channels that allow rapid osmotic water flow mainly in epithelial tissues (Deen and van Os, 1998). Although AQP isoforms such as AQP-1 are proteins dedicated to water transport, some isoforms such as AQP-3 also allow the passage of larger polar compounds such as glycerol and urea (Borgnia et al., 1999). In mammals, AQP-3 is located on the basolateral membrane of renal collecting duct cells, suggesting a role in renal water reabsorption (Frigeri et al., 1995). AQP-3 is also present in the airway epithelium (Frigeri et al., 1995), eye conjunctiva and meningeal cells (Lee et al., 1997) and is abundant in the nasopharyngeal epithelium, suggesting a possible role in mucosal fluid excretion and allergic rhinitis (King et al., 1997; Nielsen et al., 1997). In the gastrointestinal tract, AQP-3 was detected on the plasma membranes of stratified squamous epithelial cells in the oesophagus (Koyama et al., 1999), along the basolateral membranes of cardiac gland epithelia in the lower stomach (Koyama et al., 1999), in the columnar epithelia in the villi and crypts of the small intestine from the jejunum to the ileum and along the basolateral membranes of the columnar epithelial cells in the colon (Matsuzaki et al., 1999; Ramirez-Lorca et al., 1999). As indicated by Koyama et al. (1999), the presence of AQP-3 in the oesophagus may indicate a role for maintenance of wetness of the luminal surface of the epithelium. Also, the presence of AQP-3 on the basolateral membranes of cardiac gland cells in the lower stomach may be important in cell volume regulation during rapid changes in the osmolality of the gastric contents (Frigeri et al., 1995; Koyama et al., 1999).

The aim of this study was to investigate AQP-3 protein expression and to localise this putative water/urea/glycerol channel within the major osmoregulatory organs (gill and gastrointestinal tract) of the European eel, *Anguilla anguilla*. A double-labelling method was employed using an eel AQP-3 antibody along with a specific antibody raised against the β subunit of the eel Na^+,K^+-ATPase. Since the Na^+,K^+-ATPase is known to be localised within specialised branchial ion-transporting cells (chloride cells) and intestinal enterocytes in the eel (Cutler et al., 2000), the aim of this study was to determine whether AQP-3 is also colocalised within these cells. Although the movement of salt and water are inextricably linked, the main route for water transport (either paracellular or transcellular) has not yet been fully established in either the intestinal (Ando, 1975, 1985; Alves et al., 1999) or branchial (Isaia, 1984) epithelium. Experiments were therefore designed to localise the AQP-3 water channels in both the gill epithelium and the anterior, mid and posterior regions and rectal segment of the intestine in both FW and SW-acclimated eels.

Materials and methods

Animals

Adult freshwater 'silver' eels were obtained from local fish retailers in Inverness, Blairgowrie and Kelso and transferred to laboratory aquariums at the Gatty Marine Laboratory, St Andrews, Scotland. Eels were unfed and maintained at ambient temperature (5–10 °C) in aerated natural freshwater (FW: 0–10 mOsm kg^{-1}) or seawater (SW: 960–1020 mOsm kg^{-1}). A 12 h:12 h light:dark photoperiod was maintained. Fish were decapitated and pithed before removal of tissues.

Antibody production

A region of the derived amino acid (aa) sequence of the eel AQP-3, which was located at the carboxyl end of the sequence and shared the lowest level of homology with other aquaporins, was chosen for peptide manufacture (aa 915–929; Cutler and Cramb, 2002). An amino-terminal cysteine was added to the peptide for the purposes of conjugation to the carrier protein. This 16-mer (aa sequence: CDERIKLSNVATKDAA) was manufactured, coupled to keyhole limpet haemocyanin (KLH) and used to raise AQP-3-specific polyclonal antisera in rabbits (Pepceuticals, Ltd, Leicester, UK). A second eel-specific polyclonal antserum (β233) raised in sheep against the Na^+,K^+-ATPase β subunit isoform, β233 (Cutler et al., 2000) was also used for labelling and identification of chloride cells in the branchial epithelium and the enterocytes in the intestine.

Western blotting

Gill arches and the intestine were quickly removed from FW and 3-week SW-acclimated eels. Epithelial layers were then scraped free of the underlying tissue and thoroughly homogenized in 10 vol. (w:v) ice-cold sample buffer [25 mmol l^{-1} Hepes, 0.25 mol l^{-1} sucrose, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} dithiothreitol (DTT), 0.18 mg ml^{-1} phenylmethyl sulphonyl fluoride, pH 7.4]. Plasma membrane fractions were isolated by discontinuous sucrose density gradient centrifugation as described previously (McCartney and Cramb, 1993). After centrifugation, membrane fractions banding at the 35–43 % (w:v) sucrose interface were collected, washed, and finally resuspended in 25 mmol l^{-1} Hepes, 0.25 mol l^{-1} sucrose and 0.5 mmol l^{-1} DTT, pH 7.4, before freezing in portions at –25 °C. Protein concentrations were determined by the method of Bradford (1976) and western blotting was conducted using standard techniques (Hames, 1996). In brief, membrane samples (25 μg protein) were solubilised and denaturated by incubation at 100 °C for 10 min in a buffer comprising 62.5 mmol l^{-1} Tris-HCl, 10 % glycerol, 2 % SDS and 45 mmol l^{-1} β-mercaptoethanol, pH 6.8, and denaturated proteins were separated by SDS-PAGE using 7 % acrylamide gels (Laemmli, 1970).

Proteins were electroblotted onto PVDF membranes and immediately processed for immunodetection at room temperature. After blocking the membranes for 1 h with PBS buffer containing 2.5 % BSA (to block non-specific binding sites), the membranes were incubated for 1 h with AQP-3

primary antibody diluted (1:500) in PBS containing 1 % BSA. Control blots were also run simultaneously using equivalent dilutions of either pre-immune serum or immune serum pre-incubated for 1 h at room temperature with 50 μg ml^{-1} of the peptide antigen (peptide-negated antiserum). Following washes (3× 15 min) in PBS containing 0.1 % BSA, membranes were incubated for 2 h with an alkaline phosphatase-conjugated secondary antibody diluted 1:3000 in PBS. Bound antibodies were visualized by incubating the blots in a substrate (Western Blue® substrate for alkaline phosphatase, Promega) for 1 min at room temperature. The level of immunoreactivity was then measured as peak intensity (arbitrary units) using an image capture and analysis system (Genesnap/Genetools Image Analysis, Syngene, Cambridge, UK).

Immunofluorescence light microscopy

Gill, intestinal and rectal tissues were fixed for 24 h in either Bouin's fixative or 4 % paraformaldehyde (PFA). Specimens were fully dehydrated in a graded ethanol series and embedded in paraffin. Sagittal sections (3 μm) were cut on a Leitz Wetzlar microtome and collected on poly-L-lysine-coated slides. The technique for the immunocytochemical identification of AQP-3 in eel tissues was as described previously (Lignot et al., 1999). Sections were preincubated at room temperature for 10 min in 0.01 mmol l^{-1} Tween 20, 150 mmol l^{-1} NaCl in 10 mmol l^{-1} phosphate buffer, pH 7.3, and then treated with 50 mmol l^{-1} NH_4Cl in PBS, pH 7.3, for 5 min to reduce background associated with free aldehyde groups of the fixative. The sections were washed in PBS (1× 5 min) and incubated for 10 min with a blocking solution (BS) containing 1 % bovine serum albumin (BSA) and 0.1 % gelatin in PBS. Droplets (10 μl) of primary antibody diluted (1:100) in BS were placed on the sections and incubated for 1 h at room temperature in a wet chamber. After being washed in BS (6× 5 min), the sections were incubated for 2 h in droplets of secondary antibody [1:200; FITC-conjugated donkey anti-rabbit $IgG_{H\&L}$ (Jackson ImmunoResearch)]. Following extensive washes in BS (3× 5 min) and in PBS (3× 5 min), sections were mounted with anti-bleaching mounting medium (Sigma). Sections were then examined with a fluorescence microscope (Leitz Dialux 20 coupled to a Ploemopak 1-Lambda lamp) equipped with the appropriate filter set (450–490 nm band-pass excitation filter) and a phase-contrast device. The procedure was similar for the control sections, which were incubated with the pre-immune serum at the same dilution as for the primary antibody.

Confocal laser scanning microscopy

Specimen preparation was similar to the one used for the immunofluorescence light microscopy (see above). Branchial, intestinal and rectal sections were then incubated for 1 h at room temperature in a wet chamber in droplets containing both the AQP-3 antibody raised in rabbit and the eel β233 antibody raised in sheep, each at its optimal dilution in BS (1/100 and 1/50, respectively). The β233 antibody was used as a cellular marker as it specifically labels the β subunit of the Na^+,K^+-ATPase that is expressed at high levels in gill chloride cells and intestinal enterocytes (Cutler et al., 2000). After being washed in BS (6× 5 min), the sections were incubated for 2 h in droplets of mixed FITC- and Cy3-conjugated secondary antibodies [1:200; donkey anti-rabbit $IgG_{H\&L}$ and 1:200; donkey anti-sheep $IgG_{H\&L}$ (Jackson ImmunoResearch)]. Following washes in BS (3× 5 min) and then in PBS (3× 5 min), sections were mounted with anti-bleaching mounting medium (Sigma). Appropriate controls indicating no cross-reaction of the secondary antibodies were carried out in parallel.

A confocal laser scanning microscope (Biorad MRC 600) equipped with a krypton/argon laser and a Nikon Diaphot microscope (objectives: 20×, 0.75 numerical aperture and 60×, 1.4 numerical aperture, oil immersion) was used in combination with excitation by blue light (488 nm), a 515 nm emission barrier filter (BHS) and an A2 filter (blocking emission wavelengths below 600 nm). With this set-up the emission wavelengths of Cy3- and FITC-conjugate are separated and transmitted to different photomultipliers. The pictures from each photomultiplier were subsequently merged in false colour to visualise the labels simultaneously (green colour: FITC-conjugates; red colour: Cy3-conjugates).

Transmission electron microscopy

Gill samples from SW-acclimated eels were fixed on ice for 1–2 h with 2.5 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.5. They were post-fixed in 1 % osmium tetroxide in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.5, for 1 h at 4 °C, fully dehydrated in a graded ethanol series and infiltrated with LR-White (Agar Scientific), which was polymerised for 24 h at 60 °C. Ultrathin sections were cut on a Leica ultramicrotome and collected on Formvar-coated nickel grids. Sections were stained with 1 % uranyl acetate (30 min) and Reynolds lead citrate (3 min) and observed on a Philips EM 301 transmission electron microscope.

Immuno-gold electron microscopy

A post-embedding immunostaining technique on LR White sections was applied, as previously described (Lignot et al., 1999). Gill samples from SW-acclimated silver eels were fixed for 1–2 h with 0.5 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.5, adjusted to plasma osmolality (390 mOsm kg^{-1}) by addition of NaCl to avoid osmotic shock, fully dehydrated in ethanol, infiltrated with LR-White, and polymerized for 18 h at 50 °C. Ultrathin sections were cut on a Reichert TM60 ultramicrotome and collected on Formvar-coated nickel grids. Selected grids were placed on 5 % gelatin containing 50 mmol l^{-1} glycine, pH 7.3. The grids were successively preincubated on droplets of 50 mmol l^{-1} glycine in PBS (1× 5 min) and 1 % BSA-PBS (3× 5 min). The grids were then transferred to droplets of AQP-3 antibody diluted to 1:25 with BSA-PBS and incubated for 2 h at room temperature in a wet chamber. The sections were washed in BSA-PBS (6× 5 min) and incubated for 1 h in droplets of 15 nm gold-

conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). After washing in 1% BSA-PBS (3× 5 min) and PBS (3× 5 min), sections were stained with 1% uranyl acetate (30 min) and Reynolds lead citrate (3 min) and studied using a Philips EM 301 electron microscope. For the controls, the procedure was similar but the grids were incubated with the pre-immune serum.

Results

Western blotting

Western blots using the AQP-3-specific antiserum and plasma membrane fractions from the gill of both FW and SW-acclimated eels resulted in the appearance of a strongly staining band of approximately 24 kDa (Fig. 1A–F). The immunoreactive protein ran at a smaller size than the expected molecular mass calculated from the derived aa sequence (approximately 30 kDa). In addition, a number of minor staining diffuse bands of higher molecular mass were also observed between 30 and 35 kDa. These results are similar to those reported for other mammalian aquaporins with the higher molecular mass species being differentially glycosylated forms of the protein (Calamita et al., 2001). A difference in immunoreactive intensity was also observed between the gills of FW and SW-acclimated silver eels, the relative absorbance of the 24 kDa bands being threefold higher in FW eels (Figs 1D–F, 2) compared to the SW-acclimated fish (Figs 1A–C, 2). Incubation of membranes with pre-immune serum revealed no equivalent staining (Fig. 1G) and immunoreactivity also was lost after pre-incubation of the antibody with the synthetic peptide (Fig. 1H).

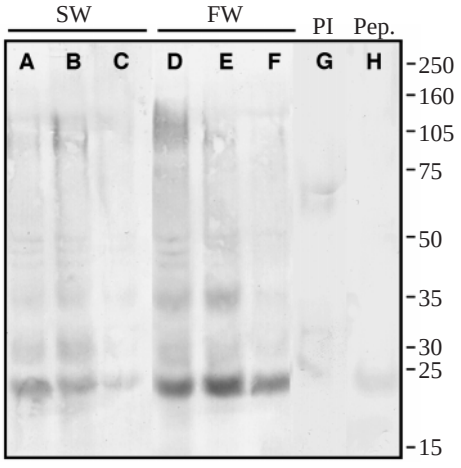

Fig. 1. Western blot analysis of aquaporin 3 (AQP-3) in branchial tissues of silver eels maintained in freshwater (FW) or acclimated for 3 weeks to a seawater (SW) environment. The blot shows AQP-3 immunoreactivity in plasma membrane fractions isolated from the gills of SW-acclimated eels (lanes A–C) and FW eels (lanes D–F). Controls include the use of both pre-immune serum (FW eel membranes; PI, lane G) and peptide-negated antiserum (FW eel membranes; Pep., lane H), see Materials and methods. Individual lanes represent protein samples from separate membrane preparations from each experimental group. Gill tissue was removed from two fish for each membrane preparation. Size markers (right) are in kDa.

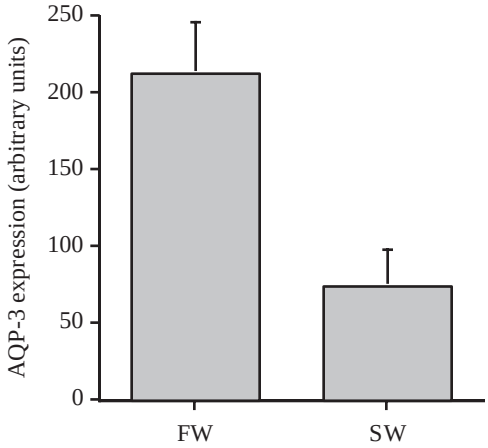

Fig. 2. Quantitative analysis of branchial AQP-3 protein expression in freshwater (FW) and 3-week seawater (SW)-acclimated silver eels. Values are means ± S.D., derived from three different preparations each using two fish.

Immunofluorescence light microscopy

Bouin and/or 4% PFA fixation and paraffin embedding procedures yielded good antigenicity and good structural preservation (Figs 3, 4). In the gill, although AQP-3-specific staining was apparent throughout the epithelium, the chloride cells within the primary filaments and at the proximal ends of secondary lamellae in SW-acclimated eels (Fig. 3A,B) and along both the primary filaments and secondary lamellae in FW fish, exhibited much stronger immunoreactivity (Fig. 3D,E). Although chloride cells were present throughout the branchial epithelium, a much higher cell density, and therefore immuno-positive staining, was found near the trailing edge of the filaments (results not shown). Immunoreactivity was present throughout the entire chloride cell although heavier staining was always noticeable towards the apical surface (Fig. 3A,B,D,E). A much lighter general staining was also observed throughout the other epithelial cells where immunoreactivity was predominantly located towards the cell periphery, indicating that AQP-3 was mainly localised on or very close to the plasma membrane. In FW eels, cells near the central cavity of the primary filament epithelium (Fig. 3G) and basal layer cells within the gill arch epithelium (Fig. 3H) exhibited strong immunoreactivity near the plasma membrane. This staining was present but not as intense in the SW-acclimated group of fish (results not shown). In both FW and SW-acclimated fish, controls using pre-immune serum showed no positive immunoreactivity with only non-specific auto-fluoresence being restricted to red blood cells (Fig. 3C,F).

In the intestinal epithelium of FW and SW-acclimated eels, neither the numerous goblet cells, nor the columnar enterocytes with their apical brush border, stained with the AQP-3

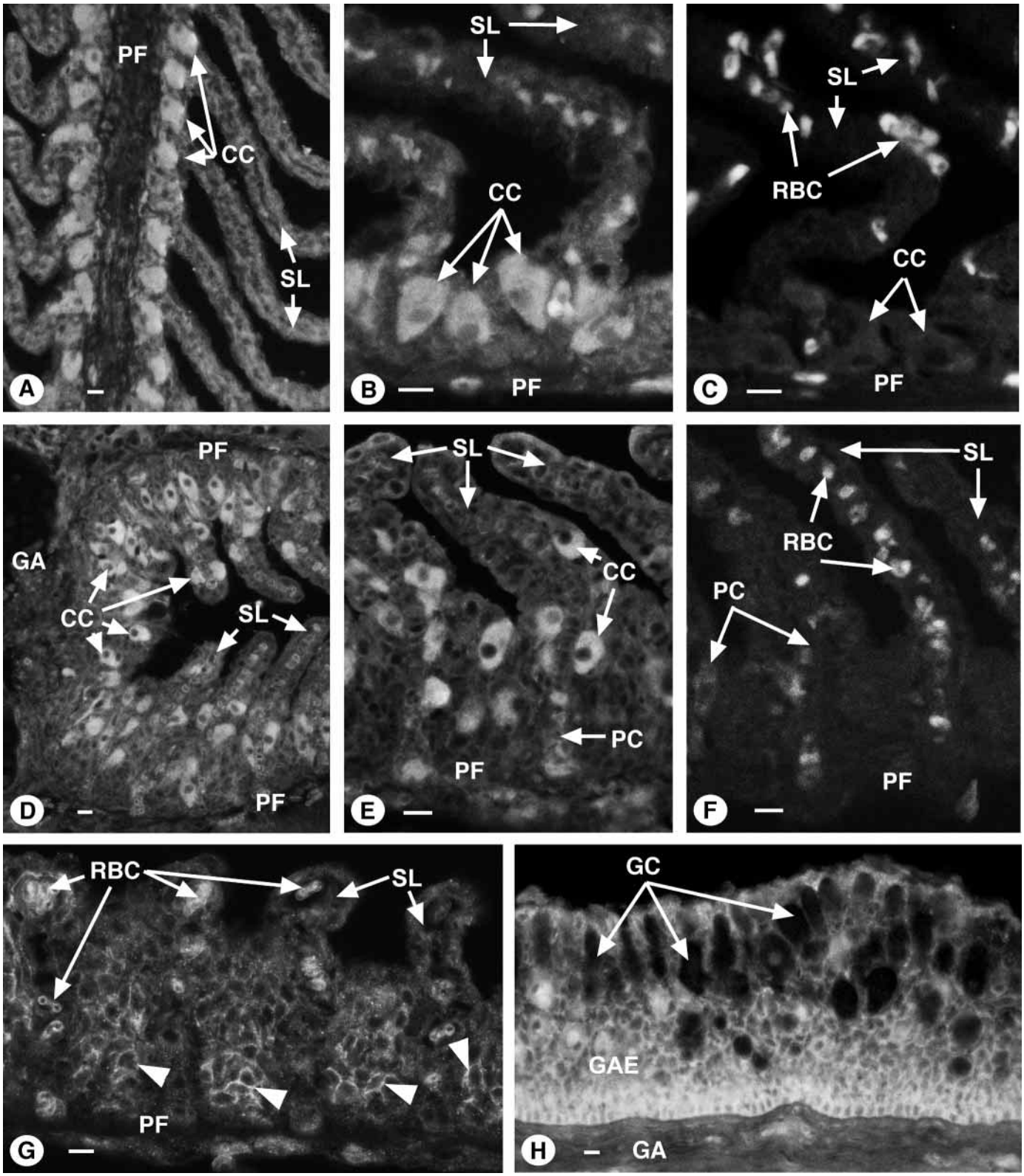

Fig. 3. Immunolocalisation of AQP-3 in the gill. Typical immuno-histochemistry of the branchial epithelium in 3-week seawater (SW)-acclimated (A–C) and freshwater (FW; D–H) silver eels. (C,F) Control sections incubated with pre-immune serum. PF, primary filaments; SL, secondary lamellae; GA, gill arch; GAE, gill arch epithelium; CC, chloride cell; GC, goblet cell; PC, pillar capillary; RBC, red blood cell. Bars, 10 μm.

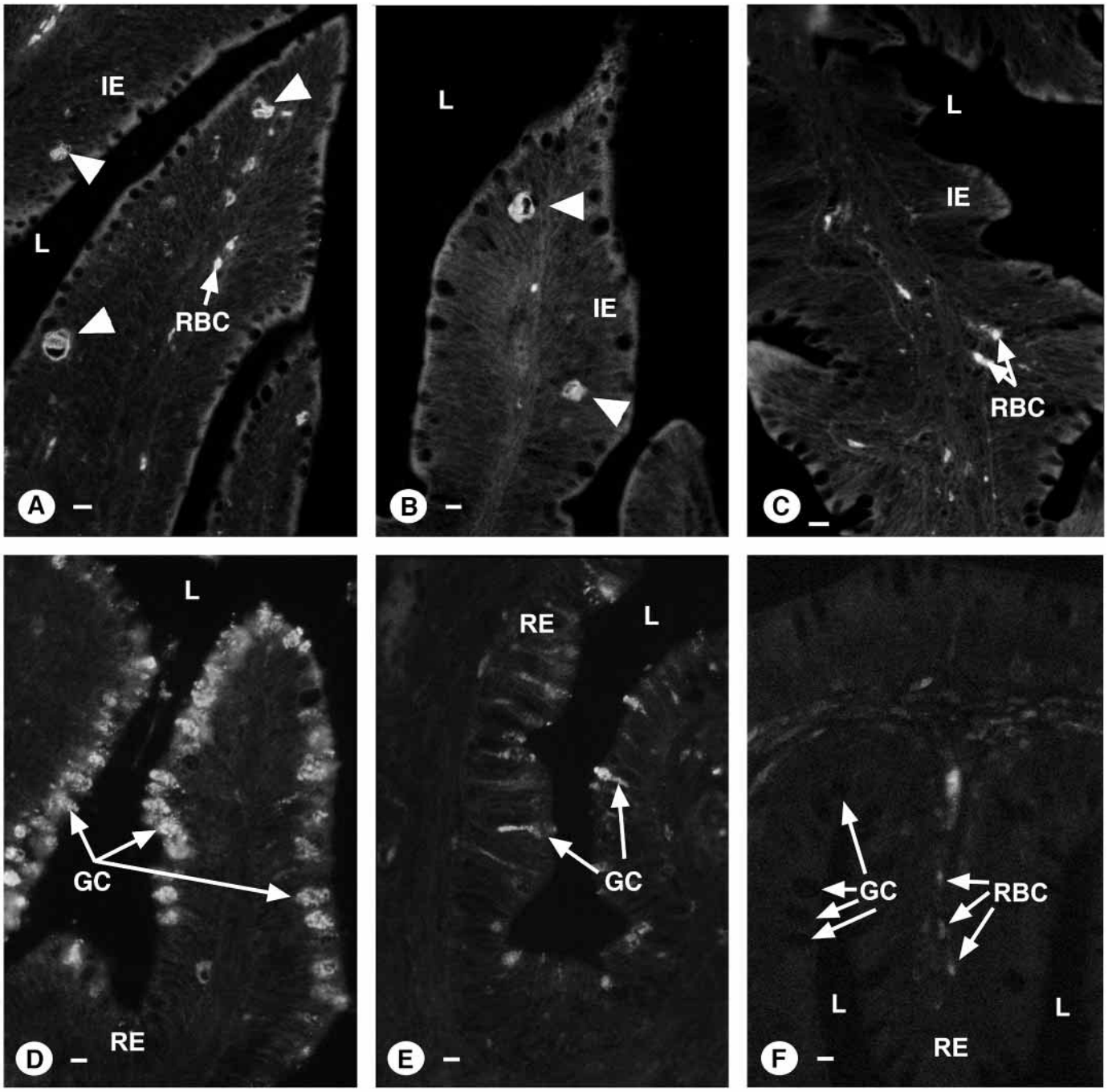

Fig. 4. Immuno-localisation of AQP-3 in the intestinal and rectal epithelia. Typical immuno-histochemistry of both the intestinal (A–C) and rectal (D–F) epithelium of 3-week seawater (SW)-acclimated (A,D,F) and freshwater (FW; B,C,E) silver eels. (C,F) Control sections were incubated with pre-immune serum. IE, intestinal epithelium; RE, rectal epithelium; L, lumen; GC, goblet cell; RBC, red blood cell. Bars, 10 μm.

antibody (Fig. 4A,B). Immunoreactivity was only found in large macrophage-like bodies found throughout the entire length of the intestinal epithelium of FW silver eels and primarily within the anterior intestine of SW-acclimated eels (Fig. 4A,B). The precise nature of these cells awaits further investigation. In the rectal epithelium of SW-acclimated eels, copious staining was also observed within the goblet cells (Fig. 4D). In FW eels, however, only a few goblet cells presented positive staining (Fig. 4E). In both FW and SW-acclimated fish, control pre-immune serum showed no positive immunofluorescence within the digestive tract, with only non-specific auto-fluorescence again restricted to red blood cells (Fig. 4C,F).

Confocal laser scanning microscopy (CLSM)

Fig. 5 shows CLSM scans of the double-labelled gill,

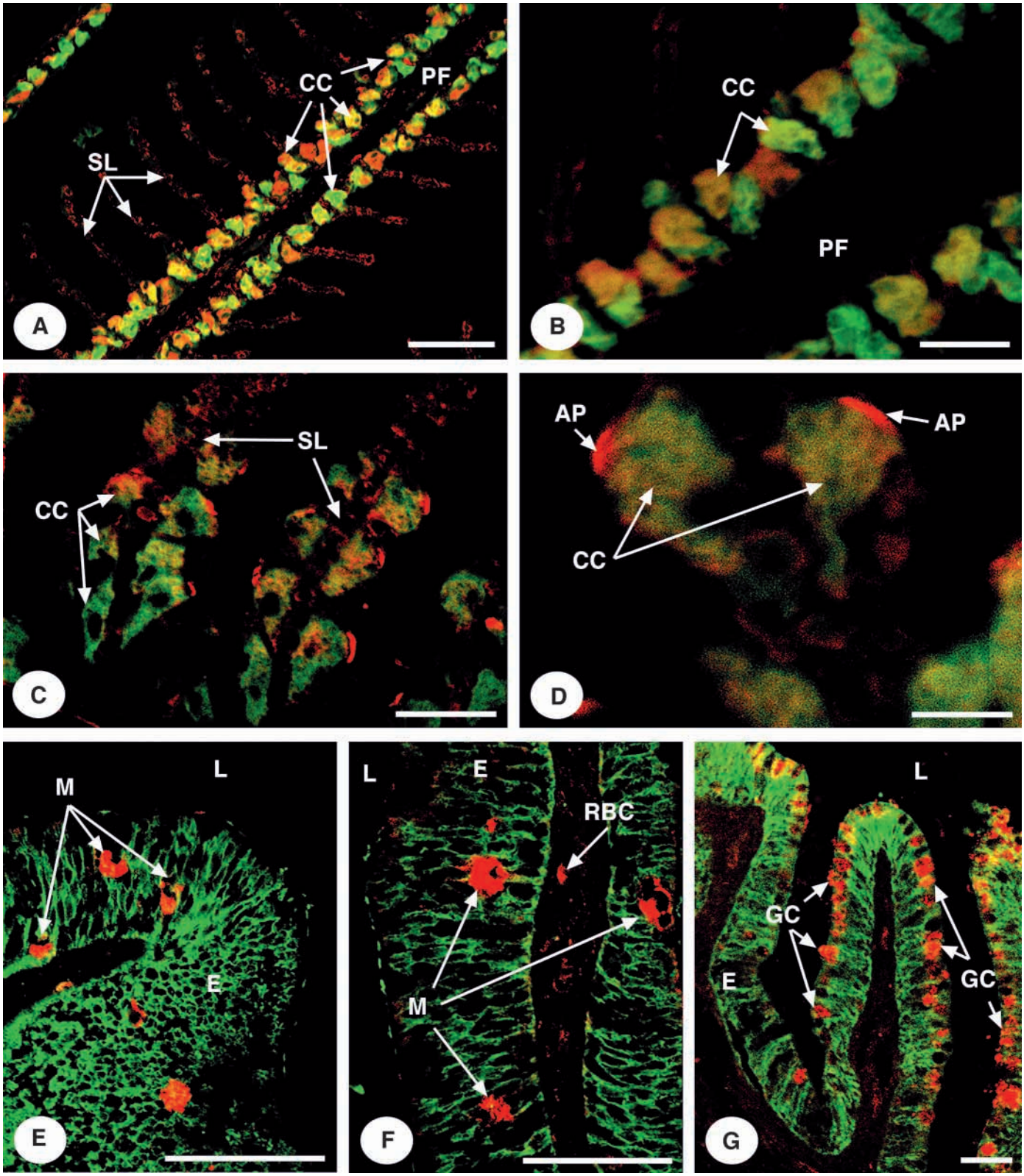

Fig. 5. Confocal laser scanning microscopy: dual immuno-localisation of AQP-3 (false colour: red) and Na^+, K^+-ATPase (β233 subunit) (false colour: green) in thin sections. Gill primary and secondary filaments of 3-week seawater (SW)-acclimated (A,B) and freshwater (FW; C,D) silver eels. Intestinal sections of SW-acclimated (E) and FW (F) silver eels. Rectal epithelium of a SW-acclimated silver eel (G). PF, primary filaments; SL, secondary lamellae; E, gut epithelium; L, gut lumen; CC, chloride cell; GC, goblet cell; M, macrophage-like body; RBC, red blood cell; AP, apical pit. Bars, 50 μm (A,E,F,G); 25 μm (B,C); 10 μm (D).

intestinal and rectal epithelia of FW and SW-acclimated eels. Intense staining, from both β233 and AQP-3 antibodies, was restricted to the chloride cells of FW and SW-acclimated eels (Fig. 5A–D). Staining corresponding to the β233 subunit of the Na^+,K^+-ATPase was restricted to the region containing the basolateral tubular network of the chloride cell (see below) but was much reduced towards the apical surface and completely absent around the apical pit region (Fig. 5A–D). In contrast, however, the AQP-3 antibody exhibited immunoreactivity throughout most of the chloride cell with increased intensity near the apical pit region, where β233 immunoreactivity was absent (Fig. 5B–D). In the intestine of both FW and SW-acclimated eels, β233 immunoreactivity was observed along the cell surface of the enterocytes (Fig. 5E,F) while AQP-3 immunoreactivity was restricted to large macrophage-like cells, which were randomly distributed within the columnar epithelium (Fig. 5E,F). Finally, in the rectum of SW-acclimated eels, while the β233 was detected within the

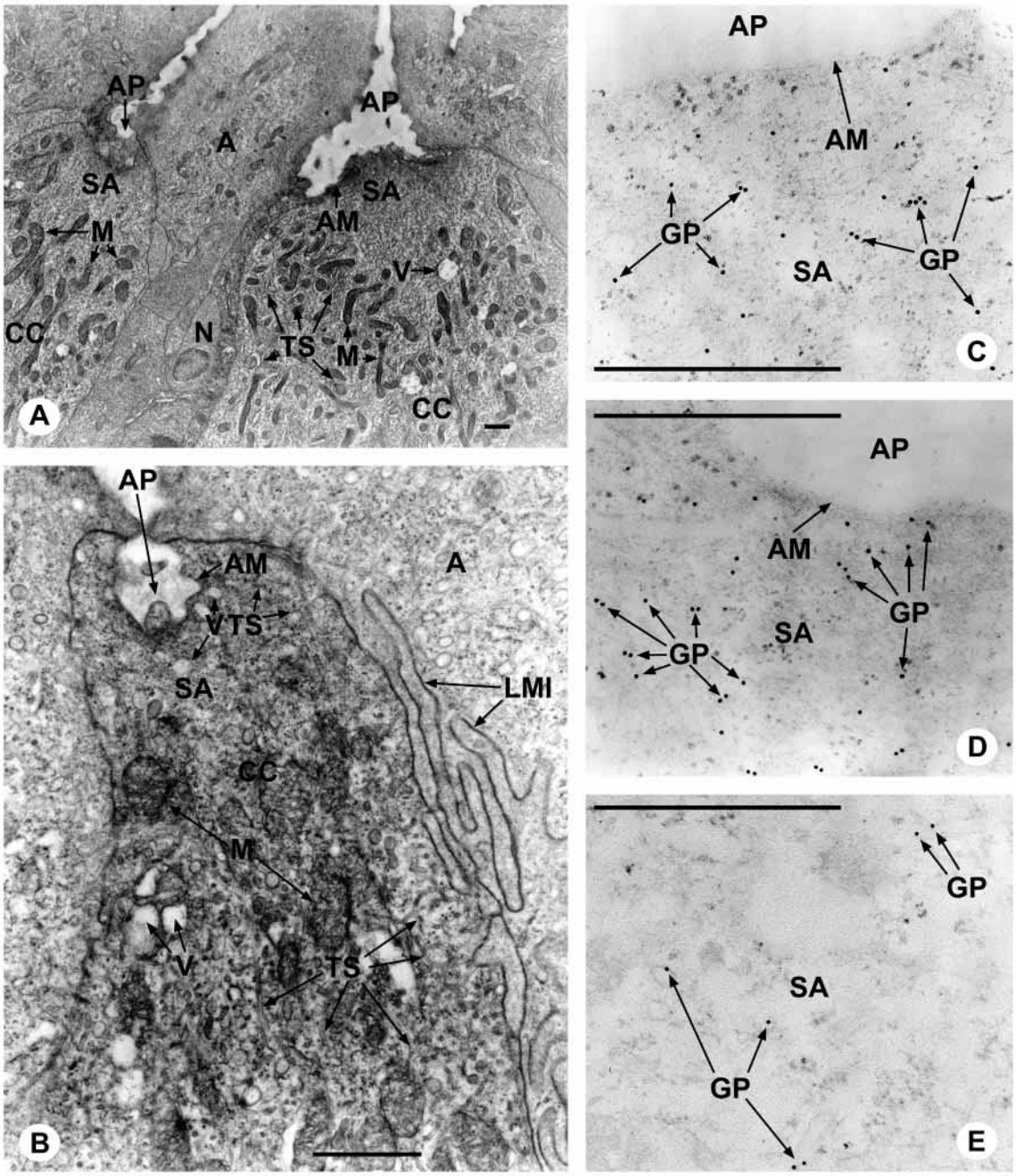

Fig. 6. Transmission electron micrograph (A,B) and immuno-gold localisation of AQP-3 (C–E) in chloride cells of the branchial epithelium of seawater (SW)-acclimated silver eels. (E) Control section incubated with pre-immune serum. CC, chloride cell; AM, apical membrane; AP, apical pit; M, mitochondria; N, nucleus; PC, pavement cell; TS, tubular system; V, vacuole; VTS, vesiculo-tubular system; A, accessory cell; SA, sub-apical region; GP, gold particles (15 nm). Bars, 1 μm.

enterocytes, AQP-3 was almost entirely confined to the goblet cells (Fig. 5G).

Transmission and immuno-gold electron microscopy

In gill chloride cells from the SW-acclimated eel, mitochondria are evenly distributed throughout the cell except for the area around the apical pit (Fig. 6A,B). An extensive smooth-surfaced tubular system is juxtaposed to the mitochondria (Fig. 6A). This tubular system forms a network from the basal to the apical part of the cell. Just below the flat or rounded apical pit, numerous small densely packed vesicles form a tubulo–vesicular system underneath the apical membrane (Fig. 6B).

On gold labelled sections, gold particles were predominantly localized in chloride cells within membranes of this apical tubulo–vesicular system and in the subjacent baso–lateral tubular network (Fig. 6C, 6D). Control sections using pre-immune serum exhibited only low levels of non-specific staining (Fig. 6E).

Discussion

During the final adult stages of the life cycle of the European eel *Anguilla anguilla*, 'yellow' eels that have grown and lived in a FW environment for several years undergo a metamorphosis and migrate as 'silver' eels back to the marine environment to breed in the Sargasso sea. Both life stages can survive either in SW or FW after acute transfer. The European eel therefore is an excellent model for the study of the mechanisms controlling the acquisition, retention and excretion of water by osmoregulatory tissues. As with most euryhaline teleosts, yellow and silver eels are able to modify the ion and water permeability of osmoregulatory surfaces following environmental salinity change, the gills and the digestive tract being the major sites of water exchange between the internal milieu and the external environment.

The leakiness of the branchial epithelium is considered to account for over 90 % of the total body water influx in FW fish (Motais et al., 1969; Haywood et al., 1977). Although the diffusional water permeability is not radically different between FW and SW-acclimated euryhaline teleosts, the osmotic water permeability is generally higher in FW fish (Isaia, 1984). The gut of marine teleosts also plays an essential part in compensating for the osmotic water loss through the gills, the oesophagus and anterior intestine being the main areas in processing the ingested seawater (Kirsch and Meister, 1982). Seawater is processed along the gut in two steps: essentially ion diffusion with little net water uptake across the oesophagus (Kirsch and Laurent, 1975; Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983; Simmonneaux et al., 1988), followed by active NaCl transport coupled to water absorption in the intestine (House and Green, 1965; Skadhauge, 1969, 1974; Field et al., 1978; Frizzell et al., 1984).

Aquaporin 3 (AQP-3), known to be a key water channel protein in mammals, has been identified in both FW and SW-acclimated silver eels using a specific polyclonal antibody directed against the C-terminal of the eel protein. Western blotting has revealed that the AQP-3 protein is predominantly expressed within the gill epithelium. SW acclimation resulted in a reduction of approximately 65 % in the expression of the branchial protein, suggesting that AQP-3 may play an important functional role in this osmoregulatory organ. Immunohistochemical studies indicated a qualitatively similar amount of AQP-3 staining within chloride cells from both FW and SW-acclimated fish. However, the results clearly demonstrated that gills from FW fish exhibited elevated expression of the protein in epithelial cells deep within the primary filaments and near the branchial arch, where the epithelial layers appeared much thicker than in the SW-acclimated fish. This staining predominated close to the plasma membrane of the cells and particularly on the serosal side of the epithelium. This staining, which was greatly reduced within the gills of SW-acclimated eels, could at least partially explain the marked downregulation of AQP-3 protein expression following SW transfer. These localisation studies also suggest that water movement *via* AQP-3 may occur across the serosal membrane into the epithelial cells in order to protect the cells from dehydration. Such a role for APQ-3 in fish epithelium could therefore be similar to that suggested for the same protein that is expressed in rat skin and urinary bladder epithelia (Matsuzaki et al., 1999, 2000). This contention is further strengthened by reports that AQP-3 expression can be upregulated by increasing the osmolality of the extracellular medium (Matsuzaki et al., 2001). AQP-3 water channel expression was also observed within the chloride cells of both FW and SW-acclimated eels, where it was co-expressed along with the β233-isoform of the Na^+,K^+-ATPase. AQP-3 immunoreactivity was also abundant towards the apical surface and particularly around the apical pit of the chloride cells, where Na^+,K^+-ATPase is absent. This apical location for AQP-3 in the chloride cells was unexpected, as in mammals AQP-3 is mainly expressed in the basolateral membranes of both the renal collecting duct cells and of epithelial cells lining the villus tip of the small intestine and colon (Frigeri et al., 1995; Ramirez-Lorca et al., 1999; Koyama et al., 1999).

The presence of high levels of expression of AQP-3 in the chloride cells correlates with physiological and morphological evidence indicating that these cells provide a major route for water as well as ion movement in SW (Isaia, 1984; Ogasawara and Hirano, 1984). The immunohistochemical localisation of AQP-3 suggests a possible association with the baso–lateral tubular network of the chloride cells, and this could be related to the osmotic water flux pathway that has been hypothesised to be operating in the system (Isaia, 1984). The possible location of AQP-3 within the tubulo–vesicular system surrounding the apical pit region also suggests that another regulated water pathway may occur within the chloride cells of both FW and SW-acclimated fish. The tubulo–vesicular system situated between the apical plasma membrane of the chloride cells and the tubular reticulum has already been hypothesized as a transient communication channel between the internal and external milieu (Sardet et al., 1979).

Another potential physiological role for AQP-3 in the branchial chloride cells is in the excretion of nitrogenous waste products. Many studies have shown that the AQP-3 isoform can be associated with the transport of small polar solutes such as urea and glycerol (Deen and van Os, 1998; Borgnia et al., 1999; Verkman and Mitra, 2000). In teleost fish, nitrogenous waste products such as urea and ammonia can be excreted by extra-renal routes, including the gill (Masoni and Payan, 1974; Isaia, 1984). In the ammoniotelic European eel, branchial and renal excretion of urea occur at the same rate and branchial urea excretion is threefold lower in SW compared to FW-acclimated eels (Masoni and Payan, 1974). Branchial urea clearance in ureotelic fish species is believed to take place through vesicular trafficking in pavement cells (Laurent et al., 2001). Ammoniotelic teleost fish, however, do not show such vesicular trafficking (Laurent et al., 2001), with branchial urea clearance believed to take place through the chloride cells (Masoni and Garcia-Romeu, 1972). The presence of AQP-3 in the baso–lateral network and within the apical vesiculo–tubular network of the chloride cells could therefore be related to the clearance of this nitrogenous waste product.

In the eel intestine, increased water absorption is observed after increasing the external salinity (Maetz and Skadhauge, 1968). Considerable ingestion of seawater occurs at the moment of transfer (Kirsch, 1972; Kirsch and Mayer-Gostan, 1973), which is suggestive of a drinking reflex that is possibly linked to the increase in chloride concentration and/or associated local cellular dehydration within the buccal cavity (Hirano, 1974; Ando and Nagashima, 1996). The osmotic water permeability of the intestine increases by two- to sixfold following SW transfer, with the highest water fluxes occurring across the mid region of the gut followed by the posterior, anterior and rectal regions, respectively (Ando and Kobayashi, 1978; Ando, 1980). The very low levels of AQP-3 protein expression observed in western blots when using intestinal extracts from both FW and SW-acclimated eels correlates with the relatively low AQP-3 mRNA expression observed in this tissue (Cutler and Cramb, 2002). Expression of mRNA and protein within the intestine is mainly associated with the positive immunoreactivity discretely localised within intra-epithelial macrophages, which were distributed throughout the intestinal epithelia, and to the goblet cells, which were mainly located near the rectum. The lack of AQP-3 expression in the eel intestinal columnar cells, however, agrees with recent studies which suggested that there was an absence of water channels in eel brush border membrane vesicles (Alves et al., 1999). However, it is highly likely that other AQP homologues are present within the teleost intestinal epithelia. In mammals a number of aquaporin isoforms, including AQP-1, AQP-4, AQP-7 and AQP-8, have been characterised in the small intestine (Nielsen et al., 1993; Kuriyama et al., 1997; Koyama et al., 1999; Ma and Verkman, 1999; Elkjaer et al., 2001). Finally, the strong expression of AQP-3 protein in the rectal goblet cells of SW-acclimated eels suggests a major role for this protein in water trafficking associated with mucus secretion. A role for maintenance of ‘wetness’ on the luminal surface of the rectal epithelium can also be hypothesised, as speculated for AQP-3 in the rat oesophagus (Koyama et al., 1999) and for AQP-5 in corneal squamous epithelial cells in the eye (Funaki et al., 1998).

In conclusion, this study indicates that the AQP-3 protein is expressed in osmoregulatory tissues of teleost fish. The cellular localisation in branchial chloride cells indicates that AQP-3 could be involved in water transport and/or with nitrogenous waste excretion hypothesised to be operating in the baso–lateral tubular and apical vesiculo–tubular systems of chloride cells. The presence of AQP-3 in the basal cells of the gill arch epithelium and of the gill primary filaments of FW-acclimated eels also suggests regulated water movement within or across other epithelial cells, particularly those associated with the serosal side of the gill epithelium. AQP-3, however, is unlikely to play any major role in the intestinal water absorption that occurs following the drinking response in SW-acclimated fish. Furthermore, the expression of AQP-3 within the rectal mucus cells may be associated with the maintenance of mucus layer fluidity in the rectum.

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References

Alves, P., Soveral, G., Macey, R. I. and Moura, T. F. (1999). Kinetics of water transport in eel intestinal vesicles. *J. Membr. Biol.* **171**, 177–182.

Ando, M. (1975). Intestinal water transport and chloride pump in relation to sea-water adaptation of the eel, *Anguilla japonica*. *Comp. Biochem. Physiol.* **52A**, 229–233.

Ando, M. and Kobayashi, M. (1978). Effects of stripping of the outer layers of the eel intestine on salt and water transport. *Comp. Biochem. Physiol.* **61A**, 497–501.

Ando, M. (1980). Chloride-dependent sodium and water transport in the seawater eel intestine. *J. Comp. Physiol.* **138B**, 87–91.

Ando, M. (1983). Potassium-dependent chloride and water transport across the seawater eel intestine. *J. Membr. Biol.* **73**, 125–130.

Ando, M. (1985). Relationship between coupled Na^+-K^+-Cl^- transport and water absorption across the seawater eel intestine. *J. Comp. Physiol.* **155B**, 311–317.

Ando, M. and Nagashima, K. (1996). Intestinal Na^+ and Cl^- levels control drinking behavior in the seawater-adapted eel *Anguilla japonica*. *J. Exp. Biol.* **199**, 711–716.

Ando, M., Kondo, K. Y. and Takei, Y. (1992). Effects of eel atrial natriuretic peptide on NaCl and water transport across the intestine of the seawater eel. *J. Comp. Physiol.* **162B**, 436–439.

Borgnia, M., Nielsen, S., Engel, A. and Agre, P. (1999). Cellular and molecular biology of the aquaporin water channels. *Annu. Rev. Biochem.* **68**, 425–458.

Bradford, M. (1976). A rapid and sensitive assay of protein utilizing the principle of dye binding. *Anal. Biochem.* **772**, 248–264.

Calamita, G., Mazzone, A., Cho, Y. S., Valenti, G. and Svelto, M. (2001) Expression and localization of the aquporin-8 water channel in rat testis. *Biol. Reprod.* **64**, 1660–1666.

Cutler, C. P. and Cramb, G. (2000). Water transport and aquaporin expression in fish. In *Molecular Biology and Physiology of Water and Solute Transport* (ed. S. Hohmann and S. Neilsen), pp. 433–441. New York, London: Kluwer Academic/Plenum Publishers.

Cutler, C. P. and Cramb, G. (2002). Branchial expression of an Aquaporin 3 (AQP-3) homologue is down-regulated in the European eel (*Anguilla anguilla*) following seawater acclimation. *J. Exp. Biol.* **205**, 2643–2651.

Cutler, C. P., Brezillon, S., Bekir, S., Sanders, I. L., Hazon, N. and Cramb,

G. (2000). Expression of a duplicate Na^+,K^+-ATPase β1 isoform in the European eel (*Anguilla anguilla*). *Am. J. Physiol.* **279**, R222–R229.

Deen, P. M. T. and van Os, C. S. (1998). Epithelial aquaporins. *Curr. Opin. Cell Biol.* **10**, 435–442.

Elkjaer, M. L., Nejsum, L. N., Gresz, V., Kwon, T. H., Jensen, U. B., Frokiaer, J. and Nielsen, S. (2001). Immunolocalization of aquaporin-8 in rat kidney, gastrointestinal tract, testis, and airways. *Am. J. Physiol.* **281**, F1047-F1057

Evans, D. H., Piermarini, P. M. and Potts, W. T. W. (1999). Ionic transport in the fish gill epithelium. *J. Exp. Zool.* **283**, 641–652.

Field, M., Karnaky, K. J., Smith, P. L., Bolton, J. E. and Kinter, W. B. (1978). Ion transport across the isolated intestinal mucosa of the winter flounder *Peudopleuronectes americanus*. I. Functinal and structural properties of cellular and paracellular pathways for Na and Cl. *J. Membr. Biol.* **41**, 265–293.

Frigeri, A., Grooper, M. A., Turck, C. W. and Verkman, A. S. (1995). Immunolocalisation of the mercurial-insensite water channel and glycerol intrinsic protein in epithelial cell plasma membranes. *Proc. Natl. Acad Sci. USA* **92**, 4328–4331.

Frizzell, R. A., Halm, D. R., Musch, M. W., Stewart, C. P. and Field, M. (1984). Potassium transport by flounder intestinal mucosa. *Am. J. Physiol.* **246**, F946–F951.

Funaki, H., Yamamoto, T., Kondo Y., Yaoita, E., Kawasaki, K., Kobayashi, H., Abe, H. and Kihara, I. (1998). Immunolocalization and expression of AQP5 in systemic organs. *Am. J. Physiol.* **44**, C1151–C1157.

Hames, B. D. (1996). One dimensional polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach*, second edition (ed. B. D. Hames and D. Rickwood), pp. 1–147. Oxford, UK: Oxford University Press.

Haywood, G. P., Isaia, J. and Maetz, J. (1977). Epinepherine effects on branchial water and urea flux in rainbow trout. *Am. J. Physiol.* **232**, R110–R268.

Hirano, T. (1974). Some factors regulating water intake by the eel, *Anguilla japonica*. *J. Exp. Biol.* **61**, 737–747.

Hirano, T. and Mayer-Gostan, N. (1976). Eel esophagus as an osmoregulatory organ. *Proc. Natl. Acad. Sci. USA* **73**, 1348–1350.

House, C. R. and Green, K. (1965). Ion and water transport in the isolated intestine of the marine telost *Cottus scorpius*. *J. Exp. Biol.* **42**, 177–189.

Isaia, J. (1984). Water and nonelectrolyte permeation. In *Fish Physiology*, vol. X. *Gills*; Part B, *Ion and water transfer* (ed. W. S. Hoar and D. J. Randall), pp. 1–38. New York, London: Academic Press.

Karnaky, K. J., Jr (1998). Osmotic and ionic regulations. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 157–176. Boca Raton, NY: CRC Press.

King, L. S., Nielsen, S. and Agre, P. (1997). Aquaporins in complex tissues. 1. Developmenal patterns in respiratory and glandular tissues of rat. *Am. J. Physiol.* **42**, C1541–C1548.

Kirsch, R. (1972). The kinetics of peripheral exchanges of water and electrolytes in the silver eel (*Anguilla anguilla L.*) in fresh water and in sea water. *J. Exp. Biol.* **57**, 489–512.

Kirsch, R. and Laurent, P. (1975). L'œsophage, organe effecteur del'osmorégulation chez un téléostéen euryhalin, l'anguille (*Anguilla anguilla* L.). *CR Acad. Sci. Paris* **280D**, 2013–2015.

Kirsch, R. and Mayer-Gostan, N. (1973). Kinetics of water and chloride exchanges during adaptation of the European eel to sea water. *J. Exp. Biol.* **58**, 105–121.

Kirsch, R. and Meister, M. F. (1982). Progressive processing of of ingested seawater in the gut of seawater teleosts. *J. Exp. Biol.* **98**, 67–81.

Koyama, Y., Yamamoto, T., Tani, T., Nihei, K., Kondo, D., Funaki, H., Yaoita, E., Kawasaki, K., Sato, N., Hatakeyama, K. and Kihara, I. (1999). Expression and localization of aquaporins in rat gastrointestinal tract. *Am. J. Physiol.* **276**, C621–C627.

Kuriyama, H., Kawamoto, S., Ishida, N., Ohno, I., Mita, S., Matsuzawa, Y., Matsubara, K. and Okubo, K. (1997). Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. *Biochem. Biophys. Res. Commun.* **241**, 53-58.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteiophage T4. *Natur*e **227,** 680–685.

Laurent, P., Wood, C. M., Yang, Y., Perry, S. F., Gilmour, K. M., Part, P., Chevalier, C., West, M. and Walsh, P. J. (2001). Intracellular vesicular trafficking in the gill epithelium of urea-excreting fish. *Cell Tissue Res.* **303**, 197–210.

Lee, M. D., King, L. S. and Agre, P. (1997). The aquaporin family of water channel proteins in clinical medicine. *Medicine* **76**, 141–156.

Lignot, J.-H., Charmantier-Daures, M. and Charmantier, G. (1999). Localization of the Na^+, K^+ ATPase in the branchial cavity of juvenile lobster, *Homarus gammarus* (Crustacea, Decapoda). *Cell Tissue Res.* **296**, 417–426.

Ma, T. and Verkman A. S. (1999). Aquaporin water channels in gastrointestinal physiology. *J. Physiol.* **517**, 317-326

Maetz, J. and Skadhauge, E. (1968). Drinking rates and gill ionic environment in relation to external salinities in the eel. *Nature* **217**, 371–373.

McCartney, S. and Cramb, G. (1993). Effects of high-salt diet on hepatic atrial natriuretic peptide receptor expression in Dahl salt-resistant and salt-sensitive rats. *J. Hypertens* **11**, 253–315.

Masoni, A. and Garcia-Romeu, F. (1972). Accumulation et excrétion de substances organiques par les cellules à chlorure de la branchie d'*Anguilla anguilla* L. adaptée à l'eau de mer. *Z. Zellforsch.* **133**, 389–398.

Masoni, A. and Payan P. (1974). Urea, inulin and para-amino-hippuric acid (PAH) excretion by the gills of the eel *Anguilla anguilla L*. *Comp. Biochem. Physiol.* **47A**, 1241–1244.

Matsuzaki, T., Suzuki, T., Koyama, H., Tanaka, S. and Takata, K. (1999). Water channel protein AQP-3 is present in epithelia exposed to the environment of possible water loss. *J. Histochem. Cytochem.* **47**, 1275–1286.

Matsuzaki, T., Suzuki, T. and Takata, K. (2000). Water channel protein, aquaporin 3, in epithelial cells. In *Molecular Biology and Physiology of Water and Solute Transport* (ed. S. Hohmann and S. Neilsen), pp. 167–171. New York, London: Kluwer Academic / Plenum Publishers.

Matsuzaki, T., Suzuki, T. and Takata, K. (2001). Hypertonicity-induced expression of aquaporin 3 in MDCK cells. *Am. J. Physiol.* **281**, C55–C63

Motais, R., Isaia, J., Rankin, J. C. and Maetz, J. (1969). Adaptive changes of the water permeability of the teleostean gill epithelium in relation to external salinity. *J. Exp. Biol.* **51**, 529–546.

Nielsen, S., King, L. S., Christensen, B. M. and Agre, P. (1997). Aquaporins in complex tissues. 2. Subcellular distribution in respiratory and glandular tissues of rat. *Am. J. Physiol.* **42**, C1549–C1561.

Ogasawara, T. and Hirano, T. (1984). Effects of prolactin and environmental calcium on osmotic water permeability of the gills in the eel, *Anguilla japonica*. *Gen. Comp. Endocrinol.* **53**, 315–324.

Parmelee, J. T. and Renfro, J. L. (1983). Esophageal desalination of seawater in flounder: role of active sodium transport. *Am. J. Physiol.* **245,** R888–R893.

Perry, S. F. (1997). The chloride cell: Structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol.* **59**, 325–347.

Pisam, M. (1981). Membranous systems in the 'chloride cell' of teleostean fish gill; their modifications in response to the salinity of the environment. *Anat. Rec.* **200**, 401–414.

Pisam, M., Prunet, P. and Rambourg, A. (1989). Accessory cells in the gill epithelium of the freshwater rainbow trout *Salmo gairdneri*. *Am. J. Anat.* **184**, 311–320.

Potts, W. T. W. (1984). Transepithelial potentials in the fish gills. In *Fish Physiology*, vol. X, *Gills*; part B, *Ion and water transfer* (ed. W. S. Hoar and D. J. Randall), pp. 105–128. Academic Press, London.

Ramirez-Lorca, R., Vizuete, M. L., Venero, J. L., Revuelta, M., Cano, J., Ilundain, A. A. and Echevarria, M. (1999). Localization of aquaporin 3 mRNA and protein along the gastrointestinal tract of Wistar rats. *Pflugers Arch. – Eur. J. Physiol.* **438**, 94–100.

Sardet, C., Pisam, M. and Maetz, J. (1979). The surface epithelium of teleostean fish gills. Cellular and functional adaptations of the chloride cell in relation to salt adaptation. *J. Cell Biol.* **80**, 96–117.

Shirai, N. and Utida, S. (1970). Development and degeneration of the chloride cell during seawater and freshwater adaptation of the Japanese eel, *Anguilla japonica*. *Z. Zellforsch.* **103**, 247–264.

Simmonneaux, V., Humbert, W. and Kirsch, R. (1988). Structure and osmoregulatory functions of the intestinal folds in the seawater eel, *Anguilla anguilla*. *J. Comp. Physiol.* **158B**, 45–55.

Skadhauge, E. (1969). The mechanism of salt and water absorption in the intestine of the eel (*Anguilla anguilla*) adapted to waters of various salinites. *J. Physiol.* **204**, 135–158.

Skadhauge, E. (1974). Coupling of transmural flows of NaCl and water in the intestine of the eel (*Anguilla anguilla*). *J. Exp. Biol.* **60**, 535–546.

Verkman, A. S. and Mitra, A. K. (2000). Structure and function of aquaporin water channels. *Am. J. Physiol.* **278**, F13-F28.

Yamamoto, M. and Hirano, T. (1978). Morphological changes in the oesophageal epithelium of the eel, *Anguilla japonica*, during adaptation to seawater. *Cell Tissue Res.* **192**, 25–38.