The Journal of Experimental Biology 206, 3495-3505

doi:10.1242/jeb.00579

Intestinal water absorption through aquaporin 1 expressed in the apical membrane of mucosal epithelial cells in seawater-adapted Japanese eel

Mayumi Aoki[1], Toyoji Kaneko[1,2,*], Fumi Katoh[1,2], Sanae Hasegawa[2], Naoaki Tsutsui[1,3] and Katsumi Aida[1]

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo, Tokyo 113-8657, Japan,* [2]*Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan* and [3]*Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305-8686, Japan*

*Author for correspondence (e-mail: kaneko@marine.fs.a.u-tokyo.ac.jp)

Accepted 2 July 2003

Summary

To elucidate the mechanisms associated with water absorption in the intestine, we compared drinking and intestinal water absorption in freshwater- and seawater-adapted Japanese eels, and investigated a possible involvement of aquaporin (AQP) in the absorption of water in the intestine. Seawater eels ingested more water than freshwater eels, the drinking rate being 0.02 ml kg^{-1} h^{-1} in fresh water and 0.82 ml kg^{-1} h^{-1} in sea water. In intestinal sacs prepared from freshwater and seawater eels, water absorption increased in time- and hydrostatic pressure-dependent manners. The water absorption rates were greater in seawater sacs than in freshwater sacs, and also greater in the posterior intestine than in the anterior. In view of the enhanced water permeability in the intestine of seawater eel, we cloned two cDNAs encoding AQP from the seawater eel intestine, and identified two eel homologues (S-AQP and L-AQP) of mammalian AQP1. S-AQP and L-AQP possessed the same amino acid sequence, except that one amino acid was lacking in S-AQP and two amino acids were substituted. Eel AQP1 was expressed predominantly in the intestine, and the expression levels were higher in seawater eel than in freshwater eel. Immunocytochemical studies revealed intense AQP1 immunoreaction in the apical surface of columnar epithelial cells in seawater eel, in which the immunoreaction was stronger in the posterior intestine than in the anterior. In contrast, the immunoreaction was faint in the freshwater eel intestine. Preferential localization of AQP1 in the apical membrane of epithelial cells in the posterior intestine of seawater eel indicates that this region of the intestine is responsible for water absorption, and that AQP1 may act as a water entry site in the epithelial cells.

Key words: Japanese eel, *Anguilla japonica*, drinking, water absorption, aquaporin 1, intestine.

Introduction

Osmoregulation in teleost fishes is achieved by integrated ion and water transport activities of the gills, kidney and intestine (for reviews, see Evans, 1993; Bentley, 2002). Freshwater teleosts face water load and ion loss through their permeable body surfaces, and therefore excrete excess water by producing dilute urine in the kidney, and absorb ions through the gill epithelia. By contrast, marine teleosts drink a large amount of sea water and absorb water in the intestine to compensate for osmotic water loss across the body surface, while actively secreting excess ions from gill chloride cells.

Although the water-absorbing mechanism in the intestine of marine or seawater-adapted fish is still poorly understood, water and ion movements during the passage of ingested water along the gastrointestinal tract were well described in early studies (Hickman, 1968; Shehadeh and Gordon, 1969). In seawater-adapted Japanese eel *Anguilla japonica* and European eel *A. anguilla*, Hirano and Mayer-Gostan (1976) have shown that ingested seawater is mainly desalted in the ion-permeable esophagus, and to a lesser extent in the stomach, by passive diffusion of the ions into the blood. The subsequent water absorption is considered to take place in the intestine following active absorption of monovalent ions. However, the precise mechanism of water absorption in the intestine is not yet elucidated in eels and other teleost species.

Aquaporins (AQPs) are a family of integral membrane proteins that function as water channels, and are importantly involved in fluid transporting mechanisms in various organs, including the gastrointestinal tract (Agre et al., 1993; Ma and Verkman, 1999; Matsuzaki et al., 2002). Many AQP isoforms have been identified in mammals, and AQP homologues have also been found in a wide variety of organisms, ranging from bacteria to mammals (Ishibashi et al., 2000; King et al., 2000; Verkman, 2002; Connolly et al., 1998; Chispeels and Agre, 1994). In mammalian species, several AQP isoforms are

expressed in the gastrointestinal tract, and are presumably involved in water transport (Ma and Verkman, 1999). In teleost species, however, only three AQP homologues have been identified to date: AQP0 from the lens of killifish *Fundulus heteroclitus* (Virkki et al., 2001) and AQP3 from the gills of European eel and Japanese dace *Tribolodon hakonensis* (Cutler and Cramb, 2002; Hirata et al., 2003). It is not clear, therefore, if AQP is involved in the water-absorbing process in the fish intestine.

In the present study, to elucidate the mechanisms associated with water absorption in the intestine of seawater-adapted eels, the following experiments were done. (1) To reinvestigate the importance of drinking in seawater-adapted fish, the drinking rate was compared in freshwater- and seawater-adapted eels. (2) Water absorption was examined in intestines isolated from freshwater- and seawater-adapted eels, with reference to the intestinal hydrostatic pressure. (3) A water-channel molecule was explored as a possible water entry site in the intestinal epithelia. We successfully identified homologues of AQP1 from the eel intestine, and compared its expression and morphological distribution in the intestines of freshwater- and seawater-adapted eels.

Materials and methods

Fish

Cultured Japanese eels *Anguilla japonica* Temminck and Schlegel, weighing approximately 200 g, were obtained from a commercial supplier in Tokyo. After acclimation to laboratory conditions in freshwater for 2–3 days, half of the fish were acclimated to 50% seawater for 2 days and then transferred to full-strength seawater, while the other half were maintained in freshwater. Both freshwater and seawater-transferred eels were reared in their respective environments at least for 1 week before use for the following experiments. The temperature was maintained at 25°C, and the fish were not fed during this period.

Measurement of drinking

To determine the amount of ingested water, freshwater- and seawater-adapted eels were immersed in 0.01% Trypan Blue in their respective environments. The fish were sampled at 0, 2, 4, 6 and 18 h after immersion in both experimental groups. At the time of sampling, the fish were anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MO, USA), and the body mass was determined. The abdominal cavity was opened and the intestine tied off at the anterior esophagus and the rectum with cotton threads to prevent the ingested water from leaking. The gastrointestinal tract tied at both ends was carefully removed, and the ingested water was collected into a conical graduated measuring container (100 ml). The remaining dye in the tract was washed out with distilled water, which was combined with the ingested water. Distilled water was added to the recovered solution to a final volume of 7 ml.

To measure the volume of the ingested water, duplicate sample solutions and serial dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) of the environmental waters (250 ml each) were placed in wells of a 96-well plate, and the absorbance at 550 nm measured using a microplate reader (DigiScan 340, Asys Hitech, Eugendorf, Austria). Since the standard curve was linear within this range, the dilution factor (DF) was calculated from the regression line. The amount of ingested water (W; ml kg^{-1}) was calculated from the following equation:

$$W = FV \times DF \times BW^{-1}, \quad (1)$$

where FV (ml) is the final volume of the sample (7 ml), and BW (kg) is the body mass of the fish.

Preparation of the intestinal sac

After anesthesia with 0.1% 3-aminobenzoic acid ethyl ester, the intestines were isolated from freshwater- and seawater-adapted eels. After rinsing out the contents of the intestines with balanced salt solution (BSS: 140 mmol l^{-1} NaCl, 3.0 mmol l^{-1} KCl, 1.25 mmol l^{-1} $MgSO_4$, 0.4 mmol l^{-1} NaH_2PO_4, 2.0 mmol l^{-1} $NaHCO_3$, 1.5 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} Hepes, pH 7.4), the intestines were preincubated in BSS for 1–2 h at 25°C. A polyethylene tube (inner diameter, 1.4 mm; outer diameter, 1.9 mm), the tip of which was slightly flamed to make a bulge, was inserted into the anterior end of the intestine, and fixed with a cotton thread. The bulge prevents the tube from slipping off the intestine. The other end of the tube was connected to a plastic bottle containing BSS, which was equipped with a handy air pump and a manometer (Manostar Gage, 81FN-10E, Okano, Osaka, Japan) so that the pressure could be regulated. After flushing the air in the lumen with BSS supplied from the bottle, the posterior end of the intestine was tied with a thread.

The eel intestine could be divided into anterior and posterior parts by its external appearance. In macroscopic observations, the anterior intestine appeared thick and short, while the posterior intestine was thin and long. For the measurement of water absorption in anterior and posterior intestines, sacs were prepared in the same way as described above after separating the intestine into the two parts.

Measurement of water absorption

The intestinal sac was filled with BSS to a hydrostatic pressure of 1.0 kPa, when the length of the intestine between the ties at both ends was recorded. The pressure was then re-adjusted to 0.5 kPa, 1.0 kPa or 3.0 kPa, and the tube was sealed and cut with flamed cutting pliers at a site close to the anterior end of the intestine. The intestinal sac filled with BSS was lightly blotted on a Kimwipe, weighed, and incubated in O_2-bubbled BSS with shaking at 25°C. The intestinal sac was weighed to the nearest mg every 30 min for up to 3 h during incubation. The amount of water absorbed across the intestinal wall was estimated as decrease in mass, normalized to give net water absorption per cm of the intestine by dividing by the length measured at 1.0 kPa.

Regional differences in water absorption were examined in sacs prepared from the anterior and posterior intestines, filled

with BSS to a hydrostatic pressure of 3.0 kPa. The sac loss of mass during an incubation period of 1 h in BSS was determined as described above. The water absorption rate was presented as the mass loss per cm^2 of the surface area per hour. For the surface area measurement, the sac was spread serosal side down and the area was determined on the basis that the spread tissue was a rectangle.

Isolation of cDNA fragment encoding AQP

Seawater-adapted eels were used for total RNA preparation. After anesthesia, the intestines were dissected out, frozen in liquid nitrogen, and stored at –80°C until RNA extraction. Total RNA was extracted from the intestines with ISOGEN (Nippon Gene, Tokyo, Japan). Reverse transcription was carried out using dT-adaptor primer (dT-AP; 5′-ACGGACTC-GAGTCGACATCGA$(T)_{17}$-3′) and Superscript™ II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 42°C for 10 min and at 50°C for 1 h.

AQP belongs to the major intrinsic protein (MIP) family, which has two NPA (Asn-Pro-Ala) motifs that are highly conserved in the most of MIPs from various animals, plants and bacteria (Park and Saier, 1996). To obtain cDNA fragments encoding AQP, the following degenerated primers were designed based on sequences surrounding these motifs in the MIPs identified so far: sense, fAQP-I (5′-CAYATHAAYC-CIGCNGTNAC-3′); antisense, rAQP-S (5′-CCRAAISWICK-NGCNGGRTT-3′). The first-strand cDNA was amplified with *Taq* DNA polymerase (Takara *Taq*, Takara, Japan) by polymerase chain reaction (PCR) for 40 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 1 min). The PCR products were ligated into pCR 2.1-TOPO vector (Invitrogen), and sequenced using a DNA sequencer (SQ-5500, Hitachi, Tokyo, Japan).

Rapid amplification of cDNA ends (RACE)

For 3′ RACE, a gene-specific primer F0 (5′-TCTAGGA-CACCTAACAGCCAT-3′) and adaptor primer 1 (AP1; 5′-ACGGACTCGAGTCGACAT-3′) were designed. The reaction product amplified by 40 cycles of PCR (94°C for 30 s, 60°C for 30 s and 72°C for 1 min) was subcloned into pBluescript SK (–) vector (Stratagene, La Jolla, CA, USA) and sequenced.

For 5′-RACE, three gene-specific primers, R1 (5′-GCTAC-GACGTACATGA-3′), R2 (5′-TTGAGCATGCTGATCTG-GCA-3′) and R3 (5′-CAGCATGCCCAGGGTGA-3′) were designed. First-strand cDNA was synthesized from total RNA with R1, as described above. The resulting cDNA was tailed with poly(dA) by a 3′-end labeling kit (Amersham Biosciences, Piscataway, NJ, USA). Using dT-AP, AP1, R2 and poly(dA)-tailed cDNA, the first PCR was conducted in the following profiles: 3 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 2 min, and 37 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min. The second PCR was performed using AP1, R3 and a tenfold diluted reaction mixture of the first PCR product as a template with a cycle protocol as follows: 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The second PCR product was subcloned and sequenced as described above.

To confirm the nucleotide sequences of two cDNA clones encoding AQP (S-AQP and L-AQP), 41 cycles of PCR (94°C for 30 s and 68°C for 90 s) were performed with a gene-specific sense primer, AQPexF2 (5′-CAGGGATCCATGAC-GAAGGAGCTCAAGA-3′), and adaptor primer 2 (AP2; 5′-ACGGACTCGAGTCGACATCGAT-3′). The resultant PCR products containing a full-length open reading frame and 3′ un-translated region were ligated into pCR 2.1-TOPO vector and sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Analysis of the amino acid sequence

Kyte–Doolittle hydropathy profile of the deduced amino acid sequence was analyzed using GENETIX-MAC version 8.0 software (Software Development, Tokyo, Japan) at a 10-residue window. The amino acid identities between eel and other AQPs were analyzed by the BLAST search (Altschul et al., 1997).

Northern blot analysis

The heart, muscle, liver, intestine, gill, stomach, spleen, swim bladder, kidney and testis were removed from freshwater- and seawater-adapted eels. Total RNA was isolated from these tissues, as described above. 20 μg of total RNA were electrophoresed on a 1.2% formamide–agarose gel and transferred to BIODYNE B membrane (PALL, East Hills, NY, USA). A cDNA fragment corresponding to nucleotides 546–975 in S-AQP and to nucleotides 549–978 in L-AQP was labeled with [α-^{32}P]dCTP (Amersham Biosciences) using Megaprime™ DNA labeling system (Amersham Biosciences). The membrane was hybridized with the radiolabeled probe (3.7×10^5 cpm ml) in 50% formamide, 6× SSC, 2× Denhardt's solution, 0.4% SDS, and 0.1 mg ml^{-1} calf thymus DNA at 42°C for 16 h. After washing in 0.2× SSC containing 0.1% SDS at 60°C for 5 min, the membrane was exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan). RNA ladder (Invitrogen) was used as the molecular mass marker.

For quantitative analysis of AQP mRNA, intestines were isolated from freshwater- and seawater-adapted eels (N=3 each), and cut into the anterior and posterior parts. RNA extraction and northern blot analysis were carried out as described above. Hybridization signals were quantified as described previously (Tsutsui et al., 2000). The highest mRNA level determined in the posterior intestine of a seawater eel was designated an arbitrary value of 100.

Antibody

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of the C-terminal region of eel AQP1 molecules (amino acid residues 241–255 in S-AQP and 242–256 in L-AQP). The antigen designed was Gly-Pro-Asp-Gly-Asp-Tyr-Asp-Val-Asn-Gly-Pro-Asp-Asp-Val-Pro. The antigen conjugated with keyhole limpet hemocyanin (KLH) was emulsified with complete Freund's adjuvant, and immunization was performed in a New Zealand white rabbit (Sawady Technology, Tokyo, Japan).

Western blot analysis

The specificity of the antibody raised against the synthetic peptide was confirmed by western blot analysis. Prior to western blotting, the sample was subjected to immunoprecipitation. The intestine was isolated from seawater-adapted eel, and spread in lysis buffer consisting of IP buffer (pH 7.4; 140 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes and 5 mmol l^{-1} EDTA), inhibitors [10 mmol l^{-1} Benzamidine, 1 mg ml^{-1} Pepstatin and 2 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF)] and 1% Triton X-100. The mucosa and submucosa were scraped off with a blade, homogenized and left in IP buffer on ice for 40 min to lyse the cells. The lysate was centrifuged at 5000 ***g*** for 5 min at 4°C, and supernatant was incubated with 2 ml of the antibody at room temperature for 3 h. Slurry (20 ml) containing 50% protein A–sepharose beads (Amersham Biosciences, Uppsala, Sweden) was added to the sample, and the mixture was incubated for 1 h at room temperature. After washing with IP buffer and centrifugation at 10000 ***g***, 30 ml of hot Laemmli buffer containing 5% β-mercaptoethanol was added, and the mixture was incubated at 65°C for 15 min. The sample was then centrifuged at 10000 ***g*** for 2 min, and the supernatant frozen for later western blotting.

The sample was separated by SDS–polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels. After electrophoresis, the protein was transferred from the gel to a polyvinylidene difluoride membrane (Atto, Tokyo, Japan). After blocking with 2% skimmed milk at 4°C overnight, the membranes were incubated with the antibody diluted at 1:100 for 1 h at room temperature. The membranes were then incubated with protein A–gold conjugate (British Biocell International, Cardiff, UK) for 1 h and visualized by using silver-enhancing reagents (British Biocell International). The specificity of the immunoreaction was confirmed by incubating the membrane with normal rabbit serum (NRS) in place of the specific antibody.

Immunocytochemistry

To examine the localization of AQP1 and possible differences in its distribution pattern between freshwater- and seawater-adapted eels, the intestinal sections were stained immunocytochemically with the antibody specific for eel AQP1.

After anesthesia, the intestines were removed from freshwater- and seawater-adapted eels (*N*=3 each). The intestines were washed by flushing with 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4), and cut into the anterior and posterior parts. The anterior and posterior intestines were fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) for 18 h, and preserved in 70% ethanol. The intestines were then cut into small pieces (approximately 5 mm) across the axis, dehydrated in ethanol, and embedded in Paraplast. Serial sections were cut at 4 mm thickness and mounted on gelatin-coated slides. To examine the general morphology of the intestines of freshwater- and seawater-adapted eels, some sections were stained with Mayer's Hematoxylin and Eosin.

The sections were immunocytochemically stained using the avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981), using commercial reagents (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). In brief, deparaffined sections were incubated sequentially with: (1) 0.6% H_2O_2 for 30 min, (2) 2% normal goat serum for 30 min, (3) anti-eel AQP1 diluted at 1:1000 at 4°C, (4) biotinylated anti-rabbit IgG for 30 min, (5) ABC for 1 h, and (6) 0.02% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H_2O_2 for 5 min. The sections were observed under a microscope (Nikon E800, Tokyo) equipped with a differential interference contrast device. The specificity of the immunoreaction was confirmed by incubating the sections with NRS in place of the specific antibody.

Statistics

Numerical data were expressed as the mean ± S.E.M. Statistical analysis was performed using StatView 5.0 software (Hulinks, Tokyo, Japan). Significant differences at $P<0.01$ were determined by Fisher's PLSD after using two-way factorial analysis of variance (ANOVA).

Results

Drinking in freshwater- and seawater-adapted eels

Fig. 1 shows the time course of changes in amount of water ingested by freshwater- and seawater-adapted eels. The amount of ingested water was always greater in seawater-adapted eel than in freshwater-adapted eel at any sampling point. In seawater-adapted eel, the amount of ingested water increased linearly for up to 18 h after immersion, and the drinking rate was calculated to be 0.82 ml kg^{-1} h^{-1} from the linear regression. The freshwater-adapted eel, however, scarcely ingested the ambient water, and the drinking rate was calculated to be 0.02 ml kg^{-1} h^{-1}.

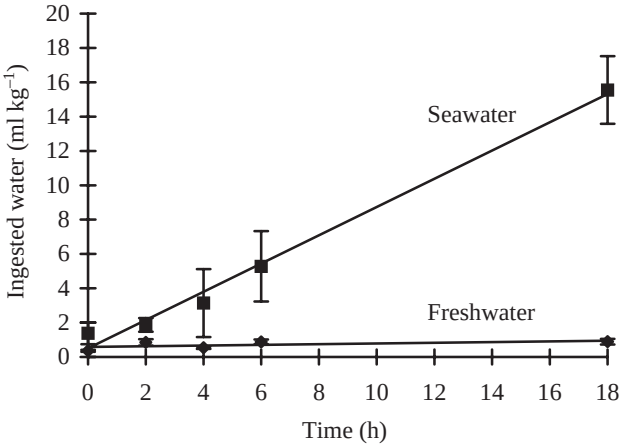

Fig. 1. Changes in the amount of water ingested by freshwater- and seawater-adapted eels with time. Drinking rates were estimated to be 0.02 ml kg^{-1} h^{-1} and 0.82 ml kg^{-1} h^{-1} in freshwater and seawater eels, respectively.

Water absorption in the intestine

In intestinal sacs prepared from both freshwater- and seawater-adapted eels, the increase in net water absorption was both time- and hydrostatic pressure-dependent (Fig. 2). The loss of sac mass was linear with time for up to 120 min in any experimental group. The water absorption rate calculated from the linear regression during the 0–120 min period was greater at higher hydrostatic pressure (Table 1). Compared at the same pressure, the water absorption rates were 2.7–3.7 times greater in seawater sacs than in freshwater sacs.

The water absorption rates tended to be higher in the posterior than in the anterior intestine in both freshwater- and seawater-adapted eels (Fig. 3). The rates were 2.6 times and 3.9 times higher in the posterior than in the anterior in freshwater and seawater eels, respectively. In the posterior intestine, water absorption was significantly greater (P<0.01) in seawater eel than in freshwater eel, whereas the difference was not significant in the anterior intestine.

cDNA cloning and sequence analysis of AQPs

Based on the DNA sequence of resultant clones, gene-specific primers were designed for 3′ and 5′ RACE. The obtained sequences were merged into a cDNA sequence, which was further confirmed by PCR to generate the full-length cDNA encoding AQP. As a result, the cDNAs for two AQPs were isolated, and were designated as S-AQP (DDBJ/EMBL/GenBank accession no. AB094502) and L-AQP (AB094501). S-AQP cDNA consisted of 1195 bp, containing 36 bp of a 5′ untranslated region (UTR), 786 bp of an open reading frame (ORF) and 373 bp of 3′ UTR, and L-AQP cDNA consisted of 1198 bp, containing 36 bp of 5′ UTR, 789 bp of an ORF and 373 bp of 3′ UTR.

The cDNAs for S-AQP and L-AQP encoded 262 and 263 amino acid residues, respectively. The deduced amino acid sequences of S- and L-AQPs are shown in Fig. 4. Both AQPs possessed the same amino acid sequence, except for a lack of one amino acid in S-AQP (corresponding to Asn^{116} in L-AQP) and substitutions of two amino acids (Ala^{117} and Glu^{125} in S-AQP, Thr^{118} and Lys^{126} in L-AQP). According to the hydropathy analysis, both S- and L-AQPs contained six putative transmembrane domains, five connecting loops, and cytoplasmic N- and C-terminal domains, all of which are widely conserved among AQP molecules. The second and fifth loops contained the NPA motifs, which is also characteristic of AQP molecules. Both S- and L-AQPs exhibited the highest identity to mouse AQP1 and frog AQP-channel forming

Table 1. *Water absorption rates of intestinal sacs prepared from freshwater- and seawater-adapted eels*

Pressure (kPa)	Rate (mg cm^{-1} h^{-1})*		Ratio†
	Freshwater	Seawater	
0.5	3.78	14.00	3.70
1.0	6.26	21.77	3.48
3.0	10.56	28.87	2.73

*Water absorption rate was calculated from the linear regression during a 0–120 min period.

†Ratio of water absorption rate in seawater-adapted eel to that in freshwater-adapted eel.

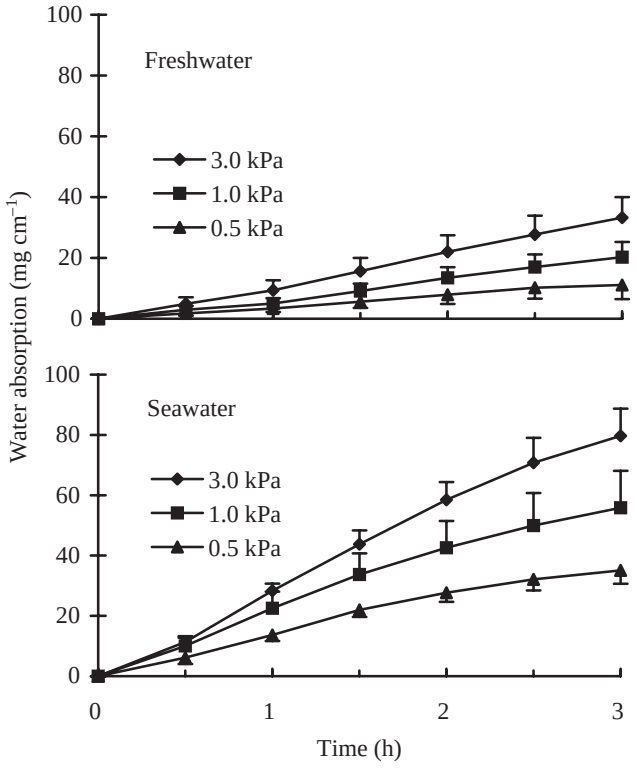

Fig. 2. Water absorption in intestinal sacs prepared from freshwater- and seawater-adapted eels. The water absorption increased with time and pressure. Water absorption rates were 2.7–3.7 times greater in seawater sacs than in freshwater sacs when compared at the same pressure.

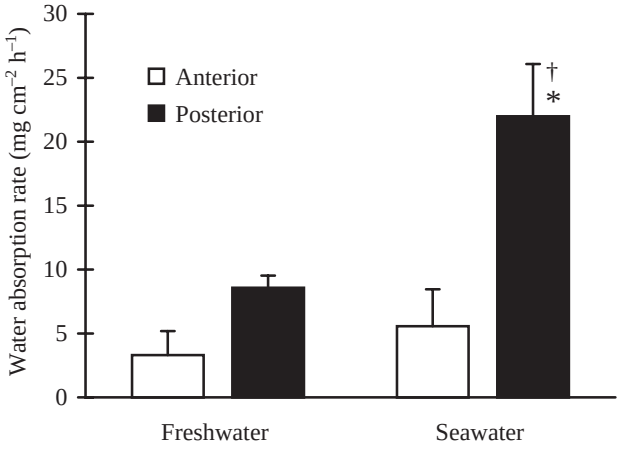

Fig. 3. Water absorption rates in the anterior and posterior intestine of freshwater- and seawater-adapted eels. The posterior intestine of seawater eel showed the highest water absorption rate. *Significantly different from the values of freshwater-adapted eels; †significantly different from the values of the anterior intestine at P<0.01.

```
S-AQP   MTKELKSKAFWRAVLAELLGMTLFIFLSIAAAIGN-----RHNSNPDQEVKVSLAFGLSIATLAQSLGHISGAHLNPAVTLGMLASCQIS   85
L-AQP   MTKELKSKAFWRAVLAELLGMTLFIFLSIAAAIGN-----RHNSNPDQEVKVSLAFGLSIATLAQSLGHISGAHLNPAVTLGMLASCQIS   85
AQP1    .AS.I.K.L.....V....A....V.I..GS.L.FNYPLE.NQTLVQDN.................V..............L.L.....   90
                                      * *       *
S-AQP   MLKAVMYVVAQMLGSSVASGIVYGVRPQNN-TALGLNSLNEISPS-QGVGVEFLATFQLVLCVIATTDKRRRDVTGSAPLAIGLSVALGH  173
L-AQP   MLKAVMYVVAQMLGSSVASGIVYGVRPQNNNTTLGLNSLNKISPS-QGVGVEFLATFQLVLCVIATTDKRRRDVTGSAPLAIGLSVALGH  174
AQP1    I.R....II..CV.AI..TA.LS.ITSSLVDNS..R.D.AHGVN.G..L.I.IIG.L......L....R....LG..............  180

S-AQP   LTAISFTGCGINPARSFGPALILGNFTNHWVYWVGPMCGGVAAALVYDFLLHPKFDDFPERMKVLVSGPDGDYDVNGPDDVPAVEMSSK   262
L-AQP   LTAISFTGCGINPARSFGPALILGNFTNHWVYWVGPMCGGVAAALVYDFLLHPKFDDFPERMKVLVSGPDGDYDVNGPDDVPAVEMSSK   263
AQP1    .L..DY...........S.VLTR.FS...IF....FI..AL.V.I...I.A.RSS..TD....WT..QVEE..LDAD.INSR...KP.   269
```

Fig. 4. Deduced amino acid sequences of eel homologues of AQP1, S-AQP and L-AQP, in comparison to the sequence of mouse AQP1. Periods and hyphens indicate identical residues and gaps introduced for alignment, respectively. Two NPA motifs are shaded. Asterisks indicate differences between S-AQP and L-AQP. The antibody was raised against a synthetic peptide corresponding to the underlined sequence.

integral protein (CHIP) (59% each). They showed generally high identity to AQP1s in other species (55–59%), whereas the identities to the other AQPs (AQP0 and 2–10) were less than 50%. Thus, S-AQP and L-AQP were considered to be eel homologues of AQP1. Among teleost species, both S-AQP and L-AQP shared 25% identity with European eel AQP3, 48% identity with killifish AQP0, and 24% identity with Japanese dace AQP3.

Tissue distribution of AQP1 mRNA

Tissue-specific expression of AQP1 mRNA was analyzed by northern hybridization (Fig. 5). In both freshwater and seawater eels, hybridized signals were detected as a single band of approximately 1.3 kb in the heart, intestine, spleen and swim bladder, but not in the other tissues examined. It was confirmed that the size of the mRNA was in agreement with that of the cDNAs for S- and L-AQPs obtained in this study. The expression levels of AQP1 mRNA in the intestine were higher in seawater eel than in freshwater eel, whereas such trend was not observed in the other AQP1-expressing tissues.

Quantitative analysis of AQP1 mRNA levels in the intestine

The expression levels of AQP1 mRNA in the anterior and posterior intestines were examined in freshwater and seawater eels (Fig. 6). Compared separately in the anterior and posterior intestines, AQP1 mRNA levels were significantly higher in seawater eel than in freshwater eel (P<0.01). There was a tendency for AQP mRNA to accumulate more highly in the posterior than in the anterior in both freshwater and seawater eels; however, the differences were not significant.

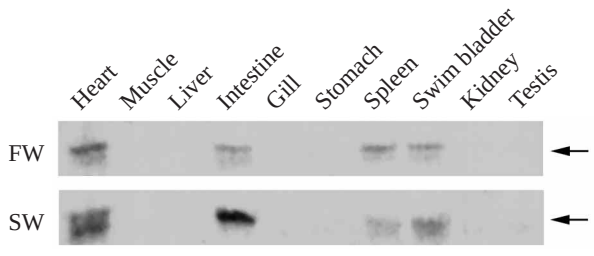

Fig. 5. Tissue distribution of eel AQP1 (S-AQP and L-AQP) mRNA determined by northern hybridization in freshwater (FW)- and seawater (SW)-adapted eels. Hybridization signals were detected as a single band of approximately 1.3 kb (arrows) in the heart, intestine, spleen and swim bladder, but not in the other tissues examined.

Western blot analysis

In western blot analysis, the antibody recognized a major protein band of approximate size 30 kDa in the intestine of seawater-adapted eel (Fig. 7). This band was not detected in the control, in which the membrane was incubated with NRS in place of the specific antibody. The other bands with higher molecular mass appeared to be non-specific reactions, and were also observed in the control lane.

General morphology of the intestine

The intestinal wall consisted of mucosa, submucosa, muscularis and serosa (Fig. 8). The mucosal epithelium was composed of a single layer of columnar epithelial cells with a basally located nucleus. Mucous cells were scattered among the columnar epithelial cells. In both freshwater- and seawater-adapted eels, mucous cells were more frequently observed in the posterior intestine than in the anterior. The muscularis

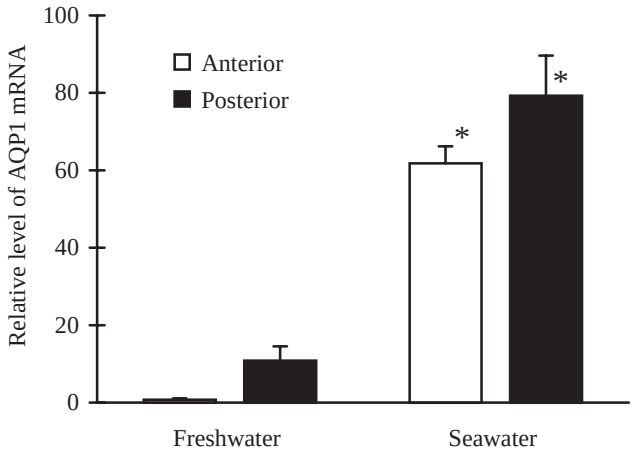

Fig. 6. Quantitative analysis of eel AQP1 (S-AQP and L-AQP) mRNA levels in the anterior and posterior intestines of freshwater- and seawater-adapted eels. *Significantly different from the freshwater value at P<0.01.

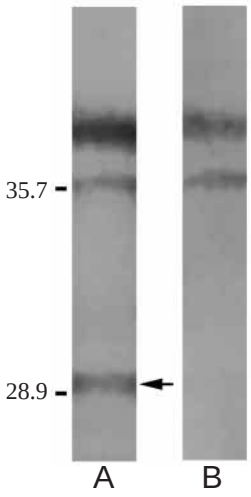

Fig. 7. Western blot analysis for eel AQP1 (S-AQP and L-AQP) expressed in the intestine of seawater-adapted eel. The membranes were incubated with anti-eel AQP1 (lane A) and normal rabbit serum (lane B). The antibody recognized one specific protein band of approximately 30 kDa (arrow), agreeing with the expected molecular mass of eel AQP1. Positions of molecular mass markers (kDa) are indicated on the left.

consisted of the inner circular and outer longitudinal muscle layers. The anterior intestine was generally thicker with the broader muscularis than the posterior. In the anterior intestine, there was no structural difference between freshwater and seawater eels. In the posterior intestine, in contrast, both inner circular and outer longitudinal muscle layers were thinner in seawater eel than in freshwater eel.

Immunocytochemical detection of AQP1 in the intestine

In seawater-adapted eel, the intense immunoreaction for AQP1 was detected in the apical surface of columnar epithelial cells in the mucosa along the posterior intestines (Fig. 9A,E). Since the apical membrane of most epithelial cells was stained with the antibody, the intestinal folds appeared to be lined with the immunoreactive product, except for the apical surface of mucous cells that were immunonegative. This was in sharp contrast with the anterior intestine, in which AQP1 immunoreaction was faint in the apical membrane of mucosal epithelial cells (Fig. 9C). AQP1 immunoreaction was also faint in the intestine of freshwater-adapted eel (Fig. 9B,D). In both anterior and posterior intestines of freshwater eel, not all epithelial cells showed the immunoreaction, but some cells were immunonegative.

In addition to columnar epithelial cells, AQP1 immunoreaction was detected in endothelial cells of the blood vessels distributed in the muscle layers and, to a lesser extent, in the submucosa and mucosa (Fig. 9A). There was no apparent difference in AQP1 immunoreactivity in the endothelia between the anterior and posterior intestines and between freshwater and seawater eels (data not shown).

Discussion

Since the early work by Smith (1930), it has become well accepted that seawater fish drink a large amount of ambient water to compensate for osmotic water loss. The present study confirmed a higher drinking rate in seawater-adapted eel than in freshwater-adapted fish; seawater eel ingested a considerable amount of sea water (0.82 ml kg^{-1} h^{-1}), while drinking in freshwater eel was considered to be almost

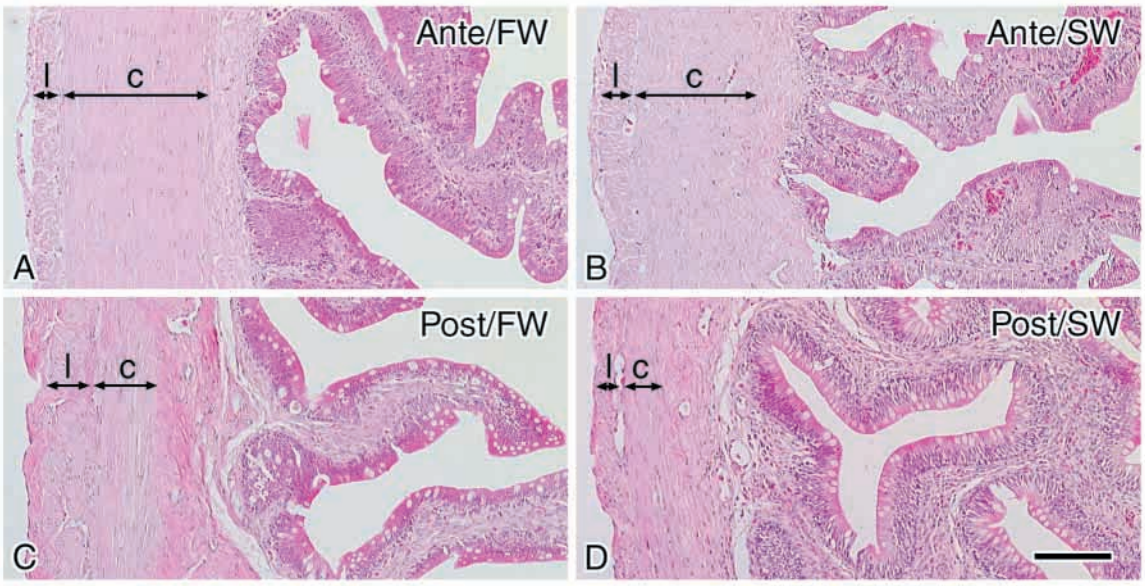

Fig. 8. Cross sections of the anterior (Ante: A,B) and posterior (Post: C,D) intestines from freshwater (FW: A,C)- and seawater (SW: B,D)-adapted eels, stained with Hematoxylin and Eosin. In the posterior intestine, both inner circular (c) and outer longitudinal (l) muscle layers were thinner in seawater eel than in freshwater eel, whereas there was no apparent structural difference in the anterior intestine of freshwater and seawater eels. Scale bar, 100 μm.

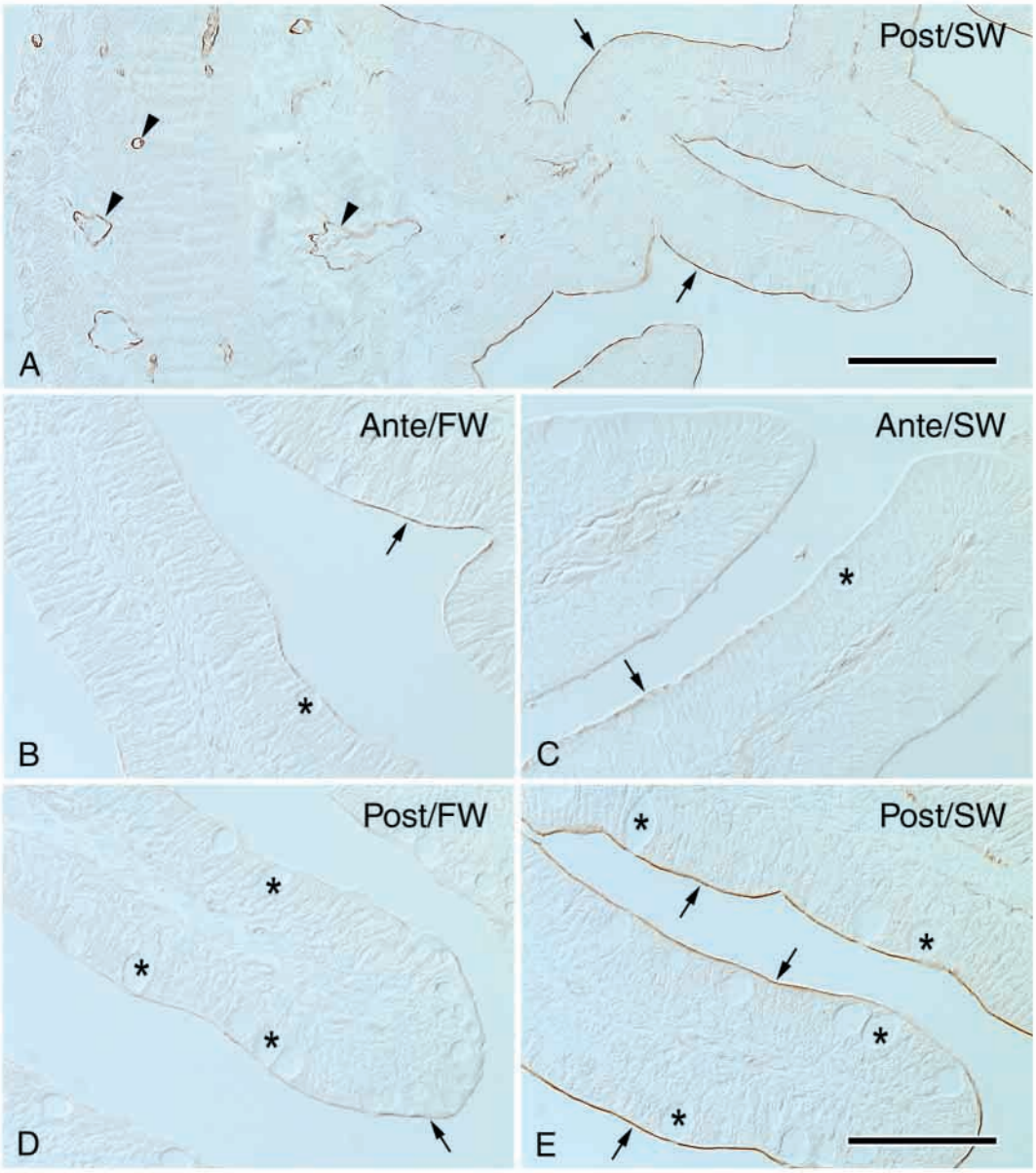

Fig. 9. Cross sections of the anterior (Ante: B,C) and posterior (Post: A,D,E) intestines from freshwater (FW: B,D)- and seawater (SW: A,C,E)-adapted eels, stained immunocytochemically with anti-eel AQP1. In seawater eel, intense immunoreaction for AQP1 was observed in the apical surface of columnar epithelial cells (arrows) in the mucosa of the posterior intestine. In addition, AQP1 immunoreaction was detected in endothelial cells of the blood vessels (arrowheads) distributed in the muscularis, submucosa and mucosa. Asterisks indicate mucous cells. Scale bars: 100 μm (A); 50 μm (B–E).

negligible (0.02 ml kg^{-1} h^{-1}). This is in accordance with previous reports for *Anguilla* species (Smith, 1930; Oide and Utida, 1968; Gaitskell and Chester Jones, 1971; Kobayashi et al., 1983).

In seawater-adapted eel, ingested water is desalted in the esophagus by passive diffusion of ions into blood, followed by water absorption in the intestine (Hirano and Mayer-Gostan, 1976). In the present study, water absorption was much higher in the intestinal sacs prepared from seawater-adapted eels than in those from freshwater-adapted eel. Increases in intestinal water absorption have been reported in eels transferred from freshwater to seawater (Oide, 1967; Oide and Utida, 1967, 1968; Kirsch and Meister, 1982), and our result is in agreement with those previous reports. Similar profiles have been observed in coho salmon (Collie and Bern, 1982) and Atlantic salmon (Veillette et al., 1993) following parr-smolt transformation.

The intestine of eels is largely divided into two anatomically and physiologically distinct regions, the anterior and posterior intestines. In seawater-adapted eel, the present study revealed higher water absorption in the posterior intestine than in the anterior. Similarly, in seawater-adapted coho and Atlantic

salmon, intestinal water transport has been shown to occur in the posterior intestine rather than in the anterior (Collie and Bern, 1982; Veillette et al., 1993). The increased water permeability in the posterior intestine correlated with an apparent morphological change, whereby the muscle layers became thinner after seawater transfer (Fig. 8.) In intestinal sacs from seawater-adapted eel, Ando and Kobayashi (1978) showed that the muscle layers acted as a barrier against water flux, and that the water flux was increased by stripping off the layers. Thus, the higher water permeability in the posterior intestine of seawater-adapted eel may be explained at least in part by the decrease in thickness of the muscle layers.

The decrease in the sac mass was linear with time up to 120 min in any condition. In seawater-adapted eel, however, the slopes were more or less decreasing during incubation after 120 min, probably due to the decrease in the intestinal volume and subsequent decrease in the hydrostatic pressure. Previous studies have not examined the effect of intestinal hydrostatic pressure. Collie and Bern (1982), working with intestinal sacs from coho salmon, did not find significant differences in loss of sac mass with time for hydrostatic pressures between 0–20 cm H_2O (0–2.0 kPa), which suggested that the initial pressure was not critical for the determination of the water absorption rate. In the present study, however, the water absorption rate was apparently greater at higher pressure. Thus, the initial hydrostatic pressure should be kept constant for the precise determination and comparison of water absorption rates.

The driving force for active ion transport linked to water absorption in the intestine is provided at least in part by Na^+/K^+-ATPase, since ouabain (a Na^+/K^+-ATPase inhibitor) has been shown to inhibit solute and water transport (Oide, 1967; Collie and Bern, 1982; Veillette et al., 1993). However, although the osmotic gradient established by active ion absorption drives water uptake in the intestine, the increased hydrostatic pressure in the intestinal lumen could also contribute to water transport from the mucosal to serosal side. Although we have not measured the intestinal hydrostatic pressure in freshwater and seawater eels in this study, we may speculate that the pressure is higher in seawater fish than in freshwater fish. The intestines were always more expanded with ingested water in seawater eel than in freshwater eel. This has also been observed in freshwater and 80% seawater-transferred medaka larvae, using confocal laser scanning microscopy in combination with FITC–dextran as an inert marker (Kaneko and Hasegawa, 1999). In mammals, movement of water is the result of the osmotic driving force created by active salt transport and hydrostatic pressure differences (Ma and Verkman, 1999). Involuntary contractile movements during peristalsis could also increase intestinal hydrostatic pressure temporally and locally, which would provide an additional driving force with which to squeeze the water out.

In the present study, we succeeded in cloning two cDNAs encoding AQP from the eel intestine. AQPs were originally defined as homologue proteins of major intrinsic protein of mammalian lens that rapidly and selectively permeate water (Agre et al., 1993). At least eleven isoforms of AQPs (AQP0–AQP10) have been identified in mammals to date. S- and L-AQPs represent the first AQP1 homologues identified in teleost species. Among AQP isoforms, AQPs 1, 2, 4, 5 and 8 are considered to be water-selective, whereas AQPs 3, 7, 9 and 10 also transport glycerol and other small solutes (Ishibashi et al., 1994, 1997, 1998, 2002; Echevarría et al., 1996). AQP6 has been proposed to function in kidney endosomes as a pH-sensitive anion channel (Yasui et al., 1999a,b). Thus, S- and L-AQPs, homologues of mammalian AQP1, are likely to function as water-specific channels in the intestine.

Amino acid sequences of S- and L-AQPs are the same, except for a lack of one amino acid in S-AQP and substitutions of two amino acids. Because of their minor differences, we performed northern hybridization and immunocytochemical studies without distinguishing the two AQP1 homologues, using probes common to S- and L-AQPs.

Northern hybridization analysis revealed that eel AQP1 was expressed predominantly in the intestine, heart, spleen and swim bladder, but not detected in the other tissues examined. Among the AQP1-expressing tissues, higher expression in seawater eel than in freshwater eel was only seen in the intestine. Further quantitative analysis showed that AQP1 expression levels in the intestine were significantly higher in seawater eel than in freshwater eel, when compared separately in the anterior and posterior intestines. Taken together, these findings indicate that AQP1 expressed in the intestine is importantly involved in water-absorbing processes in the seawater eel intestine.

A specific antibody was raised against eel AQP1. The synthetic peptide corresponding to part of the C-terminal region, common to both S- and L-AQPs, was used as the antigen, so the resulting antibody was considered to react with both molecules. In western blot analysis, the antibody recognized one specific band of approximately 30 kDa, the expected molecular mass of eel AQP1, suggesting high specificity of the antibody.

The immunocytochemical detection of AQP1 in the intestine further supports the possible involvement of AQP1 in water-absorbing processes in the seawater eel intestine. In accordance with increased water absorption and predominant expression of AQP1 in the seawater eel intestine, an intense immunoreaction for AQP1 was detected in the apical surface of columnar epithelial cells in the mucosa along the posterior intestine. By contrast, the immunoreaction was faint in the freshwater eel intestine. Preferential localization of AQP1 in the apical membrane of epithelial cells in the posterior intestine of seawater eel indicates that AQP1 may act as a water entry site in the epithelial cells. It is highly possible that another type of AQP is present in the basolateral membrane, presumably providing an exit pathway for the release of water to the serosal side. In the mammalian kidney collecting duct, AQP2 is located in the apical membrane of the principal cells, whereas AQP3 and AQP4 are present in the basolateral membrane (Marples et al., 1999). Such an arrangement of the different AQP isoforms in the apical and basolateral membranes of the

epithelial cells would generally provide a transcellular pathway for water movement. By contrast, although several AQP isoforms are expressed in the mammalian gastrointestinal tract, AQPs have not been localized to the apical membrane of mammalian enterocytes (Ma and Verkman, 1999). Thus, AQPs do not appear to be the primary pathway for water absorption in mammalian intestines.

In European eel, the expression of AQP3 has been shown in the intestine as well as in the gills, eye and esophagus (Cutler and Cramb, 2002). In contrast with Japanese eel AQP1, European eel AQP3 is not likely to be responsible for intestinal water absorption. There was no apparent difference in AQP3 expression between freshwater- and seawater-acclimated eels (Cutler and Cramb, 2002), and AQP3 immunoreactivity was not detected in epithelial cells but in 'intra-epithelial macrophage-like cells' in the intestine (Lignot et al., 2002).

In addition to epithelial cells in the intestinal mucosa, AQP1 immunoreaction was observed in endothelial cells of blood vessels distributed in the muscularis, submucosa and mucosa of the intestine. Similarly, in mammals, the expression of AQP1 has been observed in microvascular endothelia of various tissues (Verkman, 2002), including the respiratory system (Nielsen et al., 1997) and kidney (Nielsen et al., 1995). Considering that AQP1 expressed in microvascular endothelia has a role in osmotically driven water transport in mammals, it is most probable that water absorbed through the intestinal epithelium would then move extracellularly to blood vessels to flow into the blood circulation.

In the present study, a higher drinking rate was confirmed in seawater-adapted eel than in freshwater-adapted eel. The subsequent water absorption occurred mainly in the posterior intestine. In addition to decreased osmolality of ingested water through active ion absorption, increased hydrostatic pressure may drive water from the mucosal to serosal side through the water-permeable apical membrane of epithelial cells in the posterior intestine of seawater-adapted eel. Increased water permeability of the posterior intestinal wall in seawater eel is attributed to AQP1, which is extensively distributed in the apical membrane of the epithelial cells. The decrease in the thickness of the muscularis would further facilitate the water movement from the epithelial cells in the mucosa to blood vessels in the outer layers in the intestine.

References

Agre, P., Preston, G. M., Smith, B. L., Jung, J. S., Raina, S., Moon, C., Guggino, W. B. and Nielsen, S. (1993). Aquaporin CHIP: the archetypal molecular water channel. *Am. J. Physiol.* **265**, F463-F476.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**, 3389-3402.

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.

Bentley, P. J. (2002). *Endocrines and Osmoregulation*. *Zoophysiology* Vol. 39. Berlin: Springer.

Chrispeels, M. J. and Agre, P. (1994). Aquaporins: water channel proteins of plant and animal cells. *Trends Biochem. Sci.* **19**, 421-425.

Collie, N. L. and Bern, H. A. (1982). Changes in intestinal fluid transport associated with smoltification and seawater adaptation in coho salmon, *Oncorhynchus kisutch* (Walbaum). *J. Fish Biol.* **21**, 337-348.

Connolly, D. L., Shanahan, C. M. and Weissberg, P. L. (1998). The aquaporins. A family of water channel proteins. *Int. Biochem. Cell Biol.* **30**, 169-172.

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

Echevarría, M., Windhager, E. E. and Frindt, G. (1996). Selectivity of the renal collecting duct water channel aquaporin-3. *J. Biol. Chem.* **271**, 25079-25082.

Evans, D. H. (ed.) (1993). Osmotic and ionic regulation. In *The Physiology of Fishes*, pp. 315-341. Boca Raton: CRC Press.

Gaitskell, R. E. and Chester Jones, I. (1971). Drinking and urine production in the European eel (*Anguilla anguilla* L.). *Gen. Comp. Endocrinol.* **16**, 478-483.

Hickman, C. P., Jr (1968). Ingestion, intestinal absorption, and elimination of seawater and salts in the southern flounder, *Paralichthys lethostigma*. *Can. J. Zool.* **46**, 457-466.

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

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M.-H., Romero, M. and Hirose, S. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiol.* **284**, R1199-R1212.

Hsu, S. M., Raine, L. and Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**, 577-580.

Ishibashi, K., Kuwahara, M., Gu, Y., Kageyama, Y., Tohsaka, A., Suzuki, F., Marumo, F. and Sasaki, S. (1997). Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J. Biol. Chem.* **272**, 20782-20786.

Ishibashi, K., Kuwahara, M., Gu, Y., Tanaka, Y., Marumo, F. and Sasaki, S. (1998). Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. *Biochem. Biophys. Res. Commun.* **244**, 268-274.

Ishibashi, K., Kuwahara, M. and Sasaki, S. (2000). Molecular biology of aquaporins. *Rev. Physiol. Biochem. Pharmacol.* **141**, 1-32.

Ishibashi, K., Morinaga, T., Kuwahara, M., Sasaki, S. and Imai, M. (2002). Cloning and identification of a new member of water channel (AQP10) as an aquaglyceroporin. *Biochem. Biophys. Acta* **1576**, 335-340.

Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T. and Marumo, F. (1994). Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. *Proc. Natl. Acad. Sci. USA* **91**, 6269-6273.

Kaneko, T. and Hasegawa, S. (1999). Application of laser scanning microscopy to morphological observations on drinking in freshwater medaka larvae and those exposed to 80% seawater. *Fish. Sci.* **65**, 492-493.

King, L. S., Yasui, M. and Agre, P. (2000). Aquaporins in health and disease. *Mol. Med. Today* **6**, 60-65.

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

Kobayashi, H., Uemura, H., Takei, Y., Itatsu, N., Ozawa, M. and Ichinohe, K. (1983). Drinking induced by angiotensin II in fishes. *Gen. Comp. Endocrinol.* **49**, 295-306.

Lignot, J.-H., Cutler, C. P., Hazon, N. and Cramb, G. (2002). Immunolocalisation of aquaporin 3 in the gill and gastrointestinal tract of the European eel *Anguilla anguilla* (L.). *J. Exp. Biol.* **205**, 2653-2663.

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

Marples, D., Frøkiaer, J. and Nielsen, S. (1999). Long-term regulation of aquaporins in the kidney. *Am. J. Physiol.* **276**, F331-F339.

Matsuzaki, T., Tajika, Y., Tserentsoodol, N., Suzuki., T., Aoki, T., Hagiwara, H. and Takata, K. (2002). Aquaporins: a water channel family. *Anat. Sci. Int.* **77**, 85-93.

Nielsen, S., King, L. S., Christensen, B. M. and Agre, P. (1997). Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. *Am. J. Physiol.* **273**, C1549-C1561.

Nielsen, S., Pallone, T., Smith, B. L., Christensen, E. I., Agre, P. and Maunsbach, A. B. (1995). Aquaporin-1 water channels in short and long

loop descending thin limbs and in descending vasa recta in rat kidney. *Am. J. Physiol.* **268**, F1023-F1037.

Oide, M. (1967). Effects of inhibitors on transport of water and ion in isolated intestine and Na^+-K^+ ATPase in intestinal mucosa of the eel. *Annot. Zool. Japon.* **40**, 130-135.

Oide, H. and Utida, S. (1967). Changes in water and ion transport in isolated intestines of the eel during salt adaptation and migration. *Mar. Biol.* **1**, 102-106.

Oide, H. and Utida, S. (1968). Changes in intestinal absorption and renal excretion of water during adaptation to sea-water in the Japanese eel. *Mar. Biol.* **1**, 172-177.

Park, J. H. and Saier, M. H., Jr (1996). Phylogenetic characterization of the MIP family of transmembrane channel proteins. *J. Membr. Biol.* **153**, 171-180.

Shehadeh, Z. H. and Gordon, M. S. (1969). The role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* **30**, 397-418.

Smith, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Am. J. Physiol.* **93**, 480-505.

Tsutsui, N., Kawazoe, I., Ohira, T., Jasmani, S., Yang, W. J., Wilder, M. N. and Aida, K. (2000). Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. *Zool. Sci.* **17**, 651-660.

Veillette, P. A., White, R. J. and Specker, J. L. (1993). Changes in intestinal fluid transport in Atlantic salmon (*Salmo salar* L) during parr-smolt transformation. *Fish Physiol. Biochem.* **12**, 193-202.

Verkman, A. S. (2002). Aquaporin water channels and endothelial cell function. *J. Anat.* **200**, 617-627.

Virkki, L. V., Cooper, G. J. and Boron, W. F. (2001). Cloning and functional expression of an MIP (AQP0) homolog from killifish (*Fundulus heteroclitus*) lens. *Am. J. Physiol.* **81**, R1994-R2003.

Yasui, M., Hazama, A., Kwon, T. H., Nielsen, S., Guggino, W. B. and Agre, P. (1999a). Rapid gating and anion permeability of an intracellular aquaporin. *Nature* **402**, 184-187.

Yasui, M., Kwon, T. H., Knepper, M. A., Nielsen, S. and Agre, P. (1999b). Aquaporin-6: an intracellular vesicle water channel protein in renal epithelia. *Proc. Natl. Acad. Sci. USA* **96**, 5808-5813.