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ORIGINAL PAPER

The roles of acid-sensing ion channel 1a and ovarian cancer G protein-coupled receptor 1 on passive Mg^{2+} transport across intestinal epithelium-like Caco-2 monolayers

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Abstract Intestinal passive Mg^{2+} absorption, which is vital for normal Mg^{2+} homeostasis, has been shown to be regulated by luminal proton. We aimed to study the regulatory role of intestinal acid sensors in paracellular passive Mg^{2+} transport. Omeprazole enhanced the expressions of acid-sensing ion channel 1a (ASIC1a), ovarian cancer G protein-coupled receptor 1 (OGR1), and transient receptor potential vanilloid 4 in Caco-2 cells. It also inhibited passive Mg^{2+} transport across Caco-2 monolayers. The expression and activation of OGR1 resulted in the stimulation of passive Mg^{2+} transport via phospholipase C- and protein kinase C-dependent pathways. ASIC1a activation, on the other hand, enhanced apical HCO_3^- secretion that led, at least in part, by a Ca^{2+}-dependent pathway to an inhibition of paracellular Mg^{2+} absorption. Our results provided supporting evidence for the roles of OGR1 and ASIC1a in the regulation of intestinal passive Mg^{2+} absorption.

Keywords Acid sensor · Intestinal HCO_3^- secretion · Paracellular Mg^{2+} absorption · Proton pump inhibitor

Introduction

Magnesium (Mg^{2+}) is essential for many physiological processes such as muscle contraction and relaxation, energy metabolism, neuronal function, and bone formation. Dietary intake is the sole source of Mg^{2+} in human, therefore adequate intestinal absorption of Mg^{2+} is vital for normal Mg^{2+} balance. Intestinal Mg^{2+} uptake comprises saturable transcellular active and non-saturable paracellular passive mechanisms [1–3], with the latter contributing about 90 % of the total intestinal Mg^{2+} absorption [3]. This transport mechanism is driven by the electrochemical gradient set up by a higher luminal Mg^{2+} concentration and lumen positive voltage with respect to the basolateral side [1, 3, 4]. The tight junction-associated claudins (Cldn) have been reported to act as a paracellular Mg^{2+} channel within the tight junction [5, 6]. However, the regulation of the paracellular passive intestinal absorption of Mg^{2+} is unknown.

Apical proton has been shown to modulate paracellular Mg^{2+} transport [6–8]. Suppression of apical proton accumulation by a proton pump inhibitor (PPI) omeprazole altered paracellular permselectivity, suppressed Cldn-7 and -12 expressions, and inhibited paracellular passive Mg^{2+} transport across the intestinal-like Caco-2 monolayers [6, 9]. On the other hand, apical acidity (pH 7.0–5.5) was reported to increase paracellular passive Mg^{2+} uptake, affinity of paracellular channel for Mg^{2+}, and expression of Cldn-7 and -12 in both control and omeprazole-exposed epithelia [6]. However, the underlying mechanism of apical proton regulation of the passive Mg^{2+} absorption remains elusive.

Epithelial cells in the small intestine are regularly exposed to strong gastric acid. When luminal pH decreases, the intestinal epithelium cells can directly detect and modulate their cellular response through the proton sensors, e.g., ASIC1a, OGR1, and transient receptor potential vanilloid 4 (TRPV4) [10–14]. The pH of half ($pH_{0.5}$) and full activation of OGR1 are 7.4–7.2 and 6.8, respectively [15, 16]. OGR1 is associated with G_q proteins and acts

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through phospholipase C (PLC)–protein kinase C (PKC) signaling pathway to activate the epithelial Na^+/H^+ exchanger (NHE) and H^+-ATPase activity [15, 17]. ASIC1a is activated by extracellular pH below 6.9 with $pH_{0.5}$ of 6.2–6.8 [13], whereas TRPV4 requires a more acidic pH for activation (pH > 6.0) and is fully activated at pH 4.0 [18]. Both ASIC1a and TRPV4 are Ca^{2+} channels that trigger Ca^{2+} signaling to regulate epithelial HCO_3^- secretion [11, 19]. Activation of TRPV4 also modulates paracellular permeability and Cldn expressions by a Ca^{2+}-dependent mechanism [20]. It is not known whether intestinal acid sensing ASIC1a, OGR1, and TRPV4 have any role in apical acidity-induced stimulation of paracellular passive Mg^{2+} absorption. The present study investigated the role of intestinal acid sensors in the regulation of paracellular passive Mg^{2+} absorption. The results showed that OGR1 enhanced whereas ASIC1a decreased the passive intestinal Mg^{2+} absorption.

Methods

Cell culture

The human intestinal Caco-2 cells (ATCC No. HTB-37) were grown and maintained for 14 days as previously described [21]. For the experiments, the cells were plated on the permeable polyester Transwell-clear inserts (1.0×10^6 cells cm^{-2}; Corning, Corning, NY, USA), 6-well plates (5.0×10^5 cells per well; Corning), or 96-well plates (5.0×10^4 cells per well; Corning) and maintained for 7 days [6]. From days 8 to 14 of culture, cells were grown in media with or without 5 mM HCL-activated omeprazole (200 or 400 ng/mL; Calbiochem, San Diego, CA, USA). In some experiments, the apical side of Caco-2 monolayers was intermittently exposed over a 2-h period, 3 times a day, to acidic culture media (pH 6.5 or 5.5) with or without 5 mM HCL-activated omeprazole (Calbiochem) from days 8 to 14 (Fig. 1d). The cell monolayers grown on Transwell-clear inserts, 6-well plates, or 96-well plates were used for ion flux studies, western blot analysis, or MTT reduction assay, respectively. Apical pH of Caco-2 monolayers grown for 14 days in media with or without 5 mM HCL-activated omeprazole was also determined as previously described [6].

Bathing solutions

For the apical to basolateral 40 mM concentration gradient-driven passive Mg^{2+} transport studies, the apical solution contained (in mM) 40 $MgCl_2$, 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 115 D-mannitol, and 10 HEPES pH 7.4, whereas the basolateral solution contained (in mM) 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-

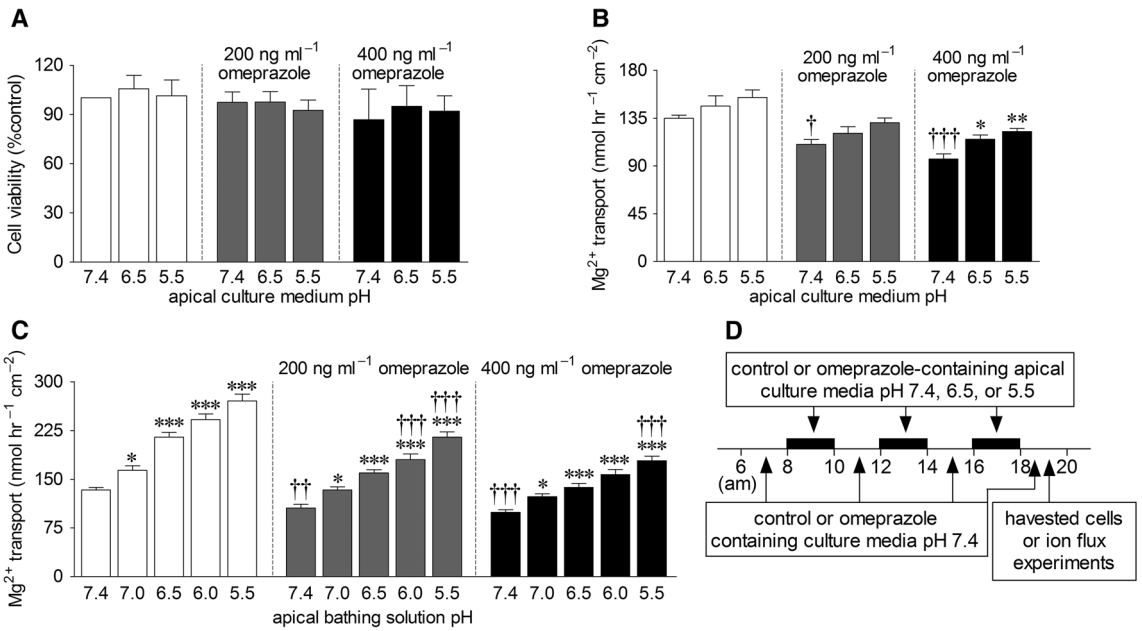

Fig. 1 Apical proton regulates intestinal passive Mg^{2+} transport. The relative cell viability of Caco-2 exposed to acidic apical culture medium, omeprazole, and omeprazole plus acidic was elucidated by MTT assay (**a**). Passive Mg^{2+} transport across Caco-2 monolayer was measured in the presence of acidic apical medium, omeprazole plus acidic apical medium (**b**), acidic apical bathing solution, and omeprazole plus acidic apical bathing solution (**c**). Representative time line of Caco-2 monolayer experiments (**d**). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding pH 7.4 group. $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with the omeprazole-free pH 7.4 group. ($n = 6$)

glutamine, 250 D-mannitol, and 10 HEPES pH 7.4. In the apical acid-activated Mg^{2+} transport studies, the apical solution of pH 7.4 was substituted with apical solution of pH 7.0, 6.5, 6.0, or 5.5 [6]. During Mg^{2+} transport studies, pH of all apical bathing solutions was simultaneously measured and not changed throughout the experiments.

For apical HCO_3^- secretion experiments, the composition of $NaHCO_3$-free apical solution was as follows (in mM) 1.25 $CaCl_2$, 4.5 KCl, 1 $MgCl_2$, 12 D-glucose, 2.5 L-glutamine, 230 D-mannitol, and 10 HEPES pH 7.4; and the $NaHCO_3$-containing basolateral solution contained (in mM) 25 $NaHCO_3$, 1.25 $CaCl_2$, 4.5 KCl, 1 $MgCl_2$, 12 D-glucose, 2.5 L-glutamine, 200 D-mannitol, and 10 HEPES pH 7.4.

All solutions were maintained at 37 °C, pre-gassed with 100 % O_2 for 30 min, and had an osmolality of 290–295 mosM as measured by a freezing-point depression-based Fiske® micro-osmometer (model 210; Fiske® Associates, Norwood, MA, USA). All chemicals were purchased from Sigma (St. Louis, MO, USA). Bioresearch grade deionized water used in the present work had a resistance of >18.3 MΩ cm, total organic compound of <10 part per billion, and pyrogen contamination of <0.005 endotoxin units/mL.

Measurements of paracellular Mg^{2+} flux

Apical to basolateral 40 mM $MgCl_2$ gradient-driven paracellular Mg^{2+} flux study and determination of Mg^{2+} concentration were performed as previously described [9]. Apical acidity-dependent passive Mg^{2+} transport was observed under apical bathing solution pH 7.0, 6.5, 6.0, or 5.5 with basolateral bathing solution remaining at pH 7.4. In some experiments, Caco-2 monolayers were pre-incubated with various compounds, namely; ASIC1a activator (1 or 10 μM amitriptyline; AMT; Sigma), ASIC1a inhibitor [30 nM psalmotoxin 1 (PcTx1); Phoenix Pharmaceuticals, Burlingame, CA, USA], TRPV4 inhibitor [10 μM ruthenium red (RR); Sigma], OGR1 inhibitor [10 μM $CuCl_2$ (Cu^{2+}) or 10 μM $ZnCl_2$ (Zn^{2+}); Sigma], polyclonal antibody raised against an extracellular domain of human OGR1 (1:10 or 1:100 OGR1 Ab; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PLC inhibitor [10 μM 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; U-73122; Calbiochem], PKC inhibitor (1 μM Bisindolylmaleimide I; Gö 6850; Calbiochem), phosphoinositide 3-kinase (PI3K) inhibitor (200 nM Wortmannin, Wort.; Calbiochem], and intracellular Ca^{2+} chelator [50 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl ester); BAPTA-AM; Calbiochem] prior to the performed experiments.

Measurements of HCO_3^- secretion

Bicarbonate secretion was measured in humidified atmosphere with 5 % CO_2 at 37 °C. After removal of the culture media, the Caco-2 monolayer was gently rinsed 3 times and incubated for 15 min in the physiological bathing solution [21]. Then, apical and basolateral solutions were substituted with apical and basolateral solutions for the HCO_3^- secretion experiment. To inhibit apical membrane-bound carbonic anhydrase (CA) activity and prevent apical HCO_3^- degradation, the selective CA IX/XII inhibitor (45 nM 4-[[[(4-fluorophenyl)amino]carbonyl]amino]-benzenesulfonamide; U-104; Sigma) was added to the apical solution. After 20 min, HCL was added to the apical solution (the final concentration of 10 mM) and incubation proceeded for 5 min. After removal of the HCL-containing apical solution, the apical side of the monolayer was gently rinsed and further incubated for 50 min in the apical solution. Aliquots of apical solution at various time points (Fig. 7a) were individually sampled. The concentration of HCO_3^- was immediately determined by using a clinical chemistry analyzer (ILab Taurus; Instrumentation Laboratory, Bedford, MA, USA). In some experiments, Caco-2 monolayers were pre-incubated with 30 nM PcTx1 (Phoenix Pharmaceuticals), 50 μM BAPTA-AM (Calbiochem) or cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor [50 μM *N*-(2-naphthalenyl)-[(3,5-dibromo-2,4-dihydroxyphenyl)methylene]glycine hydrazide; GlyH-101; Calbiochem].

MTT reduction assay

Percent viability of control Caco-2 cells and cells exposed to 200 or 400 ng/mL omeprazole (Calbiochem), and intermittently exposed to acidic apical medium with or without omeprazole was evaluated as previously described [6].

Western blot analysis

Western blot analysis was performed as previously described [6]. In brief, protein samples were prepared by using Piece® Ripa Buffer (Thermo Fisher Scientific, Rockford, IL, USA). 35 μg of protein sample or 5 μL Cruz Marker™ Molecular Weight Standards (Santa Cruz Biotechnology) were separated on 12.5 % SDS-PAGE gel, and then transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK) by electroblotting. Membranes were blocked and probed overnight at 4 °C with 1:1,000 rabbit polyclonal antibodies (Santa Cruz Biotechnology) raised against human ASIC1a, OGR1, or TRPV4. Membranes were also reprobed with rabbit polyclonal antibodies (Santa Cruz Biotechnology) raised against actin

(1:5,000), ASIC1a, OGR1, or TRPV4 antibodies. After 2 h incubation at 25 °C with 1:10,000 goat anti-rabbit IgG-HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), blots were visualized by Thermo Scientific SuperSignal® West Pico Substrate (Thermo Fisher Scientific) and captured on CL-XPosure Film (Thermo Fisher Scientific). Densitometric analysis was performed using ImageJ for Mac Os X [22].

Statistical analysis

Results were expressed as mean ± SE. Two sets of data were compared using unpaired Student's *t* test. One-way analysis of variance with Dunnett's post test was used for comparison of multiple sets of data. The level of significance was $P < 0.05$. All data were analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Apical acidity nullified omeprazole effect on passive Mg^{2+} transport

These series of experiments were performed to demonstrate the effect of direct exposure to intermittent acidic apical culture medium, apical acidity, and omeprazole on paracellular passive Mg^{2+} transport. Since an acidic extracellular pH and omeprazole have previously been reported to affect cell viability [23, 24], MTT cell proliferation assay was performed. As shown in Fig. 1a, neither exposure to intermittent acidic apical culture medium (6.5 and 5.5) nor 200 or 400 ng/mL omeprazole, concentrations resembling those found in human plasma [25], had cytotoxic effect on cell viability when compared to control. Similar to our previous report [6], 200 and 400 ng/mL omeprazole significantly increased apical pH compared to control condition (data not shown), therefore omeprazole suppressed apical acidification.

Intermittent exposure to acidic apical culture medium at pH 6.5 and 5.5 had no effect on passive Mg^{2+} transport when compared to control pH 7.4 (Fig. 1b). Both 200 and 400 ng/mL omeprazole (pH 7.4) significantly decreased the passive Mg^{2+} transport from 134.82 ± 3.02 to 110.45 ± 4.32 and 96.42 ± 4.86 nmol h^{-1} cm^{-2}, respectively (Fig. 1b). In the 200 ng/mL omeprazole-exposed groups, intermittent exposure to acidic apical culture medium of pH 6.5 and 5.5 abolished the inhibitory effect of omeprazole on passive Mg^{2+} transport (120.71 ± 6.36 and 130.70 ± 4.44 nmol h^{-1} cm^{-2}, respectively; Fig. 1b). Acidic apical culture medium of pH 6.5 and 5.5 also normalized passive Mg^{2+} transport (115.16 ± 3.98 and 122.45 ± 2.93 nmol h^{-1} cm^{-2}, respectively) in the 400 ng/mL omeprazole exposed groups (Fig. 1b).

To study the effect of apical acidity on passive Mg^{2+} transport, Caco-2 monolayers grown in culture medium pH 7.4 with or without omeprazole were used. As shown in Fig. 1c, apical acidity significantly increased passive Mg^{2+} transport from 133.60 ± 3.81 nmol h^{-1} cm^{-2} at pH 7.4 to 163.71 ± 6.94, 214.78 ± 7.43, 241.56 ± 9.07, and 270.42 ± 10.54 nmol h^{-1} cm^{-2} when apical bathing solution was changed to pH 7.0, 6.5, 6.0, and 5.5, respectively. Similar to Fig. 1b, 200 and 400 ng/mL omeprazole (apical pH 7.4) significantly decreased passive Mg^{2+} transport to 105.85 ± 5.19 and 98.95 ± 4.05 nmol h^{-1} cm^{-2}, respectively (Fig. 1c). Apical acidity abolished the inhibitory effect of 200 and 400 ng/mL omeprazole on passive intestinal Mg^{2+} transport (Fig. 1c). These results demonstrated that apical proton directly stimulated passive intestinal Mg^{2+} absorption.

Intermittent acidic apical culture medium and omeprazole altered intestinal proton sensor expression

Since previous results demonstrated the stimulatory effect of apical acidity on the intestinal passive Mg^{2+} transport (Fig. 1) [6], these experiments aimed to examine the expression of proton sensitive ASIC1a, OGR1, and TRPV4 in Caco-2 cells. Omeprazole (200 and 400 ng/mL) significantly increased the expressions of ASIC1a, OGR1, and TRPV4 (Fig. 2a–d) in Caco-2 cells. On the other hand, intermittent exposure to acidic apical culture medium (pH 6.5 and 5.5) markedly suppressed ASIC1a, OGR1, and TRPV4 expressions (Fig. 2e–h). Intermittent exposure to acidic apical culture medium also abrogated the up-regulatory effect of 400 ng/mL omeprazole on ASIC1a, OGR1, and TRPV4 expressions (Fig. 3). These findings demonstrated the regulatory role of extracellular protons on the expression of intestinal acid sensors.

Intestinal acid sensors modulated passive Mg^{2+} transport

This experiment aimed to investigate the effect of activation or inhibition of ASIC1a, OGR1, and TRPV4 on passive Mg^{2+} transport across Caco-2 monolayers. Unexpectedly, at apical pH 7.4, OGR1 inhibitors (10 μM Cu^{2+} and 10 μM Zn^{2+} [15, 17]) and 1:10 OGR1 Ab significantly decreased passive Mg^{2+} transport from 133.60 ± 3.82 to 96.93 ± 5.09, 99.20 ± 5.66, and 102.65 ± 6.18 nmol h^{-1} cm^{-2}, respectively (Fig. 4a). Neither ASIC1a inhibitor 30 nM PcTx1 [26], TRPV4 inhibitor 10 μM RR [20], nor 100 °C-heated OGR1 Ab had modulatory effect on passive Mg^{2+} transport in apical pH at 7.4 (Fig. 4a). In the presence of mild acidic apical pH 7.0, the rate of passive Mg^{2+} transport was significantly decreased from 161.18 ± 10.34 to 92.80 ± 7.58 and 96.67 ± 8.29 nmol h^{-1} cm^{-2} in the presence of Cu^{2+} and

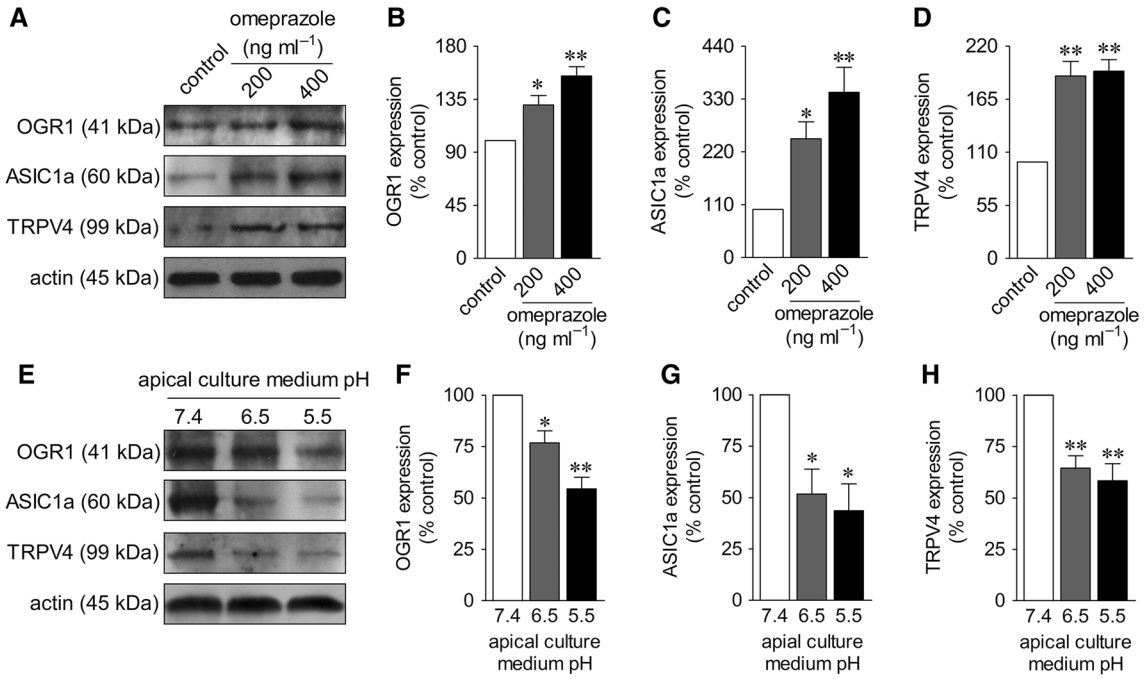

Fig. 2 Acidic apical culture medium and omeprazole regulate intestinal acid sensor expression. Representative immunoblotting and densitometric analysis of OGR1 (**a**, **b**, respectively), ASIC1a (**a**, **c**, respectively), and TRPV4 expressions (**a**, **d**, respectively) in Caco-2 cells exposed to 200 or 400 ng/mL omeprazole. Representative immunoblotting and densitometric analysis of OGR1 (**e**, **f**, respectively), ASIC1a (**e**, **g**, respectively), and TRPV4 expressions (**e**, **h**, respectively) in Caco-2 cells intermittently exposed to acidic apical culture medium. $*P < 0.05$, $**P < 0.01$ compared with the corresponding control group. ($n = 5$)

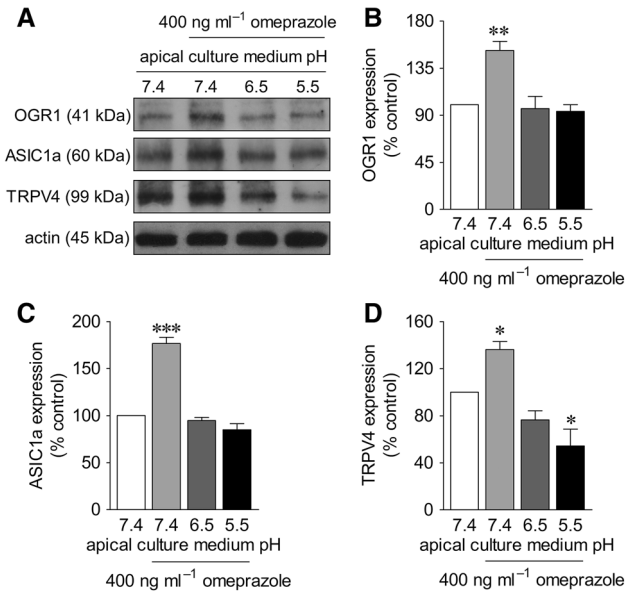

Fig. 3 Acidic apical culture medium abolishes omeprazole effect on acid sensor expression. Representative immunoblotting and densitometric analysis of OGR1 (**a**, **b**, respectively), ASIC1a (**a**, **c**, respectively), and TRPV4 expressions (**a**, **d**, respectively) in Caco-2 cells intermittently exposed to acidic apical culture medium and 400 ng/mL omeprazole. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the corresponding control group. ($n = 5$)

Zn^{2+}, respectively, and to 126.91 ± 7.44 and 107.71 ± 8.65 nmol h^{-1} cm^{-2} by 1:100 and 1:10 OGR1 Ab, respectively (Fig. 4b). In the 400 ng/mL omeprazole exposed groups, the rate of passive Mg^{2+} transport (nmol h^{-1} cm^{-2}) of 98.95 ± 4.05 was decreased by Cu^{2+} to 63.79 ± 4.36 at apical pH 7.4 (Fig. 5a) and from 122.36 ± 4.72 to 78.78 ± 3.52 at apical pH 7.0 (Fig. 5b), and by Zn^{2+} to 66.23 ± 4.63 at apical pH 7.4 and 81.39 ± 3.92 at apical pH 7.0, and by 1:10 OGR1 Ab to 67.33 ± 3.82 at apical pH 7.4 and 83.53 ± 3.58 at apical pH 7.0. These results indicated that OGR1 activation could have stimulatory effect on passive intestinal Mg^{2+} absorption.

As expected, PcTx1 significantly increased passive Mg^{2+} transport by about 14 % at acidic apical pH of 6.5 (241.56 ± 5.15 vs. 211.32 ± 5.58 nmol h^{-1} cm^{-2} of control group; Fig. 4c), by 17 % at acidic apical pH of 6.0 (280.96 ± 8.14 vs. 239.32 ± 7.58 nmol h^{-1} cm^{-2} of control group; Fig. 4d), and by 14 % at acidic apical pH of 5.5 (308.51 ± 8.74 vs. 270.42 ± 10.54 nmol h^{-1} cm^{-2} of control group; Fig. 4e). On the other hand, ASIC1a activator AMT [27] at 1 and 10 μM significantly decreased passive Mg^{2+} transport at apical pH 7.4 from 133.60 ± 3.82 to 99.41 ± 6.00 and 105.39 ± 4.92 nmol h^{-1} cm^{-2}, and at apical pH 7.0 from 165.18 ± 6.60 to 111.54 ± 9.04 and 117.26 ± 6.76

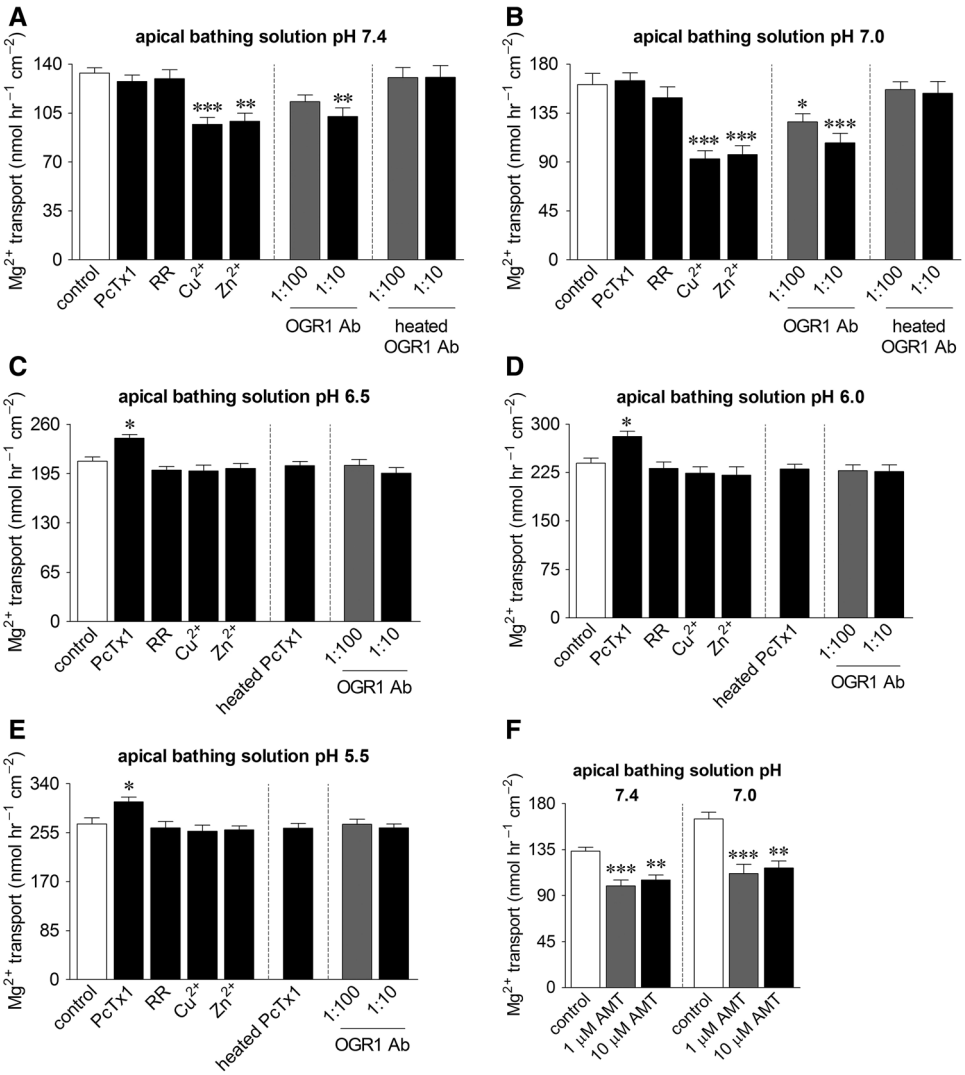

Fig. 4 OGR1 and ASIC1a regulate intestinal passive Mg^{2+} transport. In this prior of passive Mg^{2+} transport studies, Caco-2 monolayers were pre-incubated with 30 nM PcTx1, 10 μM RR, 10 μM Cu^{2+}, 10 μM Zn^{2+}, 1:10 or 1:100 OGR1 Ab, 100 °C-heated OGR1 Ab, or 100 °C-heated 30 nM PcTx1 before being exposed to apical bathing solution at pH 7.4 (**a**), 7.0 (**b**), 6.5 (**c**), 6.0 (**d**) or 5.5 (**e**). The passive Mg^{2+} transport was also studied in Caco-2 monolayers pre-incubated with 1 or 10 μM AMT (**f**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding control group. ($n = 6$)

nmol h^{-1} cm^{-2}, respectively (Fig. 4f). In the 400 ng/mL omeprazole exposed groups, PcTx1 also increased passive Mg^{2+} transport by about 42 % at acidic apical pH of 6.5 (Fig. 5c), by about 49 % at acidic apical pH of 6.0 (Fig. 5d), and by about 67 % at acidic apical pH of 5.5 (Fig. 5e). On the other hand, ASIC1a activator AMT at 1 and 10 μM significantly reduced passive Mg^{2+} transport at apical pH 7.4 from 98.95 ± 4.05 to 73.31 ± 4.14 and 75.87 ± 6.47 nmol h^{-1} cm^{-2}, and at apical pH 7.0 from 122.36 ± 4.72 to 80.89 ± 6.24 and 83.62 ± 8.05 nmol h^{-1} cm^{-2}, respectively (Fig. 5f). These results demonstrated the inhibitory effect of ASIC1A on the passive intestinal Mg^{2+} absorption.

Signaling pathway of passive Mg^{2+} transport modulation

These series of experiments were performed to elucidate the signaling pathways of OGR1 and ASIC1a activation in the regulation of passive Mg^{2+} transport. Previous reports showed that active OGR1 acted through PLC and PKC to stimulate epithelial ion transport [15, 17]. In the present study, OGR1 inhibitor 10 μM Cu^{2+}, PLC inhibitor 10 μM U-73122, and PKC inhibitor 1 μM Gö 6850 significantly decreased the rate of passive Mg^{2+} transport (nmol h^{-1} cm^{-2}) in the absence (92.80 ± 7.58, 84.55 ± 6.51, and 94.95 ± 7.20, respectively, vs. 161.18 ± 10.34 of control monolayers) and presence of 400 ng/mL omeprazole (78.78 ± 3.52, 82.44 ± 5.52, and 78.11 ± 6.53, respectively, vs. 122.36 ± 4.72 of control monolayers) (Fig. 6a). Neither PI3K inhibitor 200 nM Wort. nor intracellular Ca^{2+} chelator 50 μM BAPTA-AM had a modulatory effect on passive Mg^{2+} transport in mild acidic apical pH at 7.0 (Fig. 6a). Therefore, active OGR1 probably stimulated intestinal passive Mg^{2+} absorption by PLC- and PKC-dependent mechanisms.

At acidic apical pH 5.5, 30 nM PcTx1 and 50 μM BAPTA-AM were found to increase the rate of passive Mg^{2+} transport (nmol h^{-1} cm^{-2}) in the absence (328.46 ± 10.42 and 324.95 ± 6.31, respectively, vs. 266.68 ± 10.81 of control) and presence of 400 ng/mL

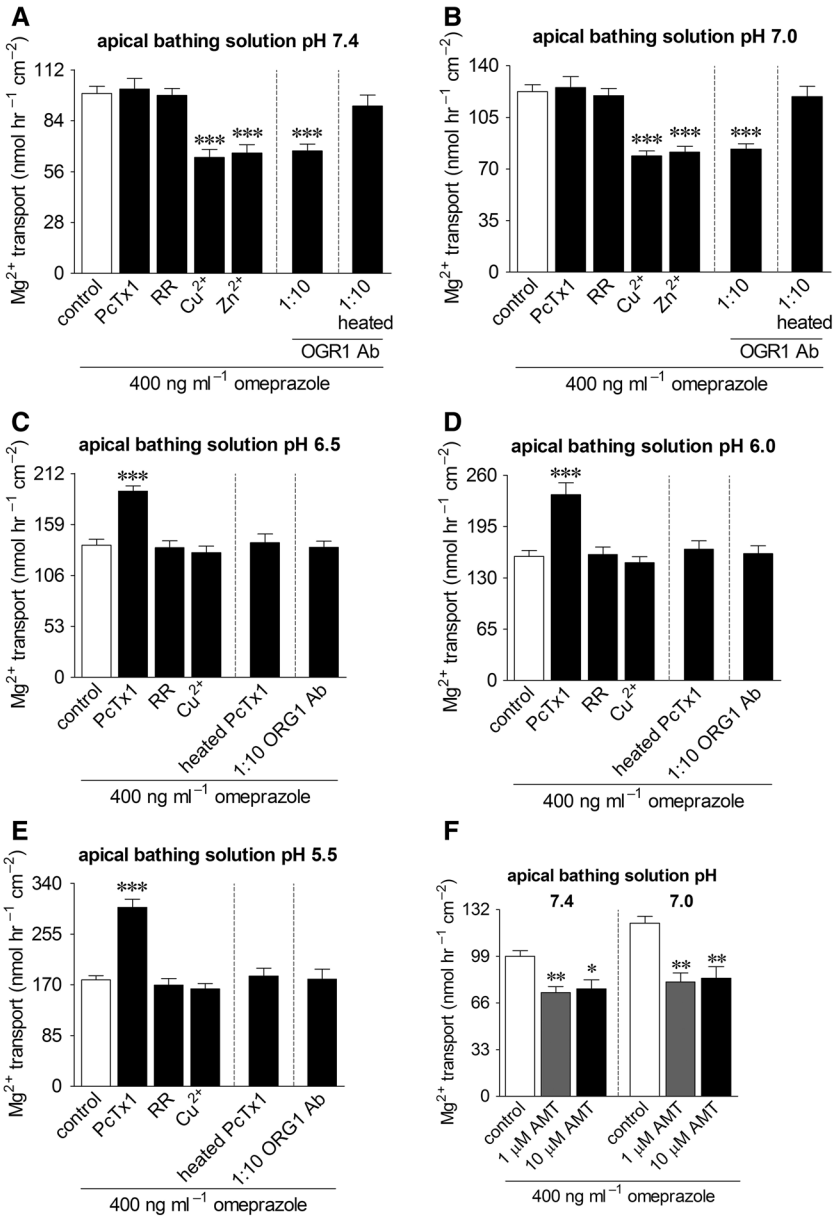

Fig. 5 OGR1 and ASIC1a regulate passive Mg^{2+} transport in omeprazole exposed epithelium. Passive Mg^{2+} transport by 400 ng/mL omeprazole-exposed Caco-2 monolayers that were pre-incubated with 30 nM PcTx1, 10 μM RR, 10 μM Cu^{2+}, 10 μM Zn^{2+}, 1:10 OGR1 Ab, 100 °C-heated OGR1 Ab, or 100 °C-heated 30 nM PcTx1 in apical bathing solution at pH 7.4 (**a**), 7.0 (**b**), 6.5 (**c**), 6.0 (**d**) or 5.5 (**e**). Passive Mg^{2+} transport was also studied in 400 ng/mL omeprazole-exposed Caco-2 monolayers that were pre-incubated with 1 or 10 μM AMT (**f**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding control group. ($n = 6$)

omeprazole (299.97 ± 13.67 and 252.97 ± 16.35, respectively, vs. 178.61 ± 6.89 of control) (Fig. 6b). Inhibitors of Ca^{2+}-sensitive signaling mediator (200 nM Wort. and 1 μM Gö 6850) had no effect on passive Mg^{2+} transport at acidic apical pH of 5.5 (Fig. 6b). Therefore, inhibition of ASIC1a and prevention of intracellular Ca^{2+} elevation could stimulate the intestinal passive Mg^{2+} absorption.

ASIC1a-stimulated HCO_3^- secretion

After having demonstrated the expression and function of ASIC1a in Caco-2 epithelium, we sought to discover the possible underlying mechanism of ASIC1a-induced decrease in the passive Mg^{2+} transport. It was previously reported that activation of ASICs stimulated duodenal HCO_3^- secretion in vivo [11]. As shown in Fig. 7a, apical acid was found to stimulate HCO_3^- secretion in both control and omeprazole-exposed monolayers at various time points. HCO_3^- secretion in response to acid peaked at 50 min in control, 200 ng/mL omeprazole-exposed, and 400 ng/mL omeprazole-exposed groups (Fig. 7a). Omeprazole at 200 and 400 ng/mL significantly increased both the basal (4.33 ± 0.31 and 5.09 ± 0.5, respectively, vs. 2.43 ± 0.48 μmol h^{-1} cm^{-2} of control group) and peak acid-stimulated HCO_3^- secretion (12.15 ± 0.55 and 13.85 ± 0.45, respectively, vs. 9.07 ± 0.54 μmol h^{-1} cm^{-2} of control group) (Fig. 7b, c). CFTR inhibitor 50 μM GlyH-101 on the other hand markedly decreased the basal HCO_3^- secretion in both

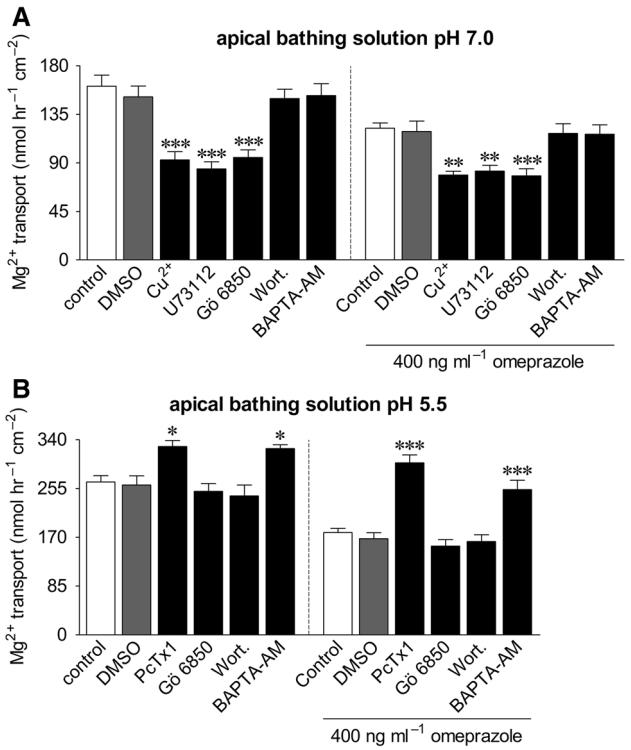

Fig. 6 Signaling pathways of OGR1 and ASIC1a regulate passive Mg^{2+} transport. Passive Mg^{2+} transport by using control or omeprazole-exposed Caco-2 monolayers that were pre-incubated with 30 nM PcTx1, 10 μM Cu^{2+}, or 10 μM Zn^{2+}, 10 μM U-73122, 1 μM Gö 6850, 200 nM Wort., or 50 μM BAPTA-AM in apical bathing solution pH at 7.0 (**a**) or 5.5 (**b**). DMSO 0.3 % (vol/vol) was used as vehicle for preparation of inhibitors. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the corresponding control group. ($n = 6$)

control and 400 ng/mL omeprazole-exposed monolayers (Fig. 7d). As expected, PcTx1, GlyH-101, and BAPTA-AM significantly reduced peak acid stimulated HCO_3^- secretion in both control (3.01 ± 0.37, 2.53 ± 0.83, and 2.90 ± 0.67 μmol h^{-1} cm^{-2}, respectively) and 400 ng/mL omeprazole exposed monolayers (5.92 ± 1.19, 5.03 ± 0.53, and 5.85 ± 1.01 μmol h^{-1} cm^{-2}, respectively) (Fig. 7e). These results suggested that omeprazole induced HCO_3^- secretion by ASIC1a-, Ca^{2+}-, and CFTR-dependent mechanisms.

Discussion

Adequate intestinal absorption of Mg^{2+} is vital for maintaining normal Mg^{2+} balance. We have previously reported a direct effect of apical acidity on passive Mg^{2+} transport across human intestinal Caco-2 epithelium. Suppression of apical proton secretion and elevation of apical pH by omeprazole decreased the paracellular cation selectivity, negative electrical field strength of paracellular pore, Mg^{2+} affinity of the paracellular channel, Cldn-7 and -12 expression, and paracellular Mg^{2+} absorption [6, 9]. On the other hand, increase in apical acidity markedly enhanced Mg^{2+} affinity of the paracellular channel, Cldn-7 and -12 expression, and paracellular Mg^{2+} absorption [6]. In the present study, we proposed that intestinal passive Mg^{2+} absorption was regulated by the proton-sensitive OGR1 and ASIC1a.

In human tissues, OGR1 has previously been detected in the small intestine, spleen, testis, brain, lung, placenta, heart, and kidney, but not in the colon, liver, or skeletal muscle [14]. OGR1 is known as a proton-sensitive G-protein-coupled receptor [15, 17] that requires extracellular histidine residues 17, 20, 84, 169, and 269 for its proton detection [15]. It is coupled to G_q proteins and PLC that triggers an increase in the intracellular Ca^{2+} transient and PKC, which in turn activates the epithelial NHE and H^+-ATPase in OGR1-transfected HEK293 cells [15, 17]. In the present study, activation of OGR1 by mild acidic apical pH of 7.0 was found to stimulate passive Mg^{2+} transport. This effect was inhibited by OGR1 inhibitors and OGR1 Ab. However, because OGR1 activity declined to an inactivation state when the extracellular pH declined to 6.5 [15], the stimulation of passive Mg^{2+} transport seen at more acidic apical pH of 6.5, 6.0, and 5.5 must be OGR1-independent. Our results also showed that the OGR1 induced stimulation of passive Mg^{2+} transport was mediated by PLC and PKC signaling pathways, but not by the intracellular Ca^{2+}-dependent mechanism. The controversial reports regarding the role of intracellular Ca^{2+} as a downstream mediator of OGR1 activation in the present and previous studies [15, 17] was probably due to the different cell types used in the studies. Moreover, the observed up-regulation of OGR1 expression was probably a compensatory response of omeprazole-exposed monolayers to oppose the inhibitory effect of omeprazole on passive intestinal Mg^{2+} absorption.

Previously, the expression and function of ASIC1a has been extensively studied in the nervous system [12, 13, 28]. Although ASICs were cloned from the human small intestine [29], expression and function of ASICs were mainly observed in neurons in the gastrointestinal tract [12, 13]. Dong et al. [11] demonstrated the expression and function of ASIC1a in rat duodenal epithelium and human HT29 cells. In the present study, we proposed that ASIC1a activation had an inhibitory effect on intestinal passive Mg^{2+} absorption. Since ASIC1a is active at extracellular pH below 6.9 [13], it might directly regulate the passive Mg^{2+} transport under acidic apical pH of 6.5, 6.0, and 5.5. By using specific inhibitor or activator of ASIC1a in the monolayer incubation, we were able to show the inhibitory role of activated ASIC1a on passive intestinal Mg^{2+} absorption. Since ASIC1a can act as a Ca^{2+} channel [11],

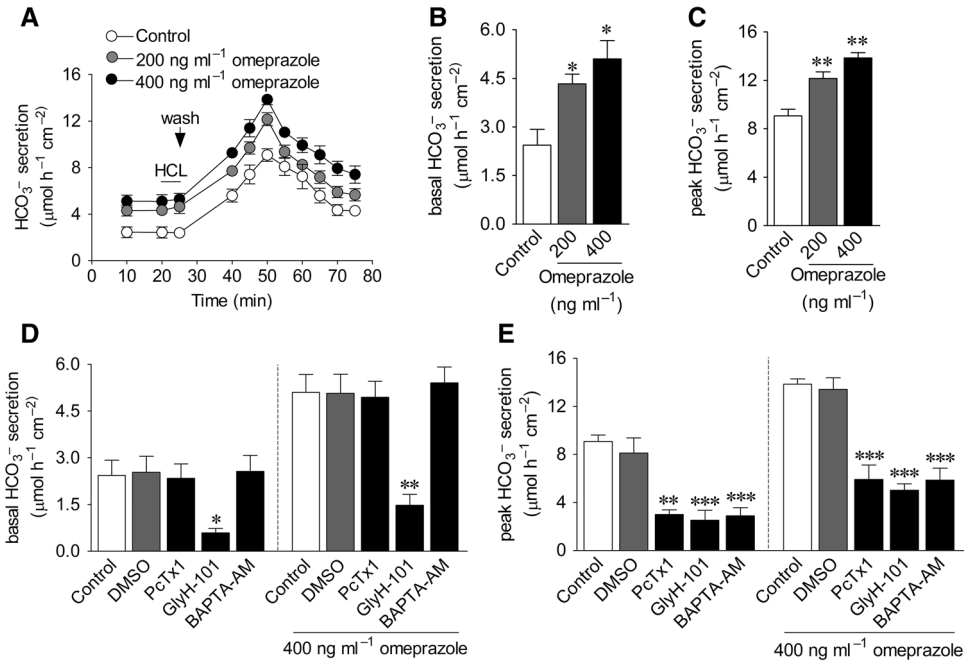

Fig. 7 ASIC1a mediates apical HCL stimulating HCO_3^- secretion. Time course of HCO_3^- secretion by control or omeprazole-exposed Caco-2 monolayers that was apically induced by 10 mM HCL (**a**). Basal (at 20 min; **b**, **d**) and peak acid-stimulated HCO_3^- secretion (at 50 min; **c**, **e**) by control or omeprazole-exposed Caco-2 monolayers that were pre-treated with 30 nM PcTx1, 50 μM BAPTA-AM, or 50 μM GlyH-101. DMSO 0.3 % (vol/vol) was used as vehicle for preparation of inhibitors. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding control group. ($n = 5$)

activation of ASIC1a by apical acidity probably involves an intracellular Ca^{2+} elevation leading to inhibition of intestinal passive Mg^{2+} absorption that in turn was relieved by PcTx1 and BAPTA-AM. However, it was possible that other acid sensors may also mediate the stimulatory effect of apical pH below 7.0 on the intestinal passive Mg^{2+} absorption.

Although TRPV4 has been reported to regulate paracellular permeability in mammary HC11 epithelium [20] and act as a proton sensor that is fully activated at pH 4.0 in Chinese hamster ovary cells [18], it was probably not involved in the apical acidity-induced paracellular passive Mg^{2+} transport in human intestinal Caco-2 epithelium. There are four possible reasons to explain this assumption: (1) being located in the basolateral membrane of human Caco-2 epithelium [10], TRPV4 cannot be directly activated by apical acidity, (2) the acidic apical pH used in the present study is not low enough to effectively activate TRPV4, (3) TRPV4 activation has not been known to affect passive Mg^{2+} transport, and (4) in the present study used different cell type from those used in the previous studies [18, 20].

It is widely accepted that duodenal mucosal HCO_3^- secretion is an important mechanism of enterocyte epithelium to defend itself against exposure to strong gastric acid [30, 31]. The duodenal epithelial cells can directly detect and regulate mucosal HCO_3^- secretion via ASIC1a and purinoceptors P2Y [11, 32]. Our results agreed with the previous study [11] that apical HCL stimulated HCO_3^- secretion in ASIC1a by an intracellular Ca^{2+}-dependent pathway. Apical HCO_3^- secretion in the small intestine occurs via: (1) anion conductive CFTR-dependent, (2) anion Cl^-/HCO_3^- exchanger-dependent, and (3) paracellular hydrostatic pressure-dependent mechanisms [31]. Because the specific CFTR inhibitor GlyH-101 could not totally suppress HCO_3^- secretion in the present study, therefore the Cl^-/HCO_3^- exchanger-dependent and paracellular pathways may also play a role in the acid-stimulated HCO_3^- secretion. Furthermore, from the finding that ASIC1a activation suppressed paracellular Mg^{2+} absorption, as well as enhanced apical HCO_3^- secretion, it was possible that apical HCO_3^- secretion could contribute to the suppression of paracellular Mg^{2+} absorption. In the small intestine, luminal proton from gastric acid was believed to play an important role in providing appropriate environment for mineral absorption by stabilizing their ionized forms [33]. Not only reducing luminal proton [30, 31], secreted HCO_3^- may also trigger the precipitation of luminal Mg^{2+} as $MgCO_3$ [34], thus reducing the intestinal Mg^{2+} absorption. In addition, secreted HCO_3^- may reduce the luminal positive voltage that is required as a driving force for passive Mg^{2+} absorption [1, 3, 4]. Therefore, apical secreted HCO_3^- may, at least in part, suppress the intestinal passive Mg^{2+} absorption by decreasing Mg^{2+} bioavailability and luminal positive voltage.

Hypomagnesemia that occurred with a long-term use of PPI probably resulted from a decrease in the intestinal absorption [35–38]. Previously, we reported an inhibitory effect of the PPI omeprazole on the paracellular passive Mg^{2+} absorption in human enterocyte-like Caco-2 monolayers [6, 9]. In the present study, we showed an enhancing effect of omeprazole on the expression of ASIC1a, an

activation of which increased the apical HCO_3^- secretion and suppressed paracellular passive Mg^{2+} absorption in CFTR- and intracellular Ca^{2+}-dependent manners. Since omeprazole increased paracellular anion selectivity [9], it was likely that paracellular HCO_3^- secretion was also involved in the omeprazole-induced HCO_3^- secretion in the present study. Our results agreed with a previous report of omeprazole stimulating human duodenal mucosal HCO_3^- secretion in vivo [39]. Therefore, the higher rate of HCO_3^- secretion by omeprazole-exposed epithelium in the present study could, at least in part, decrease the intestinal passive Mg^{2+} absorption by increasing $MgCO_3$ precipitation [34] and reducing the luminal-positive voltage that acted as a driving force for passive Mg^{2+} absorption.

In conclusion, we showed that the expression and activation of OGR1 resulted in the stimulation of paracellular intestinal Mg^{2+} absorption via PLC- and PKC-dependent pathways. ASIC1a activation in the presence of acidic apical condition, on the other hand, enhanced apical HCO_3^- secretion that led, at least in part, by a Ca^{2+}-dependent pathway to an inhibition of paracellular Mg^{2+} absorption.

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Conflict of interest The authors declare no conflicts of interest.

References

1. de Baaij JH, Hoenderop JG, Bindels RJ (2012) Regulation of magnesium balance: lessons learned from human genetic disease. Clin Kidney J 5:i15–i24
2. Konrad M, Schlingmann KP, Gudermann T (2004) Insights into the molecular nature of magnesium homeostasis. Am J Physiol Renal Physiol 286:F599–F605
3. Quamme GA (2008) Recent developments in intestinal magnesium absorption. Curr Opin Gastroenterol 24:230–235
4. Jahnen-Dechent W, Ketteler M (2012) Magnesium basics. Clin Kidney J 5:i3–i14
5. Hou J, Renigunta A, Gomes AS, Hou M, Paul DL, Waldegger S, Goodenough DA (2009) Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions and for renal reabsorption of magnesium. Proc Natl Acad Sci USA 106:15350–15355
6. Thongon N, Krishnamra N (2012) Apical acidity decreases inhibitory effect of omeprazole on Mg^{2+} absorption and claudin-7 and -12 expression in Caco-2 monolayers. Exp Mol Med 44:684–693
7. Heijnen AM, Brink EJ, Lemmens AG, Beynen AC (1993) Ileal pH and apparent absorption of magnesium in rats fed on diets containing either lactose or lactulose. Br J Nutr 70:747–756
8. Tang VW, Goodenough DA (2003) Paracellular ion channel at the tight junction. Biophys J 84:1660–1673
9. Thongon N, Krishnamra N (2011) Omeprazole decreases magnesium transport across Caco-2 monolayers. World J Gastroenterol 17:1574–1583
10. D'Aldebert E, Cenac N, Rousset P, Martin L, Rolland C, Chapman K, Selves J, Alric L, Vinel JP, Vergnolle N (2011) Transient receptor potential vanilloid 4 activated inflammatory signals by intestinal epithelial cells and colitis in mice. Gastroenterology 140:275–285
11. Dong X, Ko KH, Chow J, Tuo B, Barrett KE, Dong H (2011) Expression of acid-sensing ion channels in intestinal epithelial cells and their role in the regulation of duodenal mucosal bicarbonate secretion. Acta Physiol 201:97–107
12. Holzer P (2007) Taste receptors in the gastrointestinal tract. V. Acid-sensing in the gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol 292:G699–G705
13. Holzer P (2009) Acid-sensitive ion channels and receptors. Handb Exp Pharmacol 194:283–332
14. Xu Y, Casey G (1996) Identification of human OGR1, a novel G protein-coupled receptor that maps to chromosome 14. Genomics 35:397–402
15. Ludwig MG, Vanek M, Guerini D, Gasser JA, Jones CE, Junker U, Hofstetter H, Wolf RM, Seuwen K (2003) Proton-sensing G-protein-coupled receptors. Nature 425:93–98
16. Seuwen K, Ludwig MG, Wolf RM (2006) Receptors for protons or lipid messengers or both? J Recept Signal Transduct Res 26:599–610
17. Mohebbi N, Benabbas C, Vidal S, Daryadel A, Bourgeois S, Velic A, Ludwig MG, Seuwen K, Wagner CA (2012) The proton-activated G protein coupled receptor OGR1 acutely regulates the activity of epithelial proton transport proteins. Cell Physiol Biochem 29:313–324
18. Suzuki M, Mizuno A, Kodaira K, Imai M (2003) Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 278:22664–22668
19. Gradilone SA, Masyuk AI, Splinter PL, Banales JM, Huang BQ, Tietz PS, Masyuk TV, Larusso NF (2007) Cholangiocyte cilia express TRPV4 and detect changes in luminal tonicity inducing bicarbonate secretion. Proc Natl Acad Sci USA 104:19138–19143
20. Reiter B, Kraft R, Günzel D, Zeissig S, Schulzke J-D, Fromm M, Harteneck C (2006) TRPV4-mediated regulation of epithelial permeability. FASEB J 20:1802–1812
21. Nakkrasae L, Thongon N, Thongbunchoo J, Krishnamra N, Charoenphandhu N (2010) Transepithelial calcium transport in prolactin-exposed intestine-like Caco-2 monolayer after combinatorial knockdown of TRPV5, TRPV6 and Cav1.3. J Physiol Sci 60:9–17
22. Rasband WS (1997–2011) ImageJ. US National Institutes of Health, Bethesda. http://imagej.nih.gov/ij/
23. Capodicasa E, Cornacchione P, Natalini B, Bartoli A, Coaccioli S, Marconi P, Scaringi L (2008) Omeprazole induces apoptosis in normal human polymorphonuclear leucocytes. Int J Immunopathol Pharmacol 21:73–85
24. Parks SK, Chiche J, Pouyssegur J (2011) pH control mechanisms of tumor survival and growth. J Cell Physiol 226:299–308
25. Macek J, Klíma K, Ptácek P (2007) Rapid determination of omeprazole in human plasma by protein precipitation and liquid chromatography-tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci 852:282–287
26. Chen X, Kalbacher H, Gründer S (2005) The tarantula toxin psalmotoxin 1 inhibits acid-sensing ion channel (ASIC) 1a by increasing its apparent H^+ affinity. J Gen Physiol 126:71–79
27. Marin AN, Prica CM, Amuzescu BP, Neaga EI, Flonta ML (2008) ASIC1a activation by amitriptyline and FMRF-amide is removed by serine proteases. Channels 2:419–428

28. Lingueglia E (2007) Acid-sensing ion channels in sensory perception. J Biol Chem 282:17325–17329
29. Schaefer L, Sakai H, Mattei M, Lazdunski M, Lingueglia E (2000) Molecular cloning, functional expression and chromosomal localization of an amiloride-sensitive Na(+) channel from human small intestine. FEBS Lett 471:205–210
30. Akiba Y, Kaunitz JD (2011) Duodenal chemosensing and mucosal defenses. Digestion 83:25–31
31. Allen A, Flemström G (2005) Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. Am J Physiol Cell Physiol 288:C1–C19
32. Dong X, Smoll EJ, Ko KH, Lee J, Chow JY, Kim HD, Insel PA, Dong H (2009) P2Y receptors mediate Ca^{2+} signaling in duodenocytes and contribute to duodenal mucosal bicarbonate secretion. Am J Physiol Gastrointest Liver Physiol 296:G424–G432
33. Evenepoel P (2001) Alteration in digestion and absorption of nutrients during profound acid suppression. Best Pract Res Clin Gastroenterol 15:539–551
34. Kurita Y, Nakada T, Kato A, Doi H, Mistry AC, Chang MH, Romero MF, Hirose S (2008) Identification of intestinal bicarbonate transporters involved in formation of carbonate precipitates to stimulate water absorption in marine teleost fish. Am J Physiol Regul Integr Comp Physiol 294:R1402–R1412
35. Cundy T, Dissanayake A (2008) Severe hypomagnesaemia in long-term users of proton-pump inhibitors. Clin Endocrinol 69:338–341
36. Epstein M, McGrath S, Law F (2006) Proton-pump inhibitors and hypomagnesemic hypoparathyroidism. N Engl J Med 355:1834–1836
37. Mackay JD, Bladon PT (2010) Hypomagnesaemia due to proton-pump inhibitor therapy: a clinical case series. QJM 103:387–395
38. Shabajee N, Lamb EJ, Sturgess I, Sumathipala RW (2008) Omeprazole and refractory hypomagnesaemia. BMJ 337:173–175
39. Mertz-Nielsen A, Hillingsø J, Bukhave K, Rask-Madsen J (1996) Omeprazole promotes proximal duodenal mucosal bicarbonate secretion in humans. Gut 38:6–10