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Roles of Aquaporin-3 Water Channels in Volume-Regulatory Water Flow in a Human Epithelial Cell Line

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Abstract. Membrane water transport is an essential event not only in the osmotic cell volume change but also in the subsequent cell volume regulation. Here we investigated the route of water transport involved in the regulatory volume decrease (RVD) that occurs after osmotic swelling in human epithelial Intestine 407 cells. The diffusion water permeability coefficient (*Pd*) measured by NMR under isotonic conditions was much smaller than the osmotic water permeability coefficient (*Pf*) measured under an osmotic gradient. Temperature dependence of *Pf* showed the Arrhenius activation energy (*Ea*) of a low value (1.6 kcal/mol). These results indicate an involvement of a facilitated diffusion mechanism in osmotic water transport. A mercurial water channel blocker ($HgCl_2$) diminished the *Pf* value. A non-mercurial sulfhydryl reagent (MMTS) was also effective. These blockers of water channels suppressed the RVD. RT-PCR and immunocytochemistry demonstrated predominant expression of AQP3 water channel in this cell line. Downregulation of AQP3 expression induced by treatment with antisense oligodeoxynucleotides was found to suppress the RVD response. Thus, it is concluded that AQP3 water channels serve as an essential pathway for volume-regulatory water transport in, human epithelial cells.

Key words: Water channel — Aquaporin — Osmotic swelling — Cell volume regulation — Regulatory volume decrease — Epithelial cell

Introduction

Osmotic perturbation is one inevitable problem that all cells must cope with. Cell activities themselves produce fluctuations of osmolyte concentration by transporting and metabolizing osmotically active substances. In the small intestine, for instance, active solute absorption induces prominent increases in the osmolarity both of the cytosol within absorbing enterocytes (Okada, 1979) and of the extracellular narrow space within the lamina propria of the villi (Jodal, Hallbäck & Lundgren, 1978; Bohlen & Unthank, 1989). Animal cells respond with swelling and shrinkage to hypotonic and hypertonic stress, respectively, due to osmotic water flow through the plasma membrane. After such osmotic swelling or shrinkage, however, animal cells exhibit volume regulation. Regulatory volume decrease (RVD) and regulatory volume increase (RVI) are eventually accomplished by osmotic water flow driven mainly by KCl efflux and NaCl influx, respectively. A number of ion channels and transporters have been identified as the pathways for volume-regulatory ionic flux (*see* reviews: Hoffmann & Simonsen, 1989; Okada & Hazama, 1989; Sarkadi & Parker, 1991; Lang et al., 1998; Okada, 2004). By contrast, the pathway for volume-regulatory water flow across the plasma membrane has not been systematically investigated by biophysical/physiological approaches.

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Water crosses the cellular plasma membrane by two fundamentally distinct routes: simple diffusion through the lipid bilayer and facilitated diffusion through the membrane-spanning proteins, channels or transporters. Ionic channels possess water-filled pores (Hille, 2001) and thus are expected to facultatively pass water molecules as well. In fact, the CFTR Cl^- channel has been shown to contribute to water permeation (Hasegawa et al., 1992). Also, significant water permeability has been suggested to be provided by a variety of transporters (Zeuthen, 1996), including Na^+-glucose symporter SGLT1 (Loo et al., 1996). Recently, aquaporins (AQPs) have been found to serve most effectively as water-selective pores in many water-transporting tissues (van Os, Deen & Dempster, 1994; Verkman et al., 1996; King, Kozono & Agre, 2004) including the gastrointestinal tract (Ma & Verkman, 1999). Thus, there is a possibility that not only the lipid bilayer but also AQPs and some other channels or transporters participate in volume-regulatory water flux.

A human epithelial cell line, Intestine 407, which lacks expression of CFTR mRNA (Hazama et al., 1998), is known to respond to a hypotonic challenge with a transient osmotic swelling followed by the RVD (Hazama & Okada, 1988) induced by activation of Ca^{2+} -activated K^+ (IK1) channels; (Wang, Morishima & Okada, 2003) and volume-sensitive outwardly rectifying (VSOR) Cl^- channels (Kubo & Okada, 1992). In the present study, the pathway of water flow during osmotic swelling and during a following RVD process in this cell line was investigated by addressing the following question: Which is the main route of water transport during the RVD in the human epithelial cell, lipid bilayers, volume-regulatory ion channels or a member of the AQP family?

Materials and Methods

Cells

A human intestinal epithelial cell line, Intestine 407, was cultured in Fischer medium supplemented with 10% newborn bovine serum, as described previously (Hazama & Okada, 1988). For immunofluorescence studies, the monolayer cells grown on coverslips were provided. For other experiments, the cells were isolated to single spherical cells after detaching from the plastic substrate, and cultured in suspension with agitation for 10–15 min before use, as reported previously (Kubo & Okada, 1992).

Solutions and Chemicals

For cell volume measurements using a fast image analyzing system, three kinds of solutions of different osmolality but of the same ionic strength were used. Hypotonic solution (75%) contained (in mM) 100 NaCl, 5 KCl, 3 HEPES, 2 NaHEPES, 1 $MgCl_2$, 1 $CaCl_2$ and 11 glucose (225 mosmol/kg-H_2O), whereas normotonic (300 mosmol/kg-H_2O) and hypertonic (375 mosmol/kg-H_2O) solutions were made by adding mannitol to the hypotonic solution. Isotonic or hypotonic solution used for cell volume measurements by a Coulter-type electronic cell-sizing technique contained (in mM) 95 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES/NaOH (pH 7.3) and 100 or 0 mannitol (310 or 200 mosmol/kg-H_2O). Osmolality of these solutions was measured each day before experiments by an osmometer (Osmometer OM801, Vogel, Germany).

$HgCl_2$ and methylmethanethiosulfonate (MMTS) were added directly to the solution when necessary. Gramicidin was first dissolved in dimethylsulfoxide (DMSO) and then diluted in the solution.

Isotonic or hypotonic K^+-free Cs^+-rich bath solution employed in whole-cell patch-clamp recordings contained (in mM): 110 CsCl, 12 HEPES/CsOH (pH 7.4), 8 Tris, and 80 or 30 mannitol. Pipette solution contained (in mM): 110 CsCl, 2 $MgSO_4$, 1 Na_2ATP, 15 NaHEPES, 10 HEPES, 1 EGTA, and 50 mannitol (pH 7.4).

The D_2O-PBS (D standing for deuterium) solution used for measurements of diffusional water permeability coefficient (*Pd*) contained (in mM): 54 NaCl, 1 KD_2PO_4 and 2 Na_2DPO_4 in D_2O. To make KD_2PO_4 and Na_2DPO_4, ^1H contained in Na_2HPO_4 and KH_2PO_4 were replaced with deuterium by dissolving these chemicals in D_2O and drying at 300 °C in a dry oven. The maneuver was repeated three times.

Cell Volume Measurements

Single-cell volume was measured in Intestine 407 cells lightly attached to the coverslip by a self-made computer-aided fast image analyzing system (Morishima et al., 1998). In brief, the cell images under an inverted microscope were taken through a CCD camera (C2400, Hamamatsu Photonics, Hamamatsu, Japan) and recorded in an optical video recorder (LQ-3100A, Panasonic, Kadoma, Japan) after processing by a digital image processor (DVS-3000, Hamamatsu Photonics). Spherical cells were focused so as to show their margin most clearly. To analyze thousands of cell images that were derived from a fast sampling, we let the computer system semi-automatically define the cell margin and calculate the cell volume. The cell margin was defined according to the following strategy. First the marginal points must be one of the darkest points in its local neighborhood. Second, they must preserve continuity; i.e., in the words of digital processing, the *next* points must be close enough to the last one. Finally, they must form a closed line similar to a circle or an ellipse. Then, the cell volume was calculated from the cross-section area. After the cells were equilibrated with normotonic bathing solution over 5 min, cell images were taken every 2 s for 33 min. Osmolality of bath solution was changed by perfusing a hypotonic or hypertonic solution. 80% and 100% of the solution bathing the chamber were found to be replaced within 15 and 30 s, respectively.

The mean cell volume was measured in Intestine 407 cells in suspension by a Coulter-type electronic size analyzer (CDA-500: Sysmex, Kobe, Japan), as previously described (Hazama & Okada, 1988).

Measurements of Osmotic Water Permeability

The cell membrane osmotic water permeability coefficient (Pf) was calculated from the slope of the tangential line to the curve of osmotic cell swelling induced by a hypotonic challenge, according to previous reports (Preston et al., 1993; Sabirov, Morishima & Okada, 1998).

Table 1. Primers designed for AQP 1-9

AQP1 a	5′ TCATCAGCATCGGTTCTGC 3′	5′ ACTTCACACCATCAGCCAGG 3′
b	5′ CAGATCAGCATCTTCCGTGC 3′	5′ GCTGAAGTTGTGTGTGATCACC 3′
AQP2 a	5′ TGTGCTACAGATTGCCATGG 3′	5′ AAGCCTATGGAGAGAGCAGG 3′
b	5′ TCAATGCTCTCAGCAACAGC 3′	5′ GCAGGATTCATAGAGCAGCC 3′
AQP3 a	5′ ACCAGTTCATAGGCACAGCC 3′	5′ AGCTGGTACACGAAGACACC 3′
b	5′ CCTTATCGTGTGTGTGCTGG 3′	5′ CTCAGATCTGCTCCTTGTGC 3′
AQP4 a	5′ GAAAAGCCTTTACCGGTCGAC 3′	5′ CAACCAGGAGACCATGACCAG 3′
b	5′ AAACGGAGTGATGTCACTGGC 3′	5′ TGTCCTCCACCTCCATGTAGCT 3′
AQP5 a	5′ TGTTCGCAGAGTTCTTGGC 3′	5′ GTTGTGTTGTTGTTGAGCGC 3′
b	5′ GCTGATTCTGACCTTCCAGC 3′	5′ GTAGATTCCGACAAGGTGGC 3′
AQP6 a	5′ GGTGCTCTGTGTCTTCGCTTC 3′	5′ CACCTCTACGGTGCCTGTGAG 3′
b	5′ GAGAAGCCTCCACAGAGAGCA 3′	5′ AAGGTGATGGAATCTGTAGGAC 3′
AQP7 a	5′ ACTGCAGAGGAAGATGGTGC 3′	5′ TGATCAGGAAGGTAGGTGGC 3′
b	5′ CCTGATCACATGACATTGTGG 3′	5′ GGTGGACTGAAGATCTGTTGG 3′
AQP8 a	5′ ACCACTGGAACTTCCACTGG 3′	5′ TTGAGAAGCAAGGAAGTGGC 3′
b	5′ AGAGATAGCCATGTGTGAGCC 3′	5′ TCAGGATGATCTCTGCCACC 3′
AQP9 a	5′ ATCAACGACTGTGCTTGTCC 3′	5′ TTAGCAAGACCAGAGGTGGC 3′
b	5′ AGACTGGTCTTGAAGAGCGC 3′	5′ TGAGGAGTATCATGGTGGCC 3′

Whole-cell Patch-clamp Recordings

To record swelling-activated Cl^- currents, whole-cell patch-clamp recordings were performed, as described previously (Kubo & Okada, 1992), using a patch-clamp amplifier system (EPC-9, HEKA Electroniks, Lambrecht, Germany). After the whole-cell configuration was attained, cells were bathed in normotonic solution, which was then changed to hypotonic solution. To eliminate K^+ currents Cs^+-rich bath and pipette solutions were used. Continuously, alternative step pulses (2 s duration) from a holding potential of 0 mV to ±40 mV were repetitively applied to monitor the activation time course of volume-regulatory Cl^- channels. Step pulses from a prepotential of −60 mV (for 1 s) to test potentials of –100 to + 100 mV (for 2 s) in 20 mV increments were applied to observe the current profiles. Data were filtered at 1 kHz and sampled at 3 kHz.

Measurements of Diffusional Water Permeability

The diffusional water permeability coefficient *(Pd)* of cell membrane was measured with D_2O by NMR. A known number (about 10^7) of cells were collected in a tube. After centrifugation at 200 ×*g* for 5 min, the supernatant was removed as completely as possible. The cell pellet was then mixed rapidly with 8 ml of PBS solution made of heavy water (D_2O) in a syringe, to the orifice of which a filter of 0.22 μm (Millipore, Bedford, MA) was attached. The filtrate (about 500 μl) was squeezed out from the syringe through the filter and sampled at 5, 10, 15, 20, 30 and 60 s.

^1H nuclear magnetic resonance (NMR) spectra of each sample were measured using the JNM-EX270 FT NMR system (JEOL, Akishima, Japan) operated at 270 MHz. To each sample for the NMR measurement, a precise amount of DMSO was added in the concentration of 100 mM to 1 M. The total amount of light water (^1H_2O) in the sample was determined by taking the ratio of area of the proton signal of water to that of methyl proton signals of DMSO. The area of the proton signals was calculated using the program pre-installed in the spectrometer system. The water efflux must equal the amount of water reduced from inside of cells or that increased on the outside of cells. Thus, the following equation is obtained:

$$J = -\frac{dCi(t)}{dt} \cdot Vcell \cdot N = \frac{dCo(t)}{dt} \cdot Vout$$

where J denotes the efflux of water in a second (mol/s), $Ci(t)$ and $Co(t)$ denote intracellular and extracellular concentrations of light water (^1H_2O) at time t (mol/cm^3), $Vcell$ denotes the mean cell volume (cm^3), N denotes the number of cells in a tube, and $Vout$ denotes the volume of extracellular solution. When $Co(t)$ in each sample was plotted as the function of time, it could be well fitted by a single exponential function. Pd was calculated from the time constant τ by the following equation:

$$\tau = \frac{Vcell \cdot N}{Pd \cdot A \cdot (Vout + Vcell \cdot N)/Vout}$$

where A denotes the total surface area of cells, calculated from the product of N and the mean cell surface area, which was estimated by mean membrane capacitance obtained by whole-cell recordings, assuming the specific membrane capacitance of 1 μF/cm^2.

RT-PCR

RT-PCR (reverse transcript polymerase chain reaction) studies were done to detect the expression of aquaporins. Poly(A)$^+$ RNAs were purified from 2–3 × 10^7 Intestine 407 cells, as described previously (Tsumura et al., 1998). First-strand cDNAs were synthesized by a reverse-transcription, and PCR was performed using the SuperScript™ Preamplification System (Invitrogen, Carlsbad, CA) and Ampli Taq Gold (Perkin Elmer, Norwalk, NC). Two sets of primers for each of AQP1 to AQP9 were designed and synthesized (Table 1) according to the published sequences of cDNA encoding human AQP1 (Accession number M77829), AQP2 (D31846), AQP3 (AB001325), AQP4 (U63622), AQP5 (U46566), AQP6 (AB006190), AQP7 (AB006190), AQP8 (AB013456), and AQP9 (AB008775). The PCR protocol for thermal cycling was as follows: 12 min preheating at 94°C, followed by thirty PCR cycles at 94°C (1 min)/55°C (2 min)/72°C (3 min), and 10 min final elongation at 72°C. When we could not find a positive band in gel-electrophoresis, we changed the annealing temperature from 55 to 50, 45, and 42°C. We also changed the Mg^{2+} concentration of PCR buffer from 1.5 mM to 2.5 mM when the result was negative and tried PCR

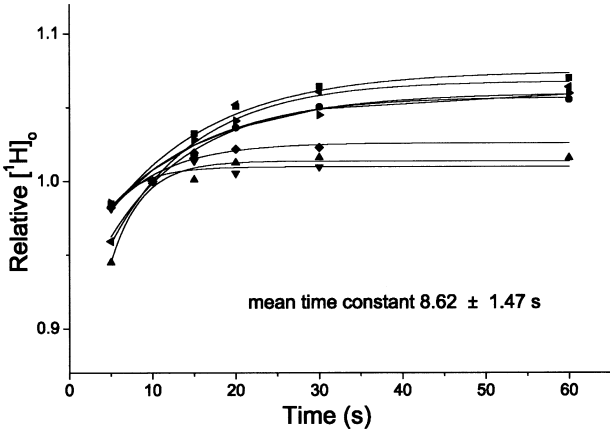

Fig. 1. Time course of NMR spectra of proton measured after resuspending Intestine 407 cells in heavy-water PBS. Each set of data ($n = 7$) was fitted to a single exponential curve. The mean time constant (8.62 ± 1.47 s) was used for evaluation of the *Pd* value.

again with various temperatures as listed above. As a positive control, we amplified a fragment of mRNA of glyceraldehyde 3-phosphate dehydrogenase (G-3-PDH) using the primers, as reported previously (Tsumura et al., 1998). Positive bands were cut out of the gel, and the cDNA was extracted, purified using the GENE CLEAN Kit (Bio 101, Carlsbad, CA) and subjected to sequencing with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA)

Antisense Knockdown of AQP3

The phosphorothioated oligodeoxynucleotides corresponding to the initiation codon region (1 to 21) of the human AQP3 mRNA (Echevarria et al., 1994) were synthesized. The antisense and sense oligodeoxynucleotides had the sequences 5′-CAGTCCT TCTGTCGACCCAT-3′ and 5′-ATGGGTCGACAGAAGGA GCTG-3′, respectively. To facilitate the uptake of the oligodeoxynucleotides by the cells, cells were cultured for 10 days in culture medium containing either of the oligodeoxynucleotides (20 μM), and then treated with a transfection reagent, Oligofectamine (Invitrogen, Carlsbad, CA), for 6 h in the same medium.

Immunocytochemistry

The cells grown on coverslips were fixed in 1% formaldehyde in PBS for 15 min at room temperature. After brief washing with PBS, the cells were permeabilized with 0.1% Triton X-100 for 10 min. After soaking in a blocking solution (1% BSA in PBS) for 10 min, the cells were then incubated with the rabbit anti-human AQP3 polyclonal antibody (1:200 dilution; CHEMICON International, Temecula, CA) as a primary antibody for 1 h. After washing with PBS three times, the cells were then incubated with the rhodamine-conjugated goat anti-rabbit IgG (1:200 dilution; CHEMICON) as a secondary antibody. The cells were again washed with PBS three times and then embedded in 90% glycerol-PBS containing 0.1% *p*-phenylendiamine and 1% *n*-propyl gallate and visualized using a fluorescence microscope (model BX50; Olympus, Tokyo). As a negative control, 1% albumin-PBS was applied instead of anti-human AQP3 antibody.

All the experiments were carried out at room temperature (20–25°C), unless otherwise noted. All the data are expressed as the mean ± SEM. Statistical differences of the data were evaluated by Student't *t*-test or ANOVA followed by Scheffe's post-hoc test, when needed, and considered significant at $P < 0.05$.

Results

Involvement of Facilitated Diffusion in Osmotic Water Transport

First we measured the diffusional water permeability coefficient *(Pd)* of the plasma membrane in Intestine 407 cells. Figure 1 shows the relative amount of ^{1}H determined by the NMR measurements of the cell suspension sampled at 5, 10, 15, 20, 30 and 60 s after the cells were resuspended in the ^2D_2O-PBS solution under normotonic conditions. From the mean time constants evaluated for curves fitted to a single exponential function, the mean *Pd* values were calculated to be 0.47 ± 0.04 μm/s ($n = 7$).

To determine the osmotic water permeability coefficient *(Pf)* for water influx and efflux under the driving force of osmotic gradients, rapid changes in cell volume were monitored by the computer-aided fast image analysis system upon osmotic challenges. Intestine 407 cells rapidly responded to hypotonic and hypertonic challenges with osmotic swelling and shrinkage, respectively, as shown in Figs. 2*A* and 2*B*. In this cell line, as reported previously (Hazama & Okada, 1988), the RVD process was found to follow swelling, whereas the RVI after shrinkage was not significant. Although the cell volume level varied to a certain extent after peak osmotic swelling or shrinkage, the initial swelling or shrinkage response was uniform. From the slope of the line tangential to either rising or falling phase of cell volume (Figs. 2*A* and *B broken lines)*, the *Pf* value was calculated and found to be identical to each other (17.5 μm/s). The *Pf* value was much larger than the *Pd* value, and the *Pf*/*Pd* ratio was 37.2. It is known that the *Pf*/*Pd* ratio is approximately unity, if membrane water transport is mediated solely by simple diffusion, whereas this ratio largely exceeds 1, if it is mediated also by facilitated diffusion (Agre et al., 1993). Thus, the present result of $Pf/Pd \gg 1$ strongly suggests an involvement of facilitated diffusion in the membrane water transport driven by osmotic gradients in Intestine 407 cells.

Since water transport via lipid bilayers is strongly affected by organization of membrane lipid molecules, simple diffusion of water molecules via lipid bilayers can be characterized by Arrhenius activation energy *(Ea)* higher than 10 kcal/mol, whereas that for facilitated diffusion is less than 6 kcal/mol (Agre et al., 1993). Thus, we next measured *Pf* values upon a hypotonic challenge at various temperatures, and

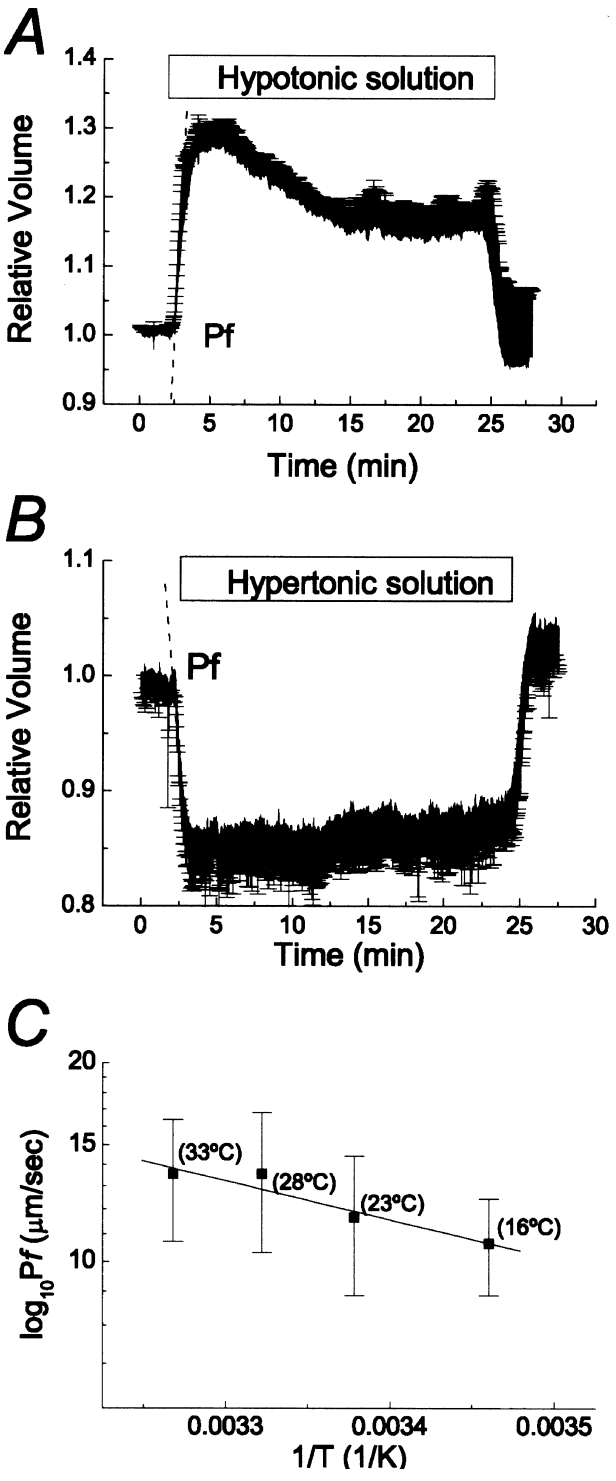

Fig. 2. Evaluation of the *Pf* value and its temperature dependence in Intestine 407 cells. *(A, B)* Changes in cell volume before, during and after application of a hypotonic *(A)* or hypertonic *(B)* solution at 23°C. The data represent the means ± SE. From the slope of the tangential line (*broken line*) for onset of osmotic swelling or shrinkage, the *Pf* value was calculated to be 17.5 μm/s. (*C*) Arrhenius plot of *Pf* values. The *Pf* values determined at different temperatures were plotted against $1/T$, where T denotes absolute temperature. The correlation coefficient of the regression line was -0.97 ± 0.17 ($P < 0.05$). From the slope (−590 ± 96), which is significantly different from zero ($P < 0.01$), the activation energy was calculated to be 1.6 kcal/mol. The data represent the means ± SE of 4 – 5 observations.

the data were plotted in the Arrhenius plot (Fig. 2*C*). The *Ea* value calculated from the slope of the Arrhenius plot is 1.6 kcal/mol. This result again indicates an involvement of facilitated diffusion in osmotic water movement via the plasma membrane of Intestine 407 cells.

Involvement of Sulfhydryl Reagent-sensitive Pathway in Osmotic Water Transport

Water transport mediated by many isoforms of aquaporin is known to be inhibited by mercurial sulfhydryl reagents such as $HgCl_2$ and pCMBS (Macey & Farmer, 1970; Agre et al., 1993; Verkman et al., 1996). AQP3-mediated water transport in the oocyte expression system was also found to be blocked by $HgCl_2$ (Ishibashi et al., 1994; Kuwahara et al., 1997). Thus, the effects of $HgCl_2$ on osmotic and regulatory volume changes were first examined by adding this mercurial sulfhydryl reagent directly to the solution. At 10 μM, $HgCl_2$ was found to reduce the *Pf* value from 19.6 ± 3.1 to 12.4 ± 2.6 μm/s ($n = 4$: $P < 0.01$). This fact suggests that some mercury-sensitive water channel serves as a pathway for osmotic water transport upon a hypotonic challenge.

Our preliminary study, which was made in *Xenopus* oocytes transfected with the AQP3 gene according to the methods described previously (Higuchi et al., 1998), demonstrated that the *Pf* value was reduced from 148 ± 33 μm/s ($n = 16$) to 43.2 ± 21 μm/s ($n = 18$) by 300 μm of MMTS. Thus, the effects of MMTS on the *Pf* value in Intestine 407 cells were next examined. The non-mercury sulfhydryl reagent, MMTS, was observed to reduce the osmotic swelling rate (Fig. 3*A*). Hence, the *Pf* value was significantly ($P < 0.01$) reduced by 3 μm, 300 μm and 3 mm MMTS from 19.5 ± 5.4 to 11.2 ± 3.2, 10.7 ± 1.6 and 7.5 ± 2.7 μm/s ($n = 4 – 5$), respectively. Thus, it is suggested that sulfhydryl reagent-sensitive cysteine residues have a role in the water transport pathway for osmotic swelling in Intestine 407 cells.

RVD was also blocked by the non-mercurial sulfhydryl reagent, MMTS, in a concentration-dependent manner (Fig. 3*A*). The MMTS effect was reversible, and non-specific cell damage observed under a microscope was insignificant. A similar blocking effect of $HgCl_2$ on the volume recovery after peak swelling was observed (*data not shown*, $n = 3$),

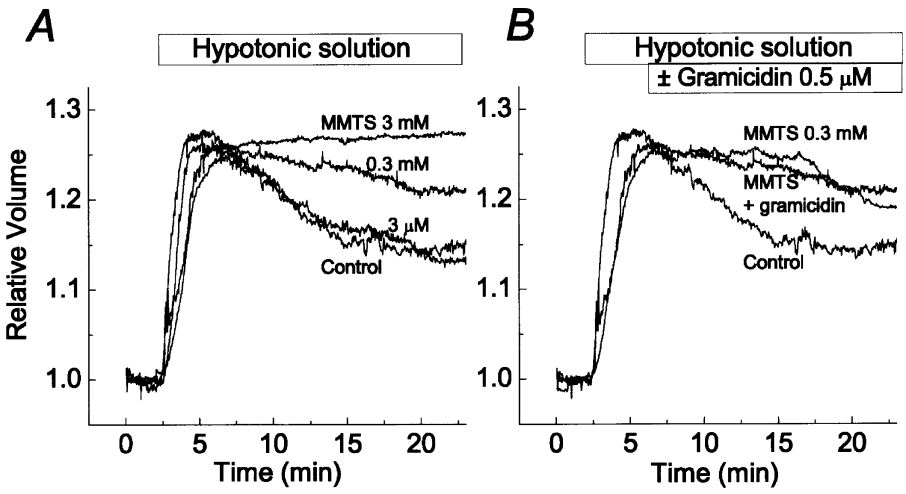

Fig. 3. Inhibitory effects of MMTS on osmotic swelling rate and RVD in the absence *(A)* and presence *(B)* of gramicidin (0.5 μM) in Intestine 407 cells. MMTS did not significantly affect the RVD time course at 3 μM, whereas MMTS significantly retarded RVD at 300 μM ($P < 0.05$ from 12 to 23 min after the hypotonic challenge) and abolished the volume recovery at 3 mM of MMTS ($P < 0.01$ vs. control from 10 to 23 min after the hypotonic challenge) *(A)*. The inhibitory effect of MMTS at 300 μM was not significantly affected by gramicidin *(B)*.

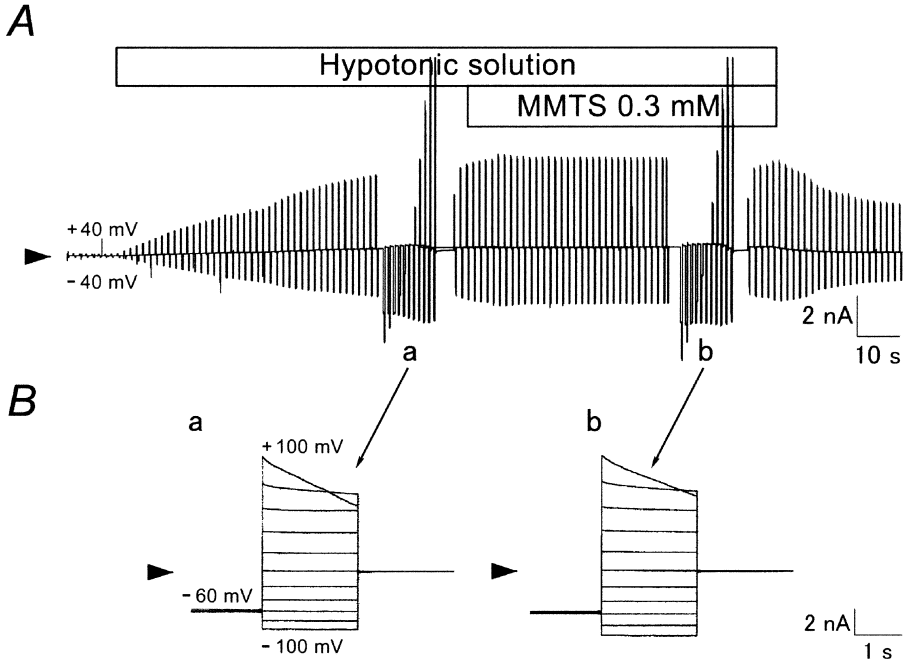

Fig. 4. Lack of effects of MMTS (300 μM) on whole-cell VSOR Cl^- currents in Intestine 407 cells. *(A)* Representative record before, during and after exposure to hypotonic solution. Currents were monitored by application of alternating pulses from 0 to ±40 mV or of step pulses from -60 mV to ±100 mV in 20 mV increments (at *a* and *b*). *(B)* Expanded traces of current responses (*a* and *b* in *A*) to step pulses.

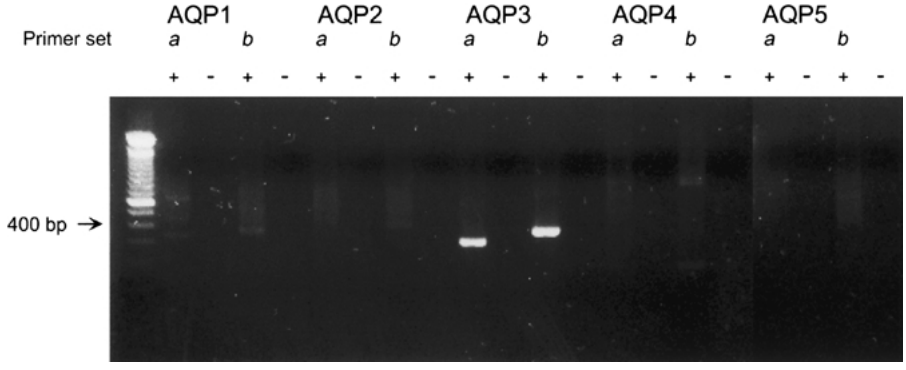

Fig. 5. RT-PCR analysis of the expression of mRNA for AQP1 to AQP5 in Intestine 407 cells. The first lane contains size markers (100 bp ladder), the following lanes represent PCR products for AQP1 to AQP5 using the primer set *a* or *b* in the presence (+) or absence (−: negative control) of reverse transcriptase. Note the detection of signals corresponding to AQP1 (327 and 360 bp for the primer set *a* and *b*, respectively) and AQP3 (301 and 371 bp for the primer sets *a* and *b*, respectively).

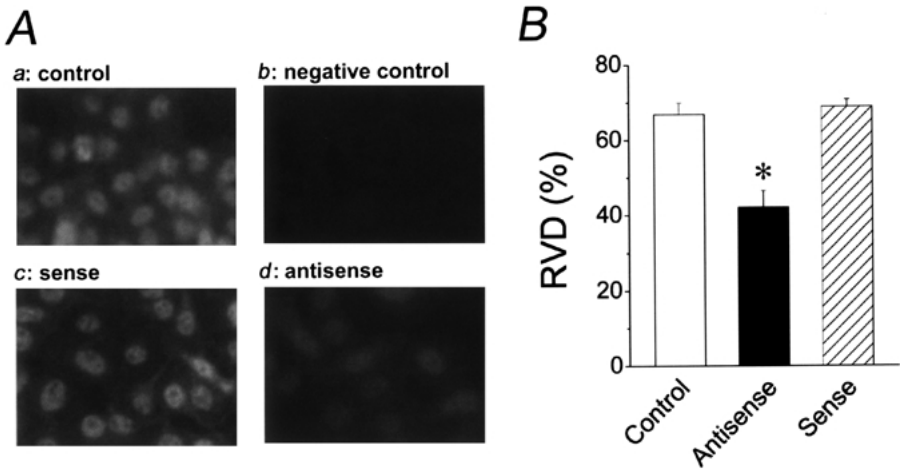

Fig. 6. Effects of antisense knockdown of AQP3 on the AQP expression and RVD in Intestine 407 cells. (*A*) Immunocytochemistry using control cells exposed to primary and secondary antibodies (*a*: positive control) and only to secondary antibodies *(b:* negative control), and using cells treated with sense oligodeoxynucleotides *(c)* and antisense oligodeoxynucleotides *(d)*. Similar data were obtained in 3 independent experiments. *(B)* Effect of treatment with antisense and sense oligodeoxynucleotides on the percent volume recovery (RVD) at 20 min after a hypotonic challenge. Each column represents the mean value of 8 observations. *Significantly different from the control.

but the cells were found to be damaged shortly thereafter.

Lack of Involvement of Volume-regulatory K^+ and Cl^- Channels in Volume-regulatory Water Transport

Since the water transport that leads to RVD is driven by KCl efflux produced by activation of K^+ and Cl^- channels in this cell line (Hazaina & Okada, 1988), there is a possibility that the MMTS effect on RVD observed in Fig. 3*A* was indirectly brought about by inhibiting either volume-regulatory K^+ or Cl^- channels. Also, it is possible that MMTS directly blocked water transport through swelling-activated K^+ and/or Cl^- channels.

We first tested whether the K^+ channel activity involved in the RVD was inhibited by MMTS. Since a cation ionophore, gramicidin, has been shown to provide an alternate pathway for volume-regulatory K^+ efflux when K^+ channel activity is impaired in a variety of cell types (Grinstein et al., 1984; Hoffman, Lambert & Simonsen, 1986; MacLeod & Hamilton, 1991; Pasantes-Morales et al., 1994), we examined whether gramicidin restores the RVD in the presence of MMTS in Intestine 407 cells. As shown in Fig. 3*B*, the MMTS-induced RVD impairment was not reversed by co-application of gramicidin. This result indicates that RVD inhibition induced by MMTS cannot be explained by inhibition of the volume-regulatory K^+ channel.

We next observed effects of MMTS on the swelling-activated whole-cell Cl^- current in Intestine 407 cells. As reported previously (Kubo & Okada, 1992), volume-sensitive outwardly rectifying (VSOR) Cl^- currents, which exhibited inactivation kinetics at large positive potentials, were activated by osmotic swelling under a whole-cell mode. MMTS did not affect the amplitude of VSOR Cl^- current, as shown in Fig. 4. The mean current density recorded at $+40$ mV was 113.2 ± 21.2 pA/pF and 108.7 ± 36.2 pA/pF ($n = 6$: $P > 0.05$) before and after application of 0.3 mm MMTS, respectively. Neither outward rectification nor inactivation kinetics was affected by MMTS, as shown in Fig. 4*B*.

Taken together, it appears that both volume-regulatory K^+ and Cl^- channels are insensitive to MMTS and not capable of serving as the MMTS-sensitive water transport pathway during the RVD in Intestine 407 cells. Thus, it is suggested that sulfhydryl reagent-sensitive water channels are functioning as the route for volume-regulatory water transport.

Involvement of Aquaporin-3 in the Water Transport During the Regulatory Volume Decrease

Molecular expression of the aquaporin water channel was examined in Intestine 407 cells by RT-PCR using two different sets of primers for AQP1 to AQP9 (Table 1). As demonstrated in Fig. 5, prominent expression of AQP3 was consistently observed, and a tiny amount of AQP1 mRNA was also detected. These PCR products were sequenced and confirmed to be identical to human AQP3 and AQP1. However, we never detected expression of AQP2, AQP4 and AQP5 (Fig. 5). Also, we could not amplify fragments of mRNAs of AQP6 to AQP9, while the PCR product of G-3-PDH was consistently detected (*data not shown*).

Immunocytochemistry also confirmed significant expression of AQP3 protein. Figure 6*A* shows that all the Intestine 407 cells were stained by rhodamine-conjugated goat anti-rabbit IgG after incubation with anti-human AQP3 antibody *(a)* but not after incubation with albumin *(b)*. Expression of AQP3 protein was partially suppressed by treatment with antisense oligodeoxynucleotides of AQP3 (Fig. 6*Ad)*, but not by treatment with the sense oligodeoxynucleotides (Fig. 6*Ac)*.

Next we examined the effect of antisense treatment on the RVD. Since the degree of AQP3 knockdown varied from cell to cell, we made mean cell volume measurements by an electronic cell-sizing technique instead of single-cell volume measurements by a fast image analysis technique. By this method the RVD process was found to be markedly suppressed in antisense-treated cells compared to control or sense-treated cells (Fig. 6*B)*. Thus, it is concluded that AQP3 water channels serve as a major pathway for water transport associated with the RVD in Intestine 407 cells.

Discussion

Water transport across the biological cell membrane takes place via lipid bilayers and transmembrane transport proteins (Finkelstein, 1987). Simple diffusion of water through lipid bilayers is characterized *by* $Pf/Pd \cong 1$, $Ea > 10$ kcal/mol and pharmacological insensitivity, whereas facilitated diffusion of water through channels and/or transporters is by $Pf/Pd >> 1$ and $Ea < 5$ kcal/mol (Agre et al., 1993). The present study showed a high Pf/Pd value (around 37: Fig. 1 and Fig. 2*A*, *B*) and a low *Ea* value (1.6 kcal/mol: Fig. 2*C*) in Intestine 407 cells. These observations completely fit the above criteria of water transport by facilitated diffusion.

A variety of ion channels and transporters, when activated, have been implicated in serving as water transport pathways. The candidates proposed so far include a cAMP-activated Cl^- channel, CFTR (Hasegawa et al., 1992), a glucose transporter, GLUT1 (Fischbarg et al., 1989) and a Na^+-gulcose cotransporter, SGLT1 (Loo et al., 1996), and a Na^+-glutamate cotransporter, EAAT1 (MacAulay et al., 2001). However, Intestine 407 cells lack expression of CFTR (Hazama et al., 1998), and osmotic water transport was measured, in the present study, without adding substrates (glucose and glutamate) for these transporters.

Animal cell membranes contain the proteins called aquaporins (AQPs) that serve as water channels (Preston et al., 1992; Agre et al., 1993; Verkman et al., 1996). Some AQP family members, such as AQP1, AQP2 and AQP3, are known to be sensitive to mercurial sulfhydryl reagents, because of the presence of a critical sulfhydryl residue (cysteine) close to the pore (Kuwahara et al., 1997). In the present study, osmotic water transport was found to be significantly suppressed either by a mercurial sulfhydryl reagent, $HgCl_2$, or by a non-mercurial sulfhydryl reagent, MMTS (Fig. 3), in Intestine 407 cells. Also, the present RT-PCR (Fig. 5) and immunocytochemical studies (Fig. 6*A*) demonstrated that AQP3, which was cloned from kidney (Echevarria et al., 1994; Ishibashi et al., 1994; Ma et al., 1994), is the major aquaporin expressed in Intestine 407 cells. This is in good agreement with a previous report that AQP3 mRNA and protein are expressed throughout the intestine (Ramirez-Lorca et al, 1999). Therefore, it appears that AQP3 is involved in osmotic water transport. However, it must be noted that osmotic swelling could finally be attained even in the presence of a high concentration of water channel blocker, though the osmotic swelling rate was markedly reduced (Fig. 3). Thus, osmotic cell swelling can be accomplished by water movement via both, water channels and lipid bilayers. Similarly, recent studies in AQP1-, AQP4- and AQP5-deficient mice suggested that AQP1, AQP4 and AQP5 provide major pathways for osmotic water transport in proximal tubular membrane vesicles (Ma et al., 1998), initial inner medullar collecting duct cells (Chou et al., 1998) and salivary acinar cells (Krane et al., 2001), respectively.

Water-filled pores are commonly believed to be installed in ion channels (Hille, 2001). In Intestine 407 cells the RVD is known to be induced by activation of VSOR Cl^- channels (Kubo & Okada, 1992; Okada, 1997) and the IK1 type of Ca^{2+}-activated K^+ channels (Wang et al., 2003). Thus, the possibility arises that these volume-regulatory K^+ and Cl^- channels mediate not only volume-regulatory KCl efflux but also water efflux that is a final event for the RVD. However, the present study excludes the possibility that MMTS-induced inhibition of RVD was due to inhibition of either the volume-regulatory K^+ or Cl^- channel (Figs. 3*B* and 4). Also, it was found that MMTS (300 μm) failed to affect whole-cell IK1 currents activated by elevation of intracellular free Ca^{2+} concentration in Intestine 407 cells in our preliminary study (T. Shimizu and Y. Okada, unpublished observations). Thus, it appears that these volume-regulatory K^+ and Cl^- channels are independent of the MMTS-sensitive water permeability in Intestine 407 cells.

AQP3 is the major aquaporin expressed in this cell line (Fig. 5) and possesses a mercury-sensitive residue, cysteine-11 (Kuwahara et al., 1997). Also, osmotic water permeability in AQP3-transfected oocytes was found to be sensitive to MMTS. Therefore, it is possible that AQP3 is the MMTS-sensitive water transport pathway responsible for the RVD. In the present study, indeed, downregulation of AQP3 expression by antisense treatment was

found to suppress the RVD activity of Intestine 407 cells (Fig. 6), indicating a major role of AQP3 in the RVD process. Recently, the critical role of a distinct AQP member (AQP5) in the RVD response in salivary acinar cells was suggested from studies in AQP5-knockout mice (Krane et al., 2001). Thus it appears that AQP water channels are essential for volume-regulatory water transport driven by KCl efflux during the RVD in epithelial cells. The mechanism is not clear. Since water permeability can be largely provided by lipid bilayers as well, however, it is conceivable that aquaporins play some role other than the aquapore water pathway in RVD; for instance, by acting as a sensor of mechanostress or of turgor pressure difference in the plasma membrane (Hill et al., 2004).

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