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Regulation of aquaporin 3 expression by magnesium ion

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ABSTRACT

For understanding the actions of magnesium formulations, magnesium oxide and magnesium sulfate as a constituent of antacid, in the gastrointestinal tract, the effect of magnesium ion on the water channel aquaporin 3 (AQP3) known to be permeable mainly to water and glycerol was investigated in Caco-2 cells. The mRNA and protein of aquaporin 3 were detected by real-time RT-PCR and Western blotting, respectively, and found to increase significantly after treatment with magnesium acetate. Inhibitors for signal transducers, MDL-12330A, H-89, U0126, and Ro 31-8220, were shown to repress the increase in expression of the mRNA. A luciferase reporter vector containing bp -1382 to -12 of the 5′-flanking region of the aquaporin 3 gene was constructed for a reporter gene assay. The luciferase activity in transfectants increased on treatment with magnesium acetate. Serial deletion constructs revealed two regions responsible for the magnesium ion-mediated activation, one between bps -404 and -190, and the other between bps -190 and -82. siRNA for the cAMP response element-binding protein (CREB) sequence located between bp -404 and -190 counteracted the magnesium ion-mediated activation of aquaporin 3 transcription. These results suggest that signal transducers, adenylyl cyclase, protein kinase A (PKA), mitogen-activated protein kinase 1/2 (MEK1/2), and mitogen- and stress-activated protein kinase 1 (MSK1), were involved in the signaling pathway for regulating transcription of the aquaporin 3 gene and CREB is one of the transcriptional regulators for aquaporin 3 gene expression mediated by magnesium ion.

1. Introduction

The water channel membrane protein aquaporin was first found by Preston and Agre (1991). Some 13 molecular species of aquaporin have been reported in human tissues so far (King et al., 2004). Aquaporin 3, discovered fourth, is distributed mainly in the human gastrointestinal tract, red blood cells, the kidney, skin, and the brain, especially abundant in the ileum and colon (King et al., 2004; Mobasheri et al., 2005), and reported to be expressed more on the apical membrane of villus epithelial cells in human colon (Silberstein et al., 1999), but more on the basolateral membrane of villus epithelial cells in rat colon (Frigeri et al., 1995). Aquaporin 3 is permeable not only to water, but also to water-soluble low-molecular weight molecules such as glycerol and urea, and so is also called aquaglyceroporin. The physiological roles of aquaporin 3 in the kidney, gastrointestinal tract, and skin have been discussed based on observations in aquaporin 3-deficient mice since a congenital failure of aquaporin 3 in humans is not yet found. Aquaporin 3 may be involved in renal failure caused by nephrogenic diabetes insipidus and hydronephrosis (Yang et al., 2001), the absorption of glycerol resulting from a decrease in the level of glycerol in serum (Ma and Verkman, 1999), and skin hydration from the promotion of dry skin (Hara et al., 2002).

Tsujikawa et al. (2003) reported that the expression of aquaporin 3 mRNA increased in the rat residual ileum and colon after small bowel resection. The rats were observed to have diarrhea due to poor digestive absorption of water and ingested nutrients in the immediate aftermath of resection, but the diarrhea gradually improved in about 1 week with adaptation. In this period, the expression of aquaporin 3 mRNA was found to increase 3 fold in the residual ileum and colon 1 day after resection. Itoh et al. (2003) reported that the mRNA and protein levels of aquaporin 3 in the human colonic epithelial cell line HT-29 increased on treatment with vasoactive intestinal peptide (VIP). The intravenous injection of VIP into healthy adults was reported to result in diarrhea (Kane et al., 1983) and the level of VIP in rat serum was observed to increase with chemical colitis (Kishimoto et al., 1992). Thus, aquaporin 3 seems to be involved in the progression of and/or recovery from diarrhea by taking part in the movement of water and glycerol in the intestinal tract and participates in the actions of VIP.

Magnesium, a biologically essential metal, is found predominantly in bone and muscle tissues which contain 67% and 20%, respectively, of all the magnesium in the body (Alfrey, 1999). About 1% of the total is to be found in extracellular fluid. Magnesium oxide is widely administered clinically to treat coprostasis as an antacid and a mild lapactic. Hypermagnesaemia was reported to result from the retarded clearance of magnesium due to a functional disorder of the kidney on the administration of magnesium sulfate (Garcia-Webb et al., 1984)

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and the excessive administration of magnesium oxide promoted hypermagnesaemia in patients with renal disorders (Baker and Worthley, 2002). The action of magnesium oxide is speculated to promote intestinal peristalsis with the movement of water in the intestinal tract, but the mechanism is not yet fully understood. It is still unknown how aquaporins involved in the movement of water and small molecular weight molecules in this organ are affected by magnesium ion.

We previously reported that the expression of aquaporin 3 in Caco-2 cells was repressed by insulin and this was controlled by the transcription factor forkhead box a2 (Foxa2) and proposed that aquaporin 3 was involved in fatty acid oxidation and glycometabolism given its permeability to glycerol (Higuchi et al., 2007). In this report, we investigate whether magnesium ion affects the expression of aquaporin 3, in order to clarify further the mechanism of action of magnesium formulations, and then find increased expression of aquaporin 3 mRNA on the treatment of Caco-2 cells with magnesium acetate. We also demonstrate a signal transduction pathway triggered by magnesium ion that leads to the gene expression of aquaporin 3 and the identification of a transcriptional regulator for aquaporin 3 mRNA expression responding to the magnesium ion-evoked signaling.

2. Materials and methods

2.1. Materials

Magnesium oxide, magnesium sulfate, magnesium chloride, and magnesium acetate were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). TRIzol, the Oligo $(dT)_{12-18}$ Primer, and SuperScript III reverse transcriptase were obtained from Invitrogen Corp. (Carlsbad, CA, USA). TaKaRa EX Taq, Syber Premix Ex Taq, and TaKaRa Taq, and FuGene6 were purchased from Takara Bio Inc. (Osaka, Japan) and Roche Diagnostics K. K. (Tokyo, Japan), respectively. The Dual-Luciferase Reporter Assay System, the GeneEditor *in vitro* Site-Directed Mutagenesis System, and other DNA-manipulating enzymes were acquired from Promega Corp. (Madison, WI, USA). siRNAs for the CREB and ATF1 sequences and a negative control were purchased from Ambion Inc. (Austin, TX, USA). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene (U0126), *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl hydrate (H-89), and calphostin C, *N*-(Cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride (MDL-12330A) and (8 R,9 S,11 S)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7 b,11 a -trizadibenzo-(a, g)-cycloocta-(c, d, e)-trinden-1-one (KT-5823), and Bisindolylmaleimide IX (Ro 31-8220) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan), Sigma-Aldrich Japan K. K. (Tokyo, Japan), and Merk Biosciences-Calbiochem Inc. (Darmstadt, Germany), respectively. All other chemicals and reagents were of analytical grade.

2.2. Cell culture and treatment with magnesium compounds

Caco-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids solution (Invitrogen-Gibco Corp.), 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were plated on a 35-mm dish at a density of 3×10^6 10^6 cells/ml and incubated in a CO_2 incubator for 36 h, then treated with magnesium compounds dissolved in the culture medium in a CO_2 incubator. Cells passaged 25 to 35 times were used in experiments.

2.3. Measurement of magnesium

The amount of intracellular magnesium ion was determined by the atomic absorption method using an AA-6500 atomic absorption flame emission spectrophotometer (Shimazu Corp., Kyoto, Japan). One million Caco-2 cells were incubated with 50 mM magnesium acetate for 1 h in the culture medium and washed with phosphate-buffered saline (PBS) containing 10 mM EDTA and 150 mM choline chloride, and then with 150 mM choline chloride. Cells were lysed with 2 ml of 5% trichloroacetic acid-1.75% $LaCl_3$ and stood for 20 min at room temperature. The supernatant was recovered by centrifugation and the magnesium concentration in the supernatant was determined with a standard magnesium solution.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from Caco-2 cells with TRIzol reagent. First-strand cDNAs were prepared from 5 μg of total RNA by SuperScript III reverse transcriptase and used as a template. Real-time RT-PCR was performed with the specific primers listed in Table 1 and SYBR Premix Ex Taq using a iCycler-iQ real-time PCR device (Bio-Rad Laboratories, Hercules, CA, USA). The cycle threshold signal was used to measure RT-PCR signals. At the end of the PCR, a dissociation curve was produced to examine the specificity of the PCR product. The glycelaldehyde 3-phosphate dehydrogenase (GAPD) housekeeping gene was used for normalization of target mRNA expression. RT-PCR was performed with the primers for CREB and ATF1 under optimal conditions in order to confirm inhibition of transcription for these

Table 1
Sequences of oligonucleotide primers and assay conditions for RT-PCR

Gene	Primers		PCR condition		Cycles	Co-efficiency of variation
Aquaporin 3	Forward	5′-GGAATAGTTTTTGGGCTGTA-3′	94 °C	30 s	27	0.99
	Reverse	5′-GGCTGTGCCTATGAACTGGT-3′	57 °C	30 s		
			72 °C	1 min		
CREB	Forward	5′-GGAGCTTGTACCACCGGTAA-3′	94 °C	30 s	29	0.98
	Reverse	5′-GGGCTAATGTGGCAATCTTGT-3′	52 °C	30 s		
			72 °C	1 min		
Sp1	Forward	5′-GGCTACCCCTACCTCAAAGG-3′	94 °C	30 s	29	0.96
	Reverse	5′-CACAACATACTGCCCACCAG-3′	52 °C	30 s		
			72 °C	1 min		
ATF1	Forward	5′-GAAGTGGGTAGTGAATTCGG-3′	94 °C	30 s	29	0.98
	Reverse	5′-CCTATGCTGTCGGATGATGC-3′	56 °C	30 s		
			72 °C	30 s		
GAPD	Forward	5′-CAATGACCCCTTCATTGACC-3′	94 °C	30 s	19	1.00
	Reverse	5′-GACAAGCTTCCCGTTCTCAG-3′	60 °C	30 s		
			72 °C	30 s		

A co-efficiency of variation is used to show the consistency of inter-assay and intra-assay in the real-time RT-PCR method. GAPD gene was used for normalization of target mRNA expression.

genes targeted by the siRNA. PCR products were resolved on a 1.5% agarose gel and detected with ethidium bromide using a Fluoro-Image Analyzer FLA-5100.

2.5. Western blotting

Caco-2 cells cultured with or without magnesium acetate were lysed with 1% sodium dodecyl sulfate (SDS) and 4 mM 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was carried out with a 12% polyacrylamide gel according to the method of Laemmli (1970). Western blotting was performed with goat anti-aquaporin 3 antibody, goat anti-GAPD antibody, and anti-goat IgG peroxidase conjugate (Sigma-Aldrich, Saint Louis, MO) using the method of Towbin et al. (1979). Peroxidase activity was detected using an ECL Western Blotting Detection System according to the manufacturer's instructions (GE Healthcare-Amersham Biosciences, UK). Band intensity was quantified using the Scion Image program.

2.6. Construction of reporter vector

The procedure for constructing reporter plasmid vectors was reported previously (Higuchi et al., 2007). A DNA fragment containing the regulatory region -1382 to -12 5′-upstream of aquaporin 3′s transcription initiation site was prepared by PCR and the product was ligated into the multiple cloning site of a pGL3 basic reporter vector. The plasmid obtained was named 1382pGL3. Serial deletion of the fragment was performed with nuclease and the plasmids which contained the deletion fragments spanning bp -780 to -12 and -404 to -12 were named 780pGL3 and 404pGL3, respectively. A DNA fragment containing the regulatory region -190 to -12 5′-upstream of aquaporin 3′s transcription initiation site was prepared by PCR with a forward primer containing a Kpn I recognition site, 5′-CAGGTACCGCTCCTCACACTCCATGCCC-3′, and a reverse primer containing a Xho I recognition site, 5′-CGCTCGAGAGCGCTGGTGGCTCCCTTTA-3′. After the PCR product was digested with both restriction enzymes, the fragment was ligated into the multiple cloning site of a pGL3 basic vector. The plasmid obtained was named 190pGL3. This product was also treated with restriction enzymes, Pst I and Hind III, and the digested fragment bp -82 to -12 was ligated into a pGL3 basic vector, yielding 82pGL3.

2.7. Site-directed mutagenesis

Site-directed mutagenesis in the Sp1-binding element was performed with the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Five micrograms of 190pGL3 as a template DNA and an oligonucleotide for the mutagenesis, 5′-CCTGCAGTCCCGGAGCGCCGAGG-AGTGTTTTTCCCCTCTTCCCCTTCTTACCTCGGCTGGGAGGCTGGTGCGG-3′, were used. The mutated plasmid obtained was named 190-4mSP1pGL3.

2.8. Transcriptional analysis

Caco-2 cells were plated onto a 24-well culture plate at a density of 5×10^4 10^4 cells/well and cultured for 4 h in a CO_2 incubator. After attaching to the plate, the cells were transfected with 0.4 μg of the reporter vectors prepared above and 5 ng of phRL-TK as a reference plasmid with 1.2 μL of FuGene6 according to the manufacturer's instructions. The activities of luciferases from the firefly and *Renilla* were detected with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions.

Caco-2 cells (3.5×10^5) were transfected with 0.09 and 0.045 nmol of small interfering RNA (siRNA) for CREB and ATF-1 (Ambion, Inc., Catalog ID #115021 and #115616, respectively) and a negative control sequence (Ambion, Inc., Catalog ID #4611) by using 0.9 ml of siPORT NeoFX (Ambion) according to the manufacturer's instructions after annealing in the supplied buffer Opti-MEM I medium. Transfected cells were maintained in regular culture medium for a day and treated with magnesium acetate in the culture medium.

2.9. Statistical analysis

The significance of differences between two groups was calculated with Student's *t* test, and the significance of differences between multiple groups was assessed by a one-way analysis of variance followed by Dunnet's test.

3. Results

3.1. Effect of magnesium ion on mRNA and protein expression of aquaporin 3

The expression of aquaporin 3 mRNA in Caco-2 cells treated with various concentrations of magnesium acetate for 12 h and with 50 mM magnesium acetate for the period indicated was investigated by real-time RT-PCR. The level of mRNA was observed to increase significantly after treatment of the cells with 50 mM of magnesium acetate for 9 and 12 h (Fig. 1A and B). The number of detached cells was observed to increase after 24 h when cells were incubated with more than 100 mM magnesium acetate although the number of viable cells was not different among the cells treated with 50, 100, and 150 mM magnesium acetate. The protein expression of aquaporin 3 also increased 36 h after treatment with 50 mM magnesium acetate (Fig. 1C). The intracellular concentration of magnesium ion was measured for estimating influx after treatment with 50 mM magnesium acetate (Fig. 1D). The concentration in Caco-2 cells significantly increased from 2.76 to 3.20 nmol/10^6 cells 1 h after the magnesium treatment.

Caco-2 cells are undifferentiated at sub-confluence and do not express significant levels of proteins typical of the large and small intestine (Engle et al., 1998). After confluence they express proteins characteristic of both enterocytes and colonocytes. From day 0 to day 21 after confluence, Caco-2 cells acquire morphological and functional characteristics of mature enterocytes even though they do not loose completely the colonocyte phenotype. Therefore, the cells were cultivated for 30 days after confluence and the expression of aquaporin 3 mRNA and protein in the cells treated with 50 mM magnesium acetate was investigated by real-time RT-PCR and Western blotting, respectively (Fig. 1E). The mRNA level of aquaporin 3 was found to increase almost equally among the cells before and after their differentiation and the increased protein level was also observed.

Mollapour and Piper (2007) recently reported that Fps1p, a homolog of mammalian aquaporin 3 in yeast, functioned as a channel for acetic acid. Their finding suggested us to test whether the increase in the expression of aquaporin 3 was specific to the application of magnesium ion rather than acetate. Therefore, we applied not only magnesium acetate but also magnesium chloride and magnesium sulfate to the expression of aquaporin 3 mRNA. The proliferation of Caco-2 cells was determined in culture medium containing magnesium acetate, magnesium chloride, and magnesium sulfate. Magnesium oxide was not examined because of its insolubility in the medium. Although magnesium acetate was observed not to affect the number of viable cells for 2 days up to a concentration of 300 mM, the chloride and sulfate salts decreased viable cell numbers at more than 250 mM. However, the expression of aquaporin 3 mRNA was observed to increase without change on the treatment with 50 mM of these salts for 12 h (Fig. 1F).

3.2. Effect of cell signaling inhibitors on aquaporin 3 mRNA expression

Cell signaling inhibitors, MDL-12330A for adenylyl cyclase and H-89 for PKA, were first examined to elucidate the signal transduction pathway activated by magnesium ion because magnesium ion was

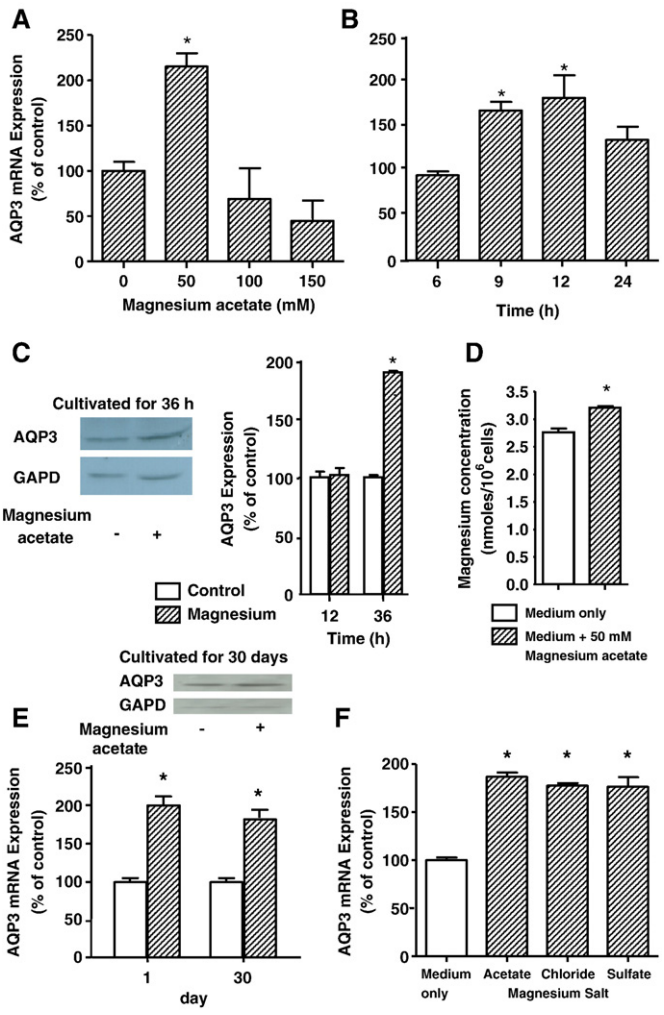

Fig. 1. Effect of magnesium ion on the mRNA and protein expression of aquaporin 3 and intracellular magnesium concentration in Caco-2 cells. (A) Caco-2 cells were incubated with various concentrations of magnesium acetate for 12 h and (B) 50 mM magnesium acetate for the period indicated. After total RNA was extracted, the expression of mRNA was quantified by the real-time RT-PCR method. The results were normalized with the glyceraldehyde 3-phosphate dehydrogenase (GAPD) mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean±S.D. from five experiments. (C) Caco-2 cells were cultivated with or without 50 mM magnesium acetate for the period indicated. Cell lysates were prepared with lysis buffer containing SDS and 2-mercaptoethanol and separated by electrophoresis with a 12% SDS-polyacrylamide gel. After proteins in the gel were electroblotted on a PVDF membrane, aquaporin 3 and GAPD were probed with anti-aquaporin 3 and anti-GAPD antibodies, respectively, and visualized using a second antibody-peroxidase conjugate and the ECL system. A typical blotting pattern is shown. The results represented as a histogram were normalized with the GAPD protein levels and the protein level of the control was taken as 100%. Data show the mean±S.D. from three experiments. (D) Caco-2 cells were incubated with 50 mM magnesium acetate for 1 h in the culture medium. Cells were lysed with 2 ml of 5% trichloroacetic acid-1.75% $LaCl_3$ after being washed with phosphate-buffered saline (PBS) containing 10 mM EDTA and 150 mM choline chloride, and then with 150 mM choline chloride. The magnesium concentration in the supernatant was determined by atomic absorption spectroscopy. (E) Caco-2 cells were cultivated for the period indicated and subsequently incubated with or without 50 mM magnesium acetate for 12 and 36 h to analyze the levels of aquaporin 3 mRNA and protein, respectively. After cells were harvested, those levels were measured as described for A, B, and C. A typical blotting pattern is shown. The results represented as a histogram were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean±S.D. from five experiments. $^{*}p<0.05$ vs. control. (F) Caco-2 cells were incubated with or without 50 mM of the magnesium salts indicated for 12 h. After total RNA was extracted, the expression of mRNA was quantified by the real-time RT-PCR method. The results were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean±S.D. from five experiments. $^{*}p<0.05$ vs. control.

reported to be necessary for the catalytic activity of adenylyl cyclase (Zimmermann et al., 1998). As shown in Fig. 2, the increase in the expression of aquaporin 3 mRNA caused by magnesium acetate was repressed by these two inhibitors, suggesting the pathway of magnesium ion signaling to be similar to that of Gs-coupled receptor signaling *via* an adenylyl cyclase. Thus, inhibitors U0126 and Ro-31-8220 for MEK1/2 and MSK1, respectively, in the MAPK pathway downstream of PKA were investigated and the increased expression was also observed to be repressed by magnesium acetate (Fig. 2). Although other inhibitors, calphostin C for PKC and KT-5823 for PKG, were studied, neither affected the aquaporin 3 mRNA expression upon stimulation with magnesium ion. Ro 31-8220 is known to inhibit not only MSK1 but also PKC. Therefore, the results obtained with Ro 31-8220 and calphostin C suggest that a signal transducer MSK1 participated in the signaling pathway triggered by magnesium ion.

3.3. Promoter activity of the aquaporin 3 gene

Aquaporin 3's promoter region from bp -1382 to -12 5′-upstream of the transcription initiation site was subcloned into the luciferase reporter vector pGL3, and this construct, 1382pGL3, was introduced into Caco-2 cells to determine whether the promoter activity of the aquaporin 3 gene was affected by 50 mM magnesium acetate (Fig. 3A). Luciferase activity significantly increased to about 3.5 times the control level on treatment for 48 and 72 h. This observation of increased aquaporin 3 promoter activity after 48 h treatment with magnesium acetate seems to be inconsistent with the result shown in Fig. 1 that aquaporin 3 mRNA expression was already increased 9 h after the addition of magnesium acetate. However, this discrepancy may be solved by the finding that enough luciferase protein for measuring the activity is accumulated after 48 h in transfected cells.

Promoter activities of a series of constructs with deletions in the aquaporin 3 promoter region were assayed to define the specific region required for the activation of aquaporin 3's transcription by magnesium ion. As shown in Fig. 3B, the transcription was significantly reduced when the regions between -404 and -190 and between -190 and -82 were removed although the deletion of -1382 to -404 did not affect the promoter activity of the aquaporin 3 gene.

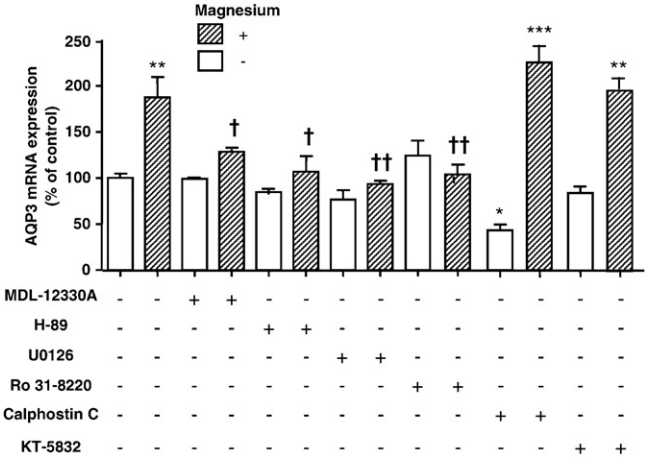

Fig. 2. Effect of cell signaling inhibitors on the mRNA expression of aquaporin 3 in Caco-2 cells. Caco-2 cells were incubated with 20 μM MDL-12330A, 25 μM H-89, 50 μM U0126, 5 μM Ro 31-8220, 1 μM calphostin C, and 10 μM KT-5823 for 1 h before the addition of magnesium acetate and subsequently treated with 50 mM magnesium acetate for 12 h. Caco-2 cells incubated without magnesium acetate and without any inhibitors were used as a control. The mRNA expression of aquaporin 3 was analyzed by the real-time RT-PCR method. The results were normalized with GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data represent the mean±S.D. from three experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.005$ vs. control. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. cells treated with 50 mM magnesium acetate and without an inhibitor.

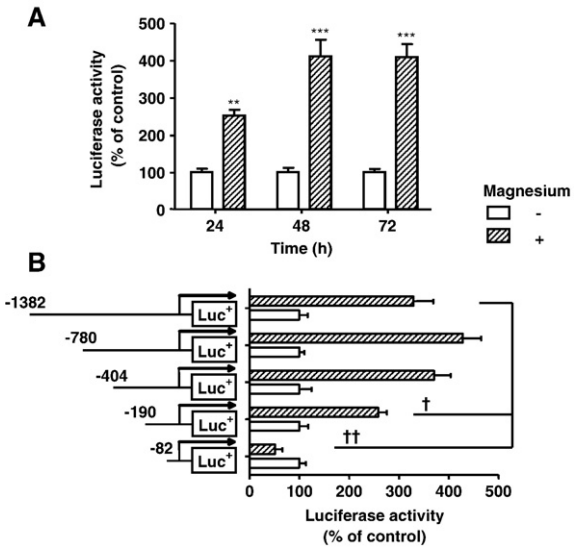

Fig. 3. Effect of magnesium ion on the promoter activity of aquaporin 3 in Caco-2 cells and promoter activity of a series of constructs with deletions of the 5′-flanking region of the aquaporin 3 gene. Caco-2 cells were simultaneously transfected with a plasmid vector containing the promoter region (bp -1382 ~ −12) of aquaporin 3 and a firefly luciferase reporter gene and the vector phRL-TK containing a *Renilla* luciferase gene. (A) Caco-2 cells were incubated with 50 mM magnesium acetate for the period indicated. (B) Serial deletion constructs were prepared to ligate various sized regions of the aquaporin 3 promoter to the reporter gene vector containing a firefly luciferase reporter gene. Caco-2 cells were simultaneously transfected with these plasmid vectors and phRL-TK containing a *Renilla* luciferase gene and incubated with 50 mM magnesium acetate for 48 h. Cell lysates were prepared after incubation and luciferase activity was measured with a Dual-Luciferase Reporter Assay System. Firefly luciferase activity was normalized with *Renilla* luciferase activity and is expressed relative to the control treated without magnesium acetate. Data represent the mean ± S.D. from four experiments. $^{**}p<0.01$, $^{***}p<0.005$ vs. control. $^{\dagger}p<0.05$ 1382pGL3 vs. 190pGL3. $^{\dagger\dagger}p<0.01$ 1382pGL3 vs. 82pGL3.

3.4. Identification of the transcriptional regulator for the aquaporin 3 gene

The binding sites of three transcriptional regulatory factors, CREB (cAMP-responsive element-binding protein), ATF1 (activated transcription factor 1), and SP1 (specificity protein 1), which were related to the signal transduction mediated by adenylyl cyclase-cAMP, were found in the promoter region of aquaporin 3 from bp -404 to -82 5′-upstream of the transcription initiation site as a result of searching the TSFSEARCH database (Fig. 4D). Whether magnesium ion affected mRNA levels of these factors was examined by real-time RT-PCR with specific primers (Fig. 4A to C). The mRNA expression of these factors was not responsive to the magnesium acetate treatment, suggesting that the signaling may not be the quantitative control of these factors.

It was further examined whether CREB and ATF1 were involved in the transcriptional control of the aquaporin 3 gene caused by magnesium ion because there were no changes in mRNA levels of these factors. Caco-2 cells were transfected with siRNA targeting the CREB and ATF1 sequences and it was determined whether the expression of aquaporin 3 mRNA was responsive to the magnesium acetate treatment. As shown in Fig. 5, the up-regulation of aquaporin 3 gene expression by magnesium ion was observed to disappear on transfection of the siRNA for CREB into Caco-2 cells (Fig. 5B and C) with the

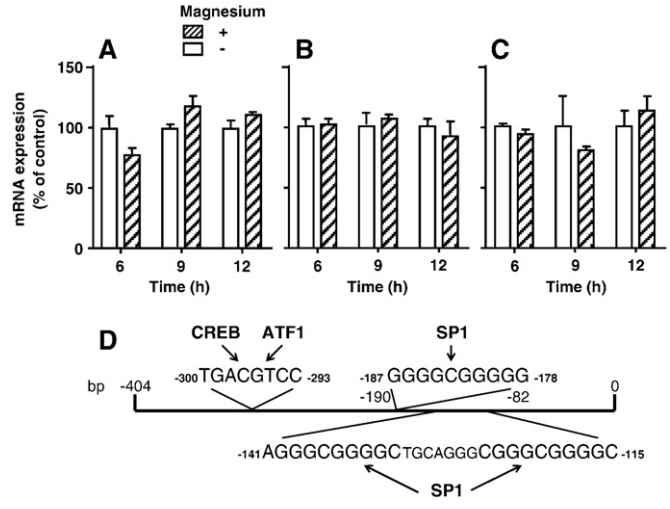

Fig. 4. Effect of magnesium ion on mRNA expression of CREB, SP1, and ATF1 in Caco-2 cells. Caco-2 cells were incubated with 50 mM magnesium acetate for the period indicated. Cells not treated with magnesium acetate were used as a control. Total RNA was isolated and subjected to a real-time RT-PCR analysis using specific primers for (A) CREB, (B) SP1, and (C) ATF1. The results were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. from five experiments. (D) CREB, ATF1, and SP1-binding motif in the promoter region of aquaporin 3.

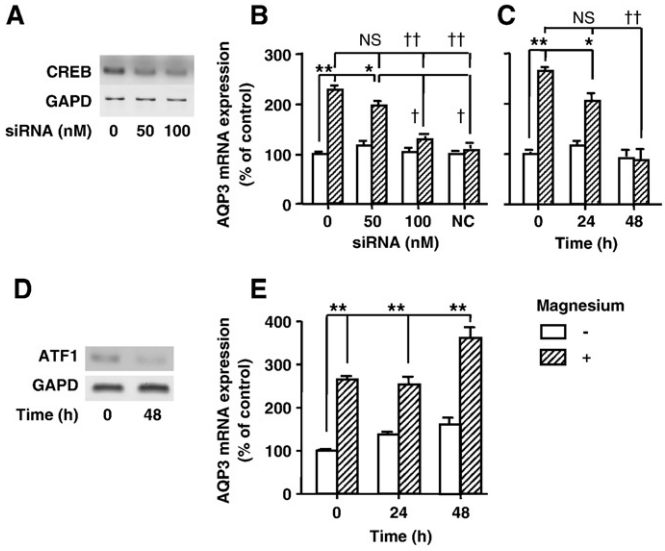

Fig. 5. Effect of siRNA for the CREB and ATF1 sequences on magnesium ion-mediated activation of the aquaporin 3 gene. Caco-2 cells were incubated with 50 mM magnesium acetate for 12 h in the culture medium after being transfected with 50 and 100 nM siRNAs for CREB and ATF1 sequences. Cells cultured without magnesium acetate and siRNA were used as a control. Total RNA was isolated and subjected to a RT-PCR analysis using specific primers for (A, B, and C) CREB and (D and E) ATF1. (A) Cells were treated with 50 mM magnesium acetate and the indicated concentrations of siRNA for CREB sequence before isolation of total RNA and subjected to a conventional RT-PCR analysis using a specific primer for CREB. The products were resolved on 1.5% agarose gels and visualized with ethidium bromide. (B) The mRNA expression of aquaporin 3 was quantified by the real-time RT-PCR method when cells were treated with 50 and 100 nM siRNA for the CREB sequence and 100 nM siRNA for the negative control sequence (NC) for 24 h. The results were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. from three experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. control, $^{\dagger\dagger}p<0.01$ vs. cells treated with magnesium acetate and without siRNA, $^{\dagger}p<0.05$ vs. cells treated with magnesium acetate and 50 nM siRNA, NS not significant. (C) Cells were incubated with 50 nM siRNA for the CREB sequence for the period indicated and the mRNA expression of aquaporin 3 was quantified by the real-time RT-PCR method. The results were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. from three experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. control, $^{\dagger\dagger}p<0.01$ vs. cells treated with magnesium acetate and without siRNA, NS not significant. (D) Cells were incubated with 100 μM siRNA for ATF1 sequence for the period indicated and subjected to treat with 50 mM magnesium acetate. Isolation of total RNA and a conventional RT-PCR analysis using a specific primer for ATF1 were performed. The products were resolved on 1.5% agarose gels and visualized with ethidium bromide. (E) Cells were transfected with 100 nM siRNA for the ATF1 sequence for the period indicated and the mRNA expression of aquaporin 3 was quantified by the real-time RT-PCR method. The results were normalized with the GAPD mRNA levels and the mRNA level of the control was taken as 100%. Data show the mean ± S.D. from three experiments. $^{**}p<0.01$ vs. control.

consequence that the amount of CREB mRNA decreased (Fig. 5A). However, siRNA for ATF1 had no effect and the expression of the aquaporin 3 gene remained increased in the transfected Caco-2 cells treated with magnesium acetate (Fig. 5E) although the amount of ATF1 mRNA decreased (Fig. 5D).

In order to examine whether SP1 is responsible for the transcriptional control of the aquaporin 3 gene in the magnesium ion signaling, the SP1-binding sequence between bp -190 and -82 in the promoter region was mutated by site-directed mutagenesis and the construct 190-4mSP1pGL3 was prepared. The promoter activity of the mutant stimulated with magnesium ion was observed to increase 2.6 fold compared to the level without magnesium ion. This increase seemed to be the same as that before the mutation was introduced, suggesting at least that the SP1-binding element in this region does not participate in the transcriptional control of the aquaporin 3 gene even though SP1 plays an important role in the transcription.

4. Discussion

In the present study, we demonstrated that signal transducers, AC, PKA, MEK1/2, and MSK1, were involved in the signaling pathway for the magnesium ion-mediated activation of aquaporin 3 expression in Caco-2 cells and a transcription factor, CREB, was one of the regulators responsible for transcription of the aquaporin 3 gene via the activation of the pathway by magnesium ion. Itoh et al. (2003) reported that the expression of aquaporin 3 mRNA increased on the treatment of HT-29 cells with VIP, an intestinal peptide inducing diarrhea, and PKA was involved in the signaling pathway. VIP was reported to activate the transcription of a major intestinal secretory mucin (MUC2) in human colonic epithelial cells via cAMP signaling to ERK and p38 and induce CREB/ATF1 phosphorylation (Hokari et al., 2005). The increased expression of aquaporin 3 controlled by the activation of CREB through the pathway of adenylyl cyclase-cAMP signaling mediated by magnesium ion seems to reflect the promotion of movement of glycerol. This suggests the additional mechanism by which magnesium formulations with lapactic action function since we speculate that aquaporin 3 is involved in glycerol metabolism (Higuchi et al., 2007).

Magnesium ion is known to be absorbed with active transport and passive diffusion in the intestinal tract, and the active transport across cells was mediated by a carrier protein. Transient receptor potential melastatin 6 (TRPM6) was recently found to be one of the transporters for magnesium ion in the intestinal tract (Voets et al., 2004). The critical role of TRPM6 in the epithelial transport of magnesium emerged from the discovery of loss-of-function mutations in patients with a severe form of hereditary hypomagnesaemia (Schlingmann and Gudermann, 2005). The intracellular concentration of magnesium ion was estimated to range from 0.1 to 1.0 mM in most mammalian cells (Altura, 1992). Under our experimental conditions without magnesium acetate, the concentration of magnesium ion was estimated at less than 1 mM from the sum of 0.8 mM in the DMEM and less than 0.1 mM in 10% fetal bovine serum. The concentration of magnesium acetate used in our experiments, 50 mM, seemed to be high enough to increase the intracellular concentration of magnesium ion in Caco-2 cells. We have not examined why increased expression of aquaporin 3 was observed at a concentration of 50 mM magnesium acetate, but not 100 or 150 mM. Since detached cells were observed to increase on treatment with 100 and 150 mM magnesium acetate compared to a concentration of 50 mM, this may concern the gene transcription of aquaporin 3. The intracellular concentration of magnesium ion was estimated to increase significantly, about 16%, with the addition of 50 mM magnesium acetate to the culture medium. Although the intracellular concentration seems to change only slightly in Caco-2 cells, this change may be necessary to trigger the signal transduction for aquaporin 3 expression. Adenylyl cyclase was reported to require magnesium ion for its catalytic activity (Zimmermann et al., 1998). Magnesium ion was essential for the cyclase to form a strong bond with the substrate ATP. Magnesium ion was reported to promote the phosphorylation of ERK through signaling from a cation-sensing G-protein-coupled receptor (Pi et al., 2005). AC, PKA, and MEK1/2 were involved in this signaling pathway and our results regarding the signal transducers activated by magnesium ion seemed to be confirmed by this observation. Wang et al. (2006) recently reported that AQP3 expression in a primary human amnion cell culture was up-regulated by the second-messenger cAMP and an adenylyl cyclase activator, forskolin. Their finding also supports our proposal that magnesium ion up-regulates aquaporin 3 expression via a MAPK pathway. The expression of aquaporin 3 was reported to be induced by osmotic pressure (Matsuzaki et al., 2001; Sugiyama et al., 2001) and is predicted to be regulated by the pressure in the intestinal tract caused by the administration of magnesium formulations as a mild lapactic. But the signal transduction initiated by osmotic pressure is unclear and will need to be studied further.

We demonstrated that CREB is one of the transcription factors responding to the up-regulation of the aquaporin 3 expression caused by magnesium ion. Magnesium ion was suggested to enhance DNA-binding of CREB to CRE (Schumacher et al., 2000). Transcriptional regulation of the aquaporin 3 gene by CREB was observed on the up-regulation of aquaporin 3 expression with VIP through a signaling pathway containing PKA (Itoh et al., 2003). CREB was reported to form a transcription complex with co-activators, SP1 (Sriraman and Richards, 2004), p300 (Anderson et al., 2005), and Egr1 (Russell et al., 2003). A SP1-binding sequence was found in aquaporin 3′s promoter region from bp -190 to -82, 5′-upstream of the transcription initiation site. SP1 mRNA expression was observed to be unaffected by magnesium acetate in Caco-2 cells and the promoter activity of an altered SP1 sequence mutated in this region was also the same as that before the mutation. These observations imply that SP1 is not a responsive co-activator for CREB or this region for binding SP1 does not respond to the regulation of aquaporin 3 expression, even if SP1 functions as a co-activator, in the transcriptional regulation of the aquaporin 3 gene by magnesium ion. Although we did not examine whether CREB underwent the phosphorylation as a result of signaling triggered by magnesium ion, the phosphorylation of Ser^{133} in CREB is known to be required for the promoter activity (Adam and Michael, 1999). Further study will be needed to elucidate the precise mechanism of transcription of the aquaporin 3 gene.

5. Conclusions

The expression of aquaporin 3 was observed to increase on the treatment of Caco-2 cells with magnesium ion produced from a magnesium formulation used as a mild lapactic. Increased expression of aquaporin 3 was induced by the treatment of HT-89 cells with VIP (Itoh et al., 2003). The expression of aquaporin 3 mRNA increased in the residual ileum and colon in rats in which a part of the intestine was resected (Tsujikawa et al., 2003). These observations all taken together suggest that the movement of water and glycerol controlled by aquaporin 3 in the intestinal tract is significantly related to the absorption and secretion of water and glycerol. Administration of the magnesium formulation may especially improve the absorption of glycerol in the intestinal tract as a result of increased expression of aquaporin 3 mediated by adenylyl cyclase-PKA signaling and subjected to control by CREB. This seems to be a benefit to stimulate the absorption of glycerol as a nutrient. Since aquaporin 3 is distributed not only in the intestinal tract but also in red blood cells, the kidney, skin, and the brain, increased concentrations of magnesium ion in these tissues appear to cause the expression of aquaporin 3. This may concern any physiological role of the movement of water and glycerol played by aquaporin 3.

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References

Adam, J.S., Micheal, E.G., 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Ann. Rev. Biochem. 68, 821–861 2003. Review. Erratum in: Ann. Rev. Biochem. 72, vii.

Alfrey, A.C., 1999. Normal and abnormal magnesium metabolism, In: Schrier, R.W. (Ed.), Renal and Electrolyte Disorders, 5th Ed. Lippincott-Raven, Philadelphia, PA, pp. 320–348.

Altura, B.M., 1992. Basic biochemistry and physiology of magnesium: a brief review. Magnes. Trace Elem. 10, 167–171.

Anderson, J., Bhandari, R., Kumar, J.P., 2005. A genetic screen identifies putative targets and binding partners of CREB-binding protein in the developing Drosophila eye. Genetics 171, 1655–1672.

Baker, S.B., Worthley, L.I., 2002. The essentials of calcium, magnesium and phosphate metabolism: part II. Disorders. Crit. Care Resusc. 4, 307–315.

Engle, M.J., Goetz, G.S., Alpers, D.H., 1998. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. J. Cell Physiol. 174, 362–369.

Frigeri, A., Gropper, M.A., Turck, C.W., Verkman, A.S., 1995. Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. Proc. Natl. Acad. Sci. USA. 92, 4328–4331.

Garcia-Webb, P., Bhagat, C., Oh, T., Hart, G., Thompson, W., 1984. Hypermagnesaemia and hypophosphataemia after ingestion of magnesium sulphate. Br. Med. J. (Clin Res Ed) 288, 759.

Hara, M., Ma, T., Verkman, A.S., 2002. Selectively reduced glycerol in skin of aquaporin-3-deficient mice may account for impaired skin hydration, elasticity, and barrier recovery. J. Biol. Chem. 277, 46616–46621.

Higuchi, S., Kubota, M., Iguchi, K., Usui, S., Kiho, T., Hirano, K., 2007. Transcriptional regulation of aquaporin 3 by insulin. J. Cell Biochem. 102, 1051–1058.

Hokari, R., Lee, H., Crawley, S.C., Yang, S.C., Gum Jr., J.R., Miura, S., Kim, Y.S., 2005. Vasoactive intestinal peptide upregulates MUC2 intestinal mucin via CREB/ATF1. Am. J. Physiol. Gastrointest. Liver Physiol. 289, G949–G959.

Itoh, A., Tsujikawa, T., Fujiyama, Y., Bamba, T., 2003. Enhancement of aquaporin-3 by vasoactive intestinal polypeptide in a human colonic epithelial cell line. J. Gastroenterol. Hepatol. 18, 203–210.

Kane, M.G., O'Dorisio, T.M., Krejs, G.J., 1983. Production of secretory diarrhea by intravenous infusion of vasoactive intestinal polypeptide. N. Engl. J. Med. 309, 1482–1485.

King, L.S., Kozono, D., Agre, P., 2004. From structure to disease: the evolving tale of aquaporin biology. Nature Rev. Mol. Cell Biol. 5, 687–698.

Kishimoto, S., Kobayashi, H., Shimizu, S., Haruma, K., Tamura, T., Kajiyama, G., Miyashi, A., 1992. Changes of colonic vasoactive intestinal peptide and cholinergic activity in rats with chemical colitis. Dig. Dis. Sci. 37, 1729–1737.

Laemmli, U.K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Ma, T., Verkman, A.S., 1999. Aquaporin water channels in gastrointestinal physiology. J. Physiol. 517, 317–326.

Mollapour, M., Piper, P.W., 2007. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol. Cell. Biol. 27, 6446–6456.

Matsuzaki, T., Suzuki, T., Takata, K., 2001. Hypertonicity-induced expression of aquaporin 3 in MDCK cells. Am. J. Physiol. Cell Physiol. 281, 55–63.

Mobasheri, A., Wray, S., Marples, D., 2005. Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J Mol Histol. 36, 1–14.

Pi, M., Faber, P., Ekema, G., Jackson, P.D., Ting, A., Wang, N., Poole, M.F., Mays, R.W., Brunden, K.R., Harrington, J.J., Quarles, L.D., 2005. Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J. Biol. Chem. 280, 40201–40209.

Preston, G.M., Agre, P., 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. Proc Natl Acad Sci USA 88, 11110–11114.

Russell, D.L., Doyle, K.M.H., Gonzales-robayna, I., Pipaon, C., Richards, J.S., 2003. Egr-1 induction in rat granulosa cells by follicle-stimulating hormone and luteinizing hormone: combinatorial regulation by transcription factors cyclic adenosine 3′,5′-monophosphate regulatory element binding protein, serum response factor, Sp1, and early growth response factor-1. Mol. Endcrinol. 17, 520–535.

Schlingmann, K.P., Gudermann, T., 2005. A critical role of TRPM channel-kinase for human magnesium transport. J. Physiol. 566, 301–308.

Schumacher, M.A., Goodman, R.H., Brennan, R.G., 2000. The structure of a CREB bZIP·somatostatin CRE complex reveals the basis for selective dimerization and divalent cation-enhanced DNA binding. J. Biol. Chem. 275, 35242–35247.

Silberstein, S., Kierbel, A., Amodeo, G., Zotta, E., Bigi, F., Berkowski, D., Ibarra, C., 1999. Functional characterization and localization of AQP3 in the human colon. Braz. J. Med. Biol. Res. 32, 1303–1313.

Sriraman, V., Richards, J.S., 2004. Cathepsin L gene expression and promoter activation in rodent granulose cells. Endocrinology 145, 582–591.

Sugiyama, Y., Ota, Y., Hara, M., Inoue, S., 2001. Osmotic stress up-regulates aquaporin-3 gene expression in cultured human keratinocytes. Biochim. Biophys. Acta 1522, 82–88.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Tsujikawa, T., Itho, A., Fukunaga, T., Satoh, J., Yasuoka, T., Fujiyama, Y., 2003. Alteration of aquaporin mRNA expression after small bowel resection in the rat residual ileum and colon. J. Gastroenterol. Hepatol. 18, 803–808.

Voets, T., Nilius, B., Hoefs, S., van der Kemp, A.W., Droogmans, G., Bindels, R.J., Hoenderop, J.G., 2004. TRPM6 forms the Mg^{2+} influx channel involved in intestinal and renal Mg^{2+} absorption. J. Biol. Chem. 279, 19–25.

Wang, S., Amidi, F., Beall, M., Gui, L., Ross, M.G., 2006. Aquaporin 3 expression in human fetal membranes and its up-regulation by cyclic adenosine monophosphate in amnion epithelial cell culture. J. Soc. Gynecol. Investig. 13, 181–185.

Yang, B., Ma, T., Verkman, A.S., 2001. Erythrocyte water permeability and renal function in double knockout mice lacking aquaporin-1 and aquaporin-3. J. Biol. Chem. 276, 624–628.

Zimmermann, G., Zhou, D., Taussig, R., 1998. Mutations uncover a role for two magnesium ions in the catalytic mechanism of adenylyl cyclase. J. Biol. Chem. 273, 19650–19655.