รายงานวิจัยฉบับสมบูรณ์

โครงการ

กลไกควบคุมระดับเซลล์ของการขนส่งแมกนีเซียมแบบไม่ใช้พลังงานผ่านช่องว่างระหว่างเซลล์ในแผ่นเซลล์เยื่อบุลำไส้ Caco-2

The cellular regulatory mechanism of paracellular passive Mg^{2+} transport in intestinal like Caco-2 monolayer

โดย

รองศาสตราจารย์ณรงค์ฤทธิ์ ทองอุ่น

มิถุนายน 2559

สัญญาเลขที่ RSA5680005

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และมหาวิทยาลัยบูรพา

Abstract

Project Code : RSA5680005

Project Title : The cellular regulatory mechanism of paracellular passive Mg^{2+} transport in intestinal like Caco-2 monolaye

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Intestinal passive Mg^{2+} absorption is the vital factor for normal Mg^{2+} homeostasis. Apical proton and omeprazole has previously been shown to modulate paracellular Mg^{2+} absorption. However the cellular regulatory mechanism of paracellular passive Mg^{2+} transport is unknown. Our results shown that omeprazole, which suppressed apical proton accumulation, enhanced the expression of acid- sensing ion channel 1a (ASIC1a), ovarian cancer G protein-coupled receptor 1 (OGR1), and transient receptor potential vanilloid 4 (TRPV4) in Caco-2 cells. It also inhibited passive Mg^{2+} transport across Caco-2 monolayers. Apical acidity and intermittent apical acidic culture medium exposure abolished omeprazole effects on passive Mg^{2+} transport and acid sensor expressions. OGR1 inhibitors, PLC inhibitor, and PKC inhibitor suppressed passive Mg^{2+} transport across control and omeprazole-exposed monolayers in apical pH at 7.4 and 7.0. In more apical acidity (apical bathing solution pH 6.5 - 5.5) ASIC1a inhibitor psalmotoxin 1 (PcTx1) and BAPTA-AM increased passive Mg^{2+} transport. On the other hand, ASIC1a activator amitriptyline suppressed passive Mg^{2+} transport in apical solution pH at 7.4 and 7.0. Moreover, omeprazole enhanced basal and acid-stimulated HCO_3^- secretion that inhibited by CFTR inhibitor NPPB, PcTx1, and BAPTA-AM. In addition, we also the effect of prolonged omeprazole administration in male Spraque-Dawley rats. The rats were administrated with 20 mg/kg omeprazole for 4 or 24 wk. Our results shown that omeprazole significantly suppressed plasma Mg^{2+} level, urinary Mg^{2+} excretion, bone and muscle Mg^{2+} content. By using Ussing chamber techniques, it was shown that omeprazole markedly suppressed duodenal transcellular and paracellular Mg^{2+} absorption and cation selectivity. Our results proposed the cellular regulatory mechanism of intestinal passive Mg^{2+} absorption.

Keyword: acid sensor, hypomagnesaemia, intestinal HCO_3^- secretion, paracellular Mg^{2+} absorption, proton pump inhibitor

บทคัดย่อ

รหัสโครงการ RSA5680005

ชื่อโครงการ กลไกควบคุมระดับเซลล์ของการขนส่งแมกนีเซียมแบบไม่ใช้พลังงานผ่านช่องว่างระหว่างเซลล์ในแผ่นเซลล์เยื่อบุลำไส้ Caco-2

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การดูดซึมแมกนีเซียม (Mg^{2+}) แบบไม่ใช้พลังงานในลำไส้มีความสำคัญอย่างยิ่งต่อการรักษาสมดุล Mg^{2+} ในร่างกายมนุษย์ มีรายงานว่าภาวะกรดด้านโพรงลำไส้ และยา omeprazole นั้นมีผลเปลี่ยนแปลงการดูดซึมแมกนีเซียม Mg^{2+} แบบไม่ใช้พลังงานในลำไส้ได้ อย่างไรก็ตามกลไกควบคุมระดับเซลล์ของการขนส่งแมกนีเซียมแบบไม่ใช้พลังงานยังไม่เป็นที่ทราบแน่ชัด ผลจากการศึกษาในโครงการวิจัยนี้พบว่า omeprazole มีฤทธิ์ลดภาวะกรดด้านโพรงลำไส้ และกดการดูดซึม Mg^{2+} แบบไม่ใช้พลังงาน แต่ omeprazole มีฤทธิ์เพิ่มการแสดงออกของโปรตีน acid- sensing ion channel 1a (ASIC1a), ovarian cancer G protein-coupled receptor 1 (OGR1), และ transient receptor potential vanilloid 4 (TRPV4) ในเซลล์เพาะเลี้ยง Caco-2 ภาวะกรดด้านโพรงลำไส้สามารถยับยั้งการออกฤทธิ์ของ omeprazole ต่อการดูดซึม Mg^{2+} แบบไม่ใช้พลังงาน และการแสดงออกของโปรตีน ASIC1a, OGR1, และ TRPV4 ได้ ในภาวะที่ด้านโพรงลำไส้มีค่า pH 7.4 และ 7.0 สารยับยั้ง OGR1, PLC, และ PKC มีฤทธิ์กดการดูดซึม Mg^{2+} แบบไม่ใช้พลังงาน แต่ในภาวะที่ด้านโพรงลำไส้มีค่า pH 6.5 และ 5.5 สารยับยั้ง ASIC1a และ intracellular chelator กลับมีฤทธิ์เพิ่มการดูดซึม Mg^{2+} แบบไม่ใช้พลังงาน omeprazole ยังมีฤทธิ์เพิ่มการขับ HCO_3^- ในแผ่นเซลล์เพาะเลี้ยง Caco-2 ทั้งนี้การออกฤทธิ์ของ omeprazole นั้นจะหมดไปเมื่อบ่มแผ่นเซลล์ด้วยสารยับยั้ง ASIC1a, CFTR และ intracellular chelator นอกจากนี้ผู้วิจัยก็ได้ศึกษาผลของ omeprazole ในหนูขาวสายพันธ์ Spraque-Dawley เพศผู้ โดยพบว่า omeprazole มีฤทธิ์ลดระดับ Mg^{2+} ในกระแสเลือด ในปัสสาวะ ในกระดูก และในกล้ามเนื้อของหนูขาว และเมื่อศึกษาการดูดซึม Mg^{2+} ในลำไส้เล็กส่วน duodenum พบว่า omeprazole มีฤทธิ์กดการดูดซึม Mg^{2+} อย่างมีนัยสำคัญทางสถิติ ผลการศึกษาจากโครงการวิจัยนี้แสดงกลไกควบคุมระดับเซลล์ของการขนส่งแมกนีเซียมแบบไม่ใช้พลังงาน

คำหลัก: ตัวรับรู้ภาวะกรด, ภาวะแมกนีเซียมในเลือดต่ำ, การขับไบคาร์บอเนตในลำไส้, การขนส่งแมกนีเซียมแบบไม่ใช้พลังงาน, ยายับยั้งการหลั่งกรดในกระเพาะอาหาร

Executive Summary

◆◆

1. Introduction to the research problem and its significance

Magnesium (Mg^{2+}) plays an important role in numerous biological functions. Mg^{2+} deficiency is associated with several diseases, e.g. Alzheimer's disease [10], osteoporosis [32], and hypertension [41]. Therefore, its plasma level is tightly regulated within a narrow range (0.7-1.1 mmol/l) by the harmonious function of the intestinal absorption and renal excretion [19]. Since dietary intake is the sole source of Mg^{2+} in human, intestinal absorption plays a vital role in the regulation of normal Mg^{2+} balance. Intestinal epithelium absorbs Mg^{2+} via both saturable transcellular and non-saturable paracellular pathways. Transcellular Mg^{2+} transport is an active process that requires the activity of transient receptor potential melastatin 6 (TRPM6), TRPM7, and basolateral Na^{+}/Mg^{2+} exchanger [28, 33]. On the other hand, paracellular Mg^{2+} transport is a passive mechanism and implicates about 90% of intestinal Mg^{2+} absorption [28]. This process is modulated by the tight junction associated protein claudin (Cldn) [35]. However the regulatory mechanism of intestinal Mg^{2+} absorption remains largely unknown.

The proton pump inhibitor (PPI) is the common therapeutic tool for acid-peptic disorders. It selectively interacts with the gastric H^{+}/K^{+}-ATPase leading to potent inhibition of gastric acid secretion [27]. There have previously been a number of case reports of severe hypomagnesemia in patients with long-term use of PPI that probably resulted from intestinal, but not renal, Mg^{2+} wasting [3, 7, 11, 17, 21, 24, 34]. The results of Mg^{2+} retention test demonstrated a very high retention (~75%) of parenteral Mg^{2+} loaded [7], thus, those cases were severe Mg^{2+} stores depletion. Interestingly, each patient experienced normalization of plasma Mg^{2+} level and probably intestinal Mg^{2+} uptake within 1 - 2 weeks after PPI cessation. However, plasma Mg^{2+} level and intestinal absorption declined again within 1 - 2 weeks after PPI resumed [3, 7, 11, 17, 21, 24, 34], suggested the rapid inhibitory effect of PPI on intestinal Mg^{2+} absorption. Therefore, intestinal Mg^{2+} flux defect could not be responsible for later developments of hypomagnesaemia in prolong PPI used patients. On the other hand, the long delay in the development of hypomagnesaemia reflected the time it took for Mg^{2+} stores to become depleted [7]. Previously, we reported an inhibitory effect of the popular PPI omeprazole on the paracellular passive, but not active, Mg^{2+} absoption in human enterocyte-like Caco-2 monolayers [39]. Omeprazole also decreased paracellular cation selectivity and increased activation energy of paracellular Mg^{2+} absorption [39]. Our recent report demonstrates that omeprazole inhibited the function of paracellular Mg^{2+} channels in the Caco-2 epithelium by decreasing Mg^{2+} affinity and claudin (Cldn) -7 and -12 expression [40]. The mechanism of these omeprazole effects has not been studied.

It is widely accepted that tight junction associated protein Cldn creates the paracellular channel for paracellular ion transport [13, 20, 36]. Cldn-16 had been proposed as paracellular channel for Mg^{2+} in kidney [35]. However Cldn-16 was not detected along the intestinal tract [12], suggesting that other Cldn might be involved in paracellular Mg^{2+} absorption. Recently, we find that omeprazole suppresses the expression of Cldn-7 and -12 as well as paracellular passive Mg^{2+} transport in Caco-2 monolayers [40]. Therefore monomeric or heteromeric combination of Cldn-7 and -12 possibly contribute to the intestinal paracellular Mg^{2+} absorption. However, the role of other intestinal related Cldn, i.e., Cldn-4, -5, -8, and 15 [12, 13, 29], on Mg^{2+} absorption has not been elucidated. Moreover, the cellular mechanism of omeprazole regulated Cldn expression is still unknown.

The acidic environment of the intestinal lumen is known to have effects on the secretary and absorption activities of the intestine. The luminal acidity along the entire human small bowel varies between pH 5.5 – 7.0 [26]. Heijnen and coworkers [15] reported that the luminal acidity increased Mg^{2+} absorption in the ileum. Our recent finding shows that apical acidity at pH 5.5, 6.0, 6.5, and 7.0 increases paracelluar passive Mg^{2+} transport, Mg^{2+} affinity of paracellular channel, and Cldn-7 and -12 expression in control and omeprazole-exposed Caco-2 epitheliums [40]. However the underlying cellular mechanism and signaling transduction pathway(s) of apical acidity enhanced paracellular Mg^{2+} absorption and Cldn expression are still elusive. In addition, apical acidity also normalizes the inhibitory effect of omeprazole on Mg^{2+} absorption and Cldn expression [40] with an unknown mechanism.

When luminal pH decreased, the intestinal epithelium cells can directly detect and modulate their cellular respond by using the acid sensors, i.e., acid sensing ion channel type 1a (ASIC1a) and transient receptor potential vanilloid 4 (TRPV4) [8, 9, 16]. Both ASIC1a and TRPV4 are Ca^{2+}-permeable channel that trigger Ca^{2+} signaling transduction pathway to regulate cellular function, e.g., bicarbonate secretion, following drop of the apical pH [9, 14, 16]. Activation of TRPV4 increased paracellular permeability and Cldn-1, -4, -5, -7, and -8 expression in Ca2+ signaling dependent manner [31]. In addition, Cldn -1, -3, -4, -5, -7, -8, -11, and -18 expression can be regulate by Ca^{2+}-dependent transcription factors, i.e., cAMP respond element binding protein (CREB), ternary complex factor (TCF), and c-Jun [1, 23, 25, 31, 42]. Several Ca^{2+} sensitive signaling mediators, i.e., mitogen-activated protein kinase (MAPK), calmodulin (CaM) / myosin light chain kinase (MLCK), protein kinase (PKA), phosphoinositide 3-kinase (PI3K), and rho-associated coiled-coil-containing protein kinase (ROCK), [2, 6] can also modulate paracellular ion permeability [4, 5, 18, 22, 37, 38]. Since, ASIC1a and TRPV4 possibly present and function in Caco-2 monolayers [N. Thongon; unpublished observation, 2012, 8] it is questionable whether ASIC1a, TRPV4, Ca^{2+}-dependent transcription factors, and Ca^{2+} sensitive signaling mediators play a role in apical acidity

regulates Cldn expression and paracellular Mg^{2+} absorption in intestinal like Caco-2 epithelium.

In the present study, I hypothesize that the apical acidity directly stimulates ASIC1a and TRPV4 that then triggers increase in intracellular Ca^{2+}. Intracellular Ca^{2+} signaling in turn activates apical HCO-3 secretions, cation selective Cldn expression, and paracellular Mg2+ absorption. Omeprazole disturbs apical acidity [40] and inhibits ASIC1a and TRPV4 activation that suppresses Cldn expression and paracellular Mg^{2+} absorption. The results from the present study provided the evidence for the regulation of the passive intestinal Mg^{2+} absorption.

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2. Objectives

2.1 To demonstrate the expression of ASIC1a and TRPV4 in Caco-2 monolayers

2.2 To demonstrate the role of ASIC1a and TRPV4 in paracellular passive Mg^{2+} transport and bicarbonate secretion in Caco-2 monolayers

2.3 To demonstrate the effect of omeprazole on ASIC1a, TRPV4, and tight junction in Caco-2 monolayer

2.4 To demonstrate the signaling transduction pathway(s) of apical acidity regulated paracellular passive Mg^{2+} transport and claudins expression in Caco-2 monolayer

2.5 To demonstrate the cellular mechanism of apical acidity normalized omeprazole effect on paracellular passive Mg^{2+} transport and claudins expression in Caco-2 monolayer

3. Methodology

3.1 Cell culture

Caco-2 cells (ATCC No. HTB-37) are grown in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 12% fetal bovine serum (FBS-Gold; PAA Laboratories GmbH, Pasching, Austria), 1% L-glutamine (Gibco, Grand Island, NY, USA), 1% non-essential amino acid (Sigma), and 1% antibiotic-antimycotic solution (Gibco) and maintained at a humidified atmosphere containing 5% CO2 at 37 °C. For Mg^{2+} flux studies, the Caco-2 monolayers are developed by seeding cells (1.0×10^6 cells/cm^2) onto the permeable polyester Transwell-clear inserts (Corning, Corning, NY, USA) and maintained for 14 days. For western blot analysis, cells are plated (5.0×10^5 cells/well) on 6-well plate and maintained for 14 days. For MTT reduction assay, cells are seeded (1.0×10^4 cells/well) on 96-well plate (Corning) and maintain for the same period of time. In the omeprazole-treated group, the monolayers are grown in media containing 200 or 400, ng/ml omeprazole (Calbiochem, San Diego, CA, USA).

3.2 Measurements of Mg^{2+} flux and HCO^-_3 secretion

Apical to basolateral paracellular Mg^{2+} flux studies are performed at a humidified atmosphere with 5% CO_2 at 37°C. After removal of the culture media, the apical and basolateral side of Caco-2 monolayers on transwell are added with apical and basolateral solutions, respectively. At 1 and 2 hours, 500 μl solution is collected from the basolateral side for measurement of Mg^{2+} concentration. Mg^{2+} flux (nmol hr^{-1} cm^{-2}) is calculated using Equation (Eq.) 1.

$$Mg^{2+} \text{ flux} = C_{Mg} / (t \times S) \quad \text{(Eq. 1)}$$

where C_{Mg} is Mg^{2+} concentration (nM); t is time (hours); and S is transport surface area (cm^2).

The Mg^{2+} permeability (P_{Mg}) of Caco-2 monolayers is calculated using Eq. 2.

$$P_{Mg} = Mg^{2+} \text{ flux} / \Delta C_{Mg} \quad \text{(Eq. 2)}$$

where ΔC_{Mg} is concentration difference of Mg^{2+} between the apical and basolateral solutions

Bicarbonate secretion is performed at a humidified atmosphere with 5% CO_2 at 37 °C. After removal of the culture media, the apical and basolateral side of monolayers on transwell are added with apical and basolateral solutions, respectively. At 15 min, 1 ml solution is collected from the apical side for measurement of bicarbonate concentration by using a clinical chemistry analyzer (ILab Taurus; Instrumentation Laboratory, Bedford, MA, USA).

3.3 Mg^{2+} transport kinetic analysis

To estimate the kinetic values of the saturable active and non-saturable passive Mg^{2+} transport, the rate of apical to basolateral Mg^{2+} transport (Mg^{2+} flux) is fitted to a modified Michaelis–Menten kinetic plus linear component as shown in Eq. 3.

$$Mg^{2+} \text{ flux} = \frac{(V_m \times C_{Mg})}{(K_m + C_{Mg})} + mC_{Mg} \quad \text{(Eq. 3)}$$

where V_m is the maximal rate of saturable active Mg^{2+} transport; K_m is the rate constant of saturable Mg^{2+} transport; m is the rate constant for non-saturable passive Mg^{2+} transport; and C_{Mg} as mentioned above. This study is performed using a nonlinear regression program of GraphPad Prism version 5.0 for Window (GraphPad Software Inc., San Diego, CA, USA).

3.4 Bathing solution

All solutions with an osmolality of 290–295 mmol/kg H_2O were gassed with 5% CO_2 in 95% O_2 30 min prior to use and maintained at 37°C. For Mg^{2+} transport study in transwell trasnsporting setup, the basolateral solution is (in mmol/l) 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose,

2.5 L-glutamine, 250 D-mannitol, and 10 HEPES pH 7.4; whereas, the apical solution is (in mmol/l) 1.25 CaCl2, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, and 10 HEPES pH 7.4, containing either 2.5 $MgCl_2$ and 242 mannitol, 5 $MgCl_2$ and 235 mannitol, 10 $MgCl_2$ and 230 mannitol, 20 $MgCl_2$ and 200 mannitol, 40 $MgCl_2$ and 115 mannitol, or 80 $MgCl_2$ and 90 mannitol.

For Bicarbonate secretion study in transwell trasnsporting setup, the basolateral solution is (in mmol/l) 1.25 $CaCl_2$, 23 $NaHCO_3$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 220 D-mannitol, and 10 HEPES pH 7.4; whereas, the apical solution is (in mmol/l) 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 250 D-mannitol, and 10 HEPES pH 7.4

In the pH dependent studies, HEPES of pH 7.4 in apical solution is substituted with same concentration of HEPES pH 7.0, HEPES pH 6.5, HEPES pH 6.0, or HEPES pH 5.5. All chemicals were purchased from Sigma (Sigma, St. Louis, MO, USA).

3.5 Measurements of Mg^{2+} concentration

The concentration of Mg^{2+} is determined by Xylidyl Blue (Sigma) colorimetric assay, modified from method of Tang and Goodenough [36]. In brief, the sample solutions are spun at 1000 × g for 10 min and 200 μl sample of the upper solution was collected. An aliquot is added to 100 μl water, gently mixed, and then 200 μl of 1.25 mM EGTA is added to the assay tube. After mixing well, 500 μl of Xylidyl Blue solution pH 10.5 is added to the assay tube. After 5 min of incubation at room temperature, the assay solution is subjected to colorimetric analysis using a spectrophotometer at 520 nm (model UV-2550; Shimadzu, Kyoto, Japan)

3.6 MTT reduction assay

Caco-2 cells were washed with PBS and treated with 1 mg/ml MTT solution (sigma) for 3 hours in a humidified atmosphere containing 5% CO_2 at 37 ˚C. Formazan crystals in cells were solubilized with DMSO and subjected to colorimetric analysis using multi-mode microplate reader at 540 nM (BioTek Instruments, Inc)

3.7 Western blot analysis

Caco-2 cells were lysed in RIPA buffer (Sigma) with gentle shaking (seesaw mode) for 20 min at 4 ˚C. After cells were scraped with Cell Scraper (Corning), lysates were sonicated, centrifuged at 12,000 g for 15 min, and then heated for 5 min at 95°C. Proteins (60 μg) or Cruz Marker™ Molecular Weight Standards were loaded and separated on 12.5% SDS-PAGE gel, then transferred to a polyvinylidene difluoride membrane (PVDF; Amersham, Buckinghamshire,UK). Membranes were blocked with 5% nonfat milk overnight at 4°C and probed overnight at 4°C with 1:500 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) raised against human ASIC1a, TRPV4, Cldn-2, -4, -5, -7, -8, -12 -15, ZO-1, CREB, phosphor-CREB, TCF, phosphor-TCF, c-Jun and phosphor-c-Jun. Membranes were also reprobed with 1:5,000 anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology). After

2h incubation at 25°C with 1:5,000 HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), blots were visualized by Pierce ECL western blotting substrate (Thermo Scientific Pierce Protein Research, Rockford, USA) and captured on Hyperfilm™ (Amersham). Densitometric analysis was performed using ImageJ for Mac Os X [30].

3.8 Transmission electron microscope (TEM) and Immunoglod TEM

Caco-2 monolayers are fixed with 2.5 % glutaraldehyde for 4 h at 4°C. They are then post-fixed in 1% OsO4 for 2 h at 4°C, dehydrated in increasing percentages of cold ethanol, and finally embedded in araldite 502 resin.

For TEM thin sections (~70 nm) are cut and mounted on formvar-coated copper grids and counterstained with lead citrate and uranyl acetate before being viewed under the transmission electron microscope (model TECNAI 20, Philips, Japan).

For immunogold TEM the sections are incubated in 3% H_2O_2 for 30 min and then in 4% BSA at 48°C for 2h to block non-specific binding. The sections are probed with primary antibody raised against human Cldn-2, -4, -5, -7, -8, -12 and -15, and then probed with biotinylated IgG and gold- conjugated streptavidin, respectively. Finally, the sections are counterstained with uranyl acetate and lead citrate, and observed in a Philips TECNAI 20 transmission electron microscope.

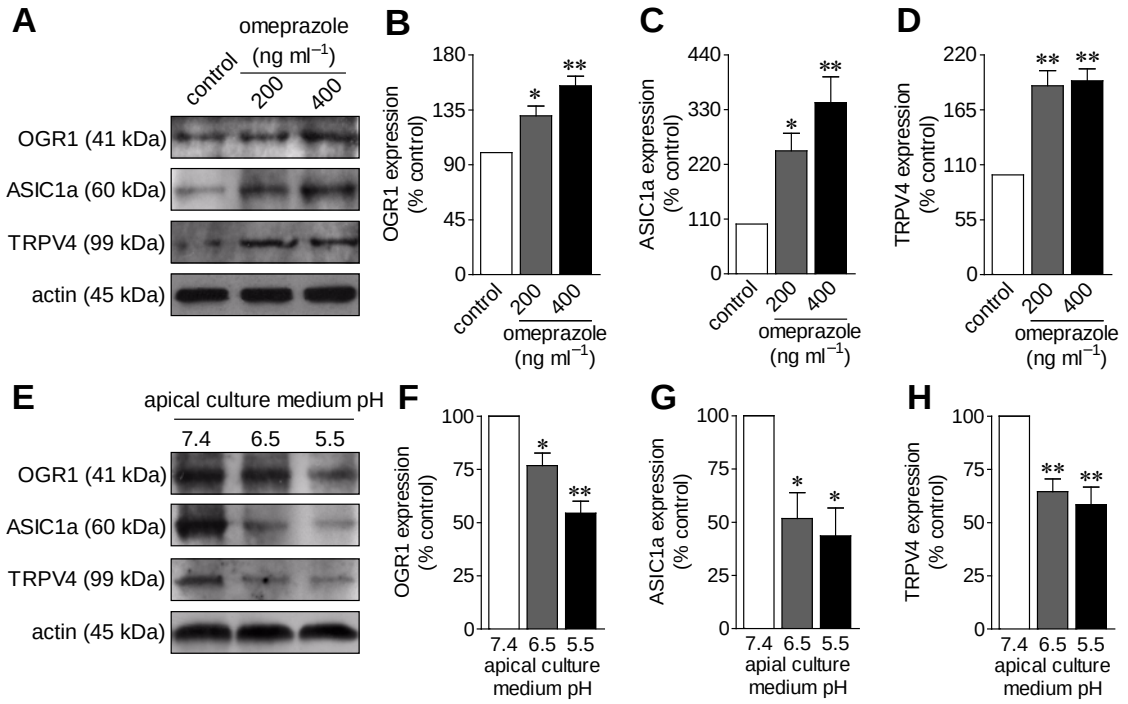
3.9 Statistical analysis

Results were expressed as means ± SE. Two sets of data were compared using the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) with Dunnett's post test was employed for multiple sets of data. Non-linear regression was performed to elucidate the %maximum - apical Mg^{2+} concentration relationship. The curve of P_{Mg}-Δmagnesium relationship was obtained using one phase exponential decay equation. The level of significance was $P < 0.05$. All data were analyzed by GraphPad Prism (GraphPad Software Inc.)

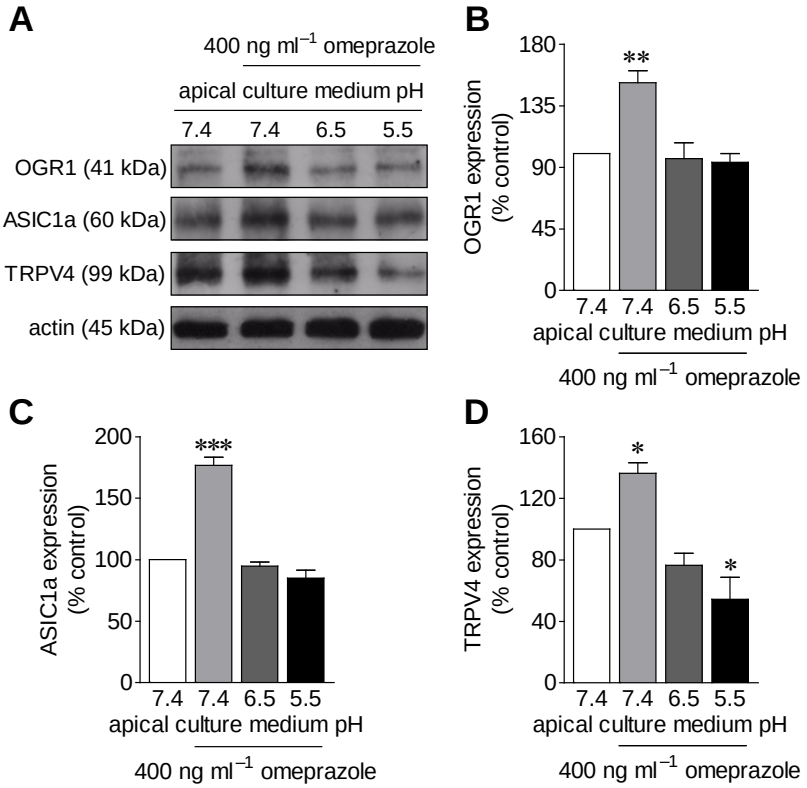
4. ผลการวิจัยที่ได้รับ

4.1 ผลของ omeprazole ภาวะกรดด้านโพรงลำไส้ และ omeprazole ร่วมกับภาวะกรดด้านโพรงลำไส้ ต่อการแสดงออกของ ASIC1a และ TRPV4 ในแผ่นเซลล์เพาะเลี้ยง Caco-2

ดังแสดงในรูปที่ 1A, 1C, และ 1D พบว่า omeprazole มีฤทธิ์เพิ่มการแสดงออกของ ASIC1a และ TRPV4 อย่างมีนัยสำคัญทางสถิติ นอกจากนั้นผู้วิจัยยังศึกษาการแสดงออกของ acid sensor อีกชนิดคือ ovarian cancer G protein-coupled receptor 1 (OGR1) ซึ่งได้ผลเช่นกัน (รูปที่ 1A และ 1B) ในทางตรงกันข้ามภาวะกรดด้านโพรงลำไส้ที่ pH 6.5 และ 5.5 มีฤทธิ์กดการแสดงออกของ OGR1 ASIC1a และ TRPV4 อย่างมีนัยสำคัญทางสถิติ (รูปที่ 1E – 1H) แต่ในกลุ่มเซลล์เพาะเลี้ยง Caco-2 ที่ได้รับ omeprazole ความเข้มข้น 400 ng/mL ร่วมกับภาวะกรดด้านโพรงลำไส้ที่ pH 6.5 และ 5.5 พบว่าระดับการแสดงออกของ OGR1 ASIC1a และ TRPV4 ไม่แตกจากกลุ่มควบคุม บ่งชี้ว่า ภาวะกรดด้านโพรงลำไส้นั้นสามารถยับยั้งผลของ omeprazole ต่อการแสดงออกของ acid sensor คือ OGR1 ASIC1a และ TRPV4 ได้



<u>รูปที่ 1</u> ผลของ omeprazole และภาวะกรดด้านโพรงลำไส้ต่อการแสดงออกของ OGR1 ASIC1a และ TRPV4. Representative immunobloting และ densitometric analysis ที่บ่งบอกระดับการแสดงออกของ OGR1 (A และ B), ASIC1a (A และ C), และ TRPV4 (A และ D) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ 200 หรือ 400 ng/mL omeprazole. Representative immunobloting และ densitometric analysis ที่บ่งบอกระดับการแสดงออกของ OGR1 (E และ F), ASIC1a (E และ G), และ TRPV4 (E และ H) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับอาหารเลี้ยงเซลล์ที่มีค่า pH 6.5 หรือ 5.5. *P < 0.05, **P < 0.01 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).



<u>รูปที่ 2</u> ผลของ omeprazole ร่วมกับภาวะกรดด้านโพรงลำไส้ต่อการแสดงออกของ OGR1 ASIC1a และ TRPV4. Representative immunobloting และ densitometric analysis ที่บ่งบอกระดับการแสดงออกของ OGR1 (A และ B), ASIC1a (A และ C), และ TRPV4 (A และ D) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ 400 ng/mL omeprazole หรือได้รับ 400 ng/mL omeprazole ร่วมกับอาหารเลี้ยงเซลล์ที่มีค่า pH 6.5 หรือ 5.5.

*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

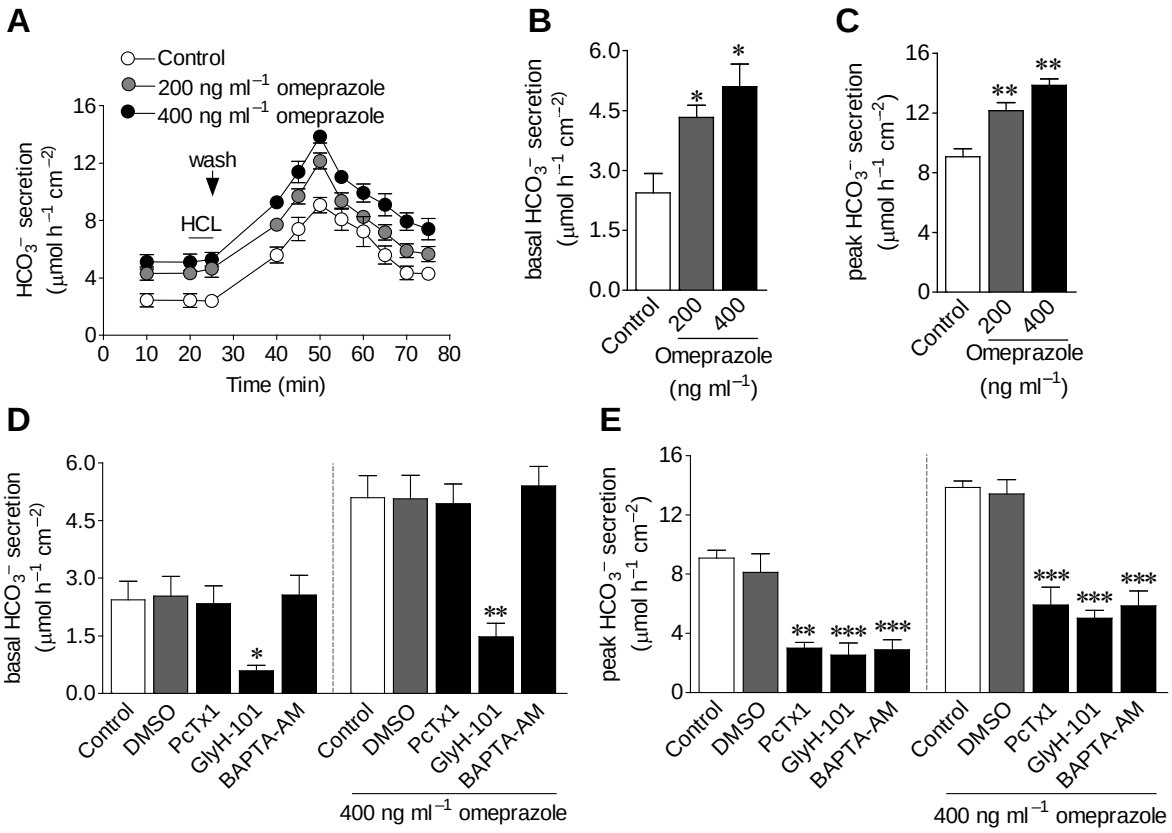
4.2 การขับไบคาร์บอเนตในแผ่นเซลล์เพาะเลี้ยง Caco-2

ผลการศึกษาการขับไบคาร์บอเนตในแผ่นเซลล์เพาะเลี้ยง Caco-2 พบว่า 200 หรือ 400 ng/mL omeprazole มีฤทธิ์เพิ่มการขับไบคาร์บอเนต ทั้งในระดับพัก (basal HCO_3^- secretion) (รูปที่ 3A และ 3B) และระดับสูงสุดหลังกระตุ้นด้วย 10 mM HCl (peak HCO_3^- secretion) (รูปที่ 3A และ 3C) ในแผ่นเซลล์เพาะเลี้ยง Caco-2

ผลการศึกษา basal HCO_3^- secretion (รูปที่ 3D) เมื่อบ่มแผ่นเซลล์ด้วย ASIC1a inhibitor (PcTx1), TRPV4 inhibitor (ruthenium red; RR), CFTR inhibitor (GlyH-101), MEK/MAPK inhibitor (U0126), PI3K inhibitor (Wortmannin; Wort.), PLC inhibitor (U-73122), PKA inhibitor (H89), PKC inhibitor (Gö 6850), หรือ intracellular Ca^{2+} chelator (BAPTA-AM) พบว่ามีเพียง GlyH-101 เท่านั้นที่มีฤทธิ์เปลี่ยนแปลงการขับไบคาร์บอเนต โดยลดระดับ basal HCO_3^- secretion ทั้งในแผ่นเซลล์เพาะเลี้ยงควบคุม และได้รับ 400 ng/mL omeprazole บ่งชี้ว่า omeprazole มีฤทธิ์เพิ่มการขับขับไบคาร์บอเนตผ่าน CFTR ในภาวะพัก

เมื่อศึกษา peak HCO_3^- secretion (รูปที่ 3E) โดยบ่มแผ่นเซลล์เพาะเลี้ยงด้วยสารต่างๆเช่นเดียวกันกับการศึกษาในรูปที่ 3D พบว่า PcTX1, GlyH-101, และ BAPTA-AM มีฤทธิ์เปลี่ยนแปลงการขับ

ไบคาร์บอเนต โดยลดระดับ peak HCO_3^- secretion ทั้งในแผ่นเซลล์เพาะเลี้ยงควบคุม และได้รับ 400 ng/mL omeprazole บ่งชี้ว่า 10 mM HCl ออกฤทธิ์กระตุ้นการทำงานของ ASIC1a ซึ่งเป็น Ca^{2+} channel จึงเพิ่มระดับ Ca^{2+} อิสระภายในเซลล์ และกนระตุ้นการทำงานของ CFTR ในการขับไบคาร์บอเนต



<u>รูปที่ 3</u> การขับไบคาร์บอเนตในแผ่นเซลล์เพาะเลี้ยง Caco-2. ระดับการขับ HCO_3^- ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ omeprazole ในแต่ละช่วงเวลาทั้งก่อนและหลังได้รับการกระตุ้นด้วย 10 10 mM HCl ที่ด้านโพรงลำไส้ (A). Basal HCO_3^- secretion (ที่เวลา 20 นาที; B และ D) และ peak acid-stimulated HCO_3^- secretion (ที่เวลา 50 นาที; C และ E) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ omeprazole

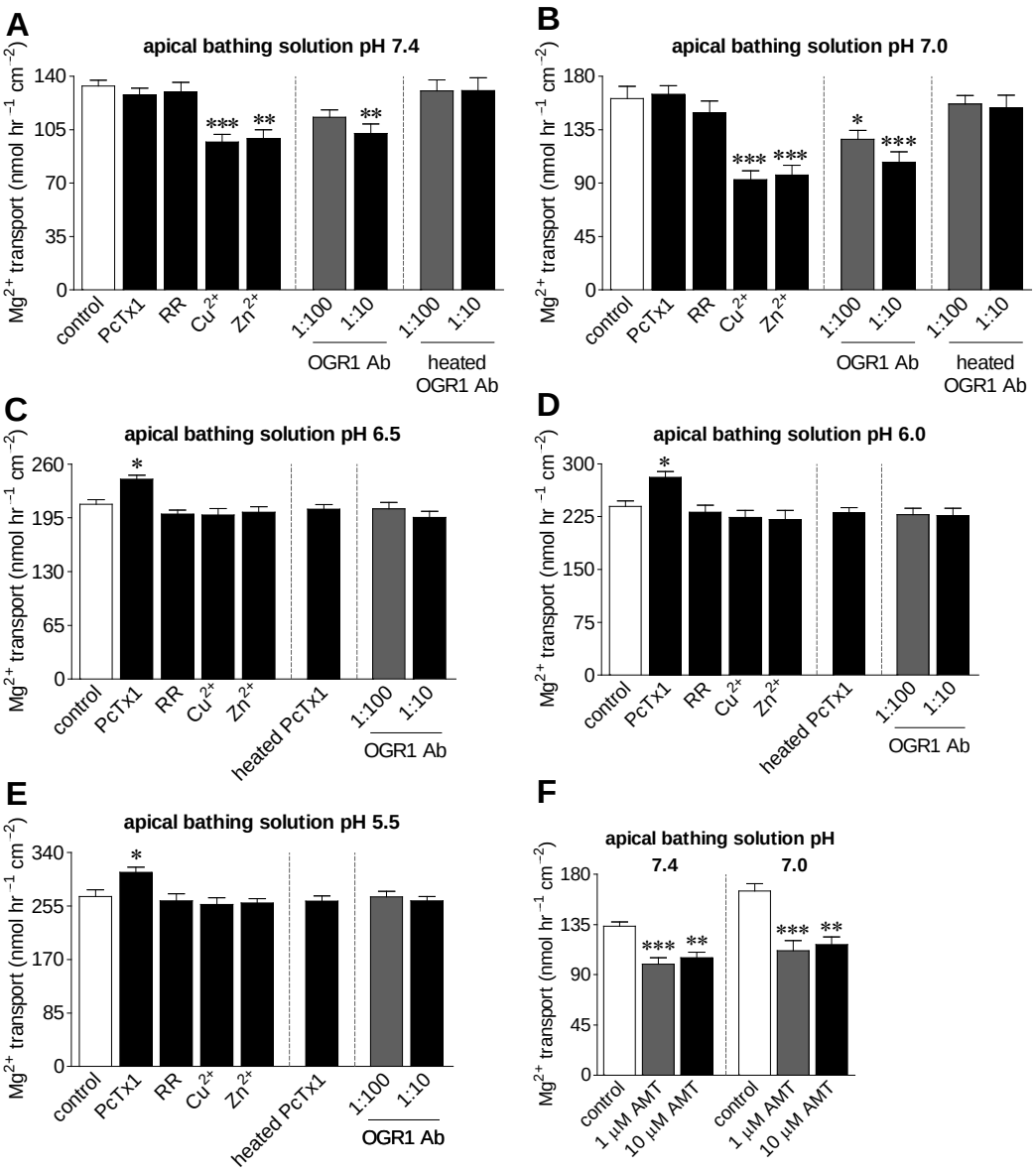
*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

4.3 กลไกการส่งสัญญาณภายในเซลล์ในภาวะกรดด้านโพรงลำไส้ควบคุมการขนส่ง Mg^{2+} ผ่านแผ่นเซลล์เพาะเลี้ยง Caco-2

ในการศึกษานี้แผ่นเซลล์เพาะเลี้ยง Caco-2 ถูกบ่มด้วย ASIC1a agonist (Amitriptyline; AMT), ASIC1a inhibitor (PcTx1), TRPV4 inhibitor (ruthenium red; RR), OGR1 inhibitor (Cu^{2+} และ Zn^{2+}), หรือ OGR-1 antibody (OGR1 Ab) ก่อนนำมาศึกษาการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานโดยมีความเข้มข้น $MgCl_2$ ด้านโพรงลำไส้ที่ 40 mmol/l แต่ไม่มี $MgCl_2$ ด้าน basolateral เพื่อให้เกิดความลาดเอียงความเข้มข้นของ Mg^{2+} และมีการเปลี่ยนค่า pH ด้านโพรงลำไส้จาก 7.4 เป็น 7.0 6.5 6.0 หรือ 5.5 จากผลการทดลองที่ค่า pH ด้านโพรงลำไส้ 7.4 (รูปที่ 4A) และ 7.0 (รูปที่ 4B) Cu^{2+}, Zn^{2+}, และ OGR1 Ab ลดอัตรา

การขนส่ง Mg^{2+} อย่างมีนัยสำคัญทางสถิติ บ่งชี้ว่าที่ระดับค่า pH ด้านโพรงลำไส้ 7.4 และ 7.0 การทำงานของ OGR-1 จะมีผลกระตุ้นการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน

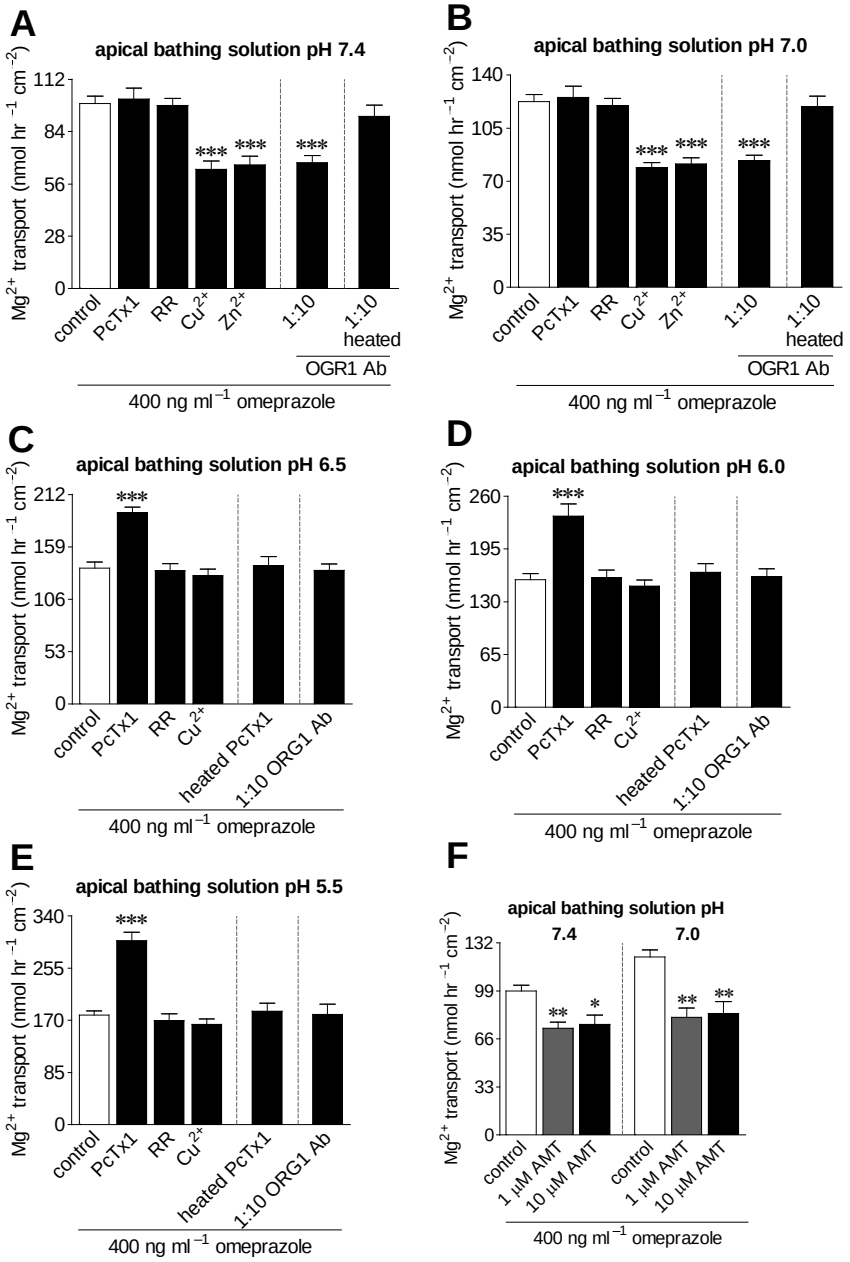
ในทางตรงกันข้ามค่า pH ด้านโพรงลำไส้ 6.5 (รูปที่ 4C), 6.0 (รูปที่ 4D), และ 5.5 (รูปที่ 4E) นั้น PcTx1 มีฤทธิ์เพิ่มอัตราการขนส่ง Mg^{2+} อย่างมีนัยสำคัญทางสถิติ บ่งชี้ว่าในภาวะกรดด้านโพรงลำไส้ (pH 6.5, 6.0, และ 5.5) การทำงานของ ASIC1a จะกดการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน ยิ่งไปกว่านั้นค่า pH ด้านโพรงลำไส้ 7.4 และ 7.0 (รูปที่ 4F) ซึ่งเป็นระดับที่ไม่กระตุ้นการทำงานของ ASIC1a แต่เมื่อให้ ASIC1a agonist AMT กลับลดอัตราการขนส่ง Mg^{2+} อย่างมีนัยสำคัญทางสถิติ เป็นการยืนยันว่า การทำงานของ ASIC1a จะกดการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน



<u>รูปที่ 4</u> อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานผ่านแผ่นเซลล์เพาะเลี้ยง Caco-2. อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานโดยมีความลาดเอียงความเข้มข้นของ Mg^{2+} ที่ 40 mmol/L และมีการเปลี่ยนค่า pH ด้านโพรงลำไส้จาก 7.4 (A) เป็น 7.0 (B), 6.5 (C), 6.0 (D) หรือ 5.5 (E)

*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

ในทางเดียวกันกับที่ผลการทดลองในแผ่นเซลล์เพาะเลี้ยง Caco-2 กลุ่มควบคุมในรูปที่ 4 บ่งชี้ว่าที่ระดับค่า pH ด้านโพรงลำไส้ 7.4 และ 7.0 การทำงานของ OGR-1 จะมีผลกระตุ้นการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน แต่ในภาวะกรดด้านโพรงลำไส้ (pH 6.5, 6.0, และ 5.5) การทำงานของ ASIC1a จะกดการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน

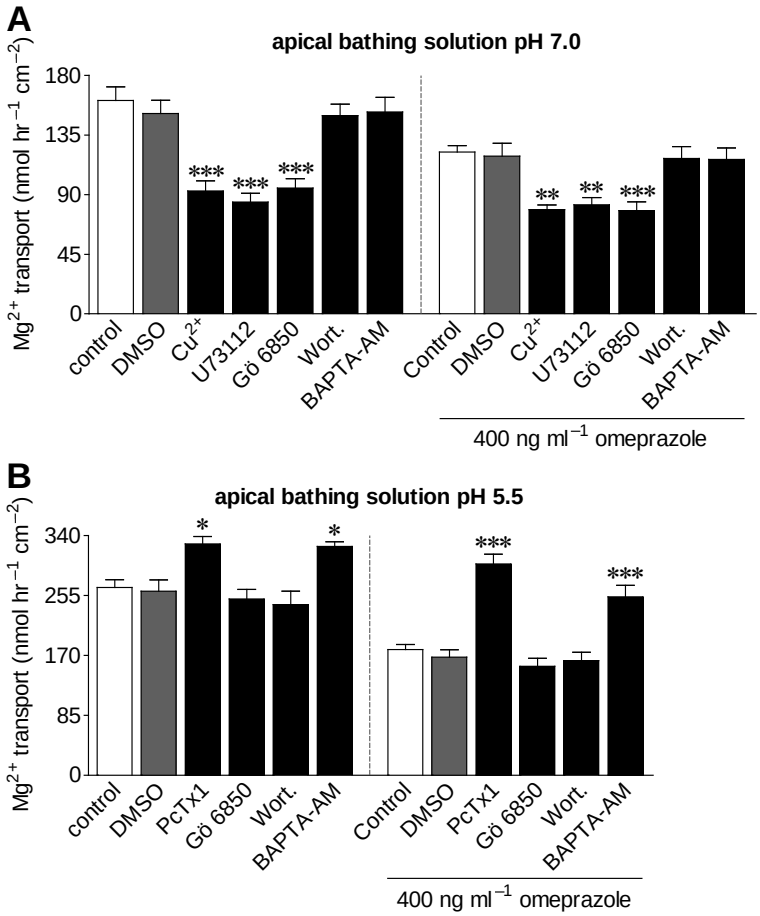


<u>รูปที่ 5</u> อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานผ่านแผ่นเซลล์เพาะเลี้ยง Caco-2 ที่ได้รับ omeprazole 400 ng/mL. อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานโดยมีความลาดเอียงความเข้มข้นของ Mg^{2+} ที่ 40 mmol/L และมีการเปลี่ยนค่า pH ด้านโพรงลำไส้จาก 7.4 (A) เป็น 7.0 (B), 6.5 (C), 6.0 (D) หรือ 5.5 (E)

*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

จากผลการศึกษาข้างต้นผู้วิจัยจึงศึกษากลไกการส่งสัญญาณภายในเซลล์ที่ค่า pH ด้านโพรงลำไส้ 7.0 ซึ่งการทำงานของ OGR-1 จะมีผลกระตุ้นการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน โดยบ่มแผ่นเซลล์เพาะเลี้ยง Caco-2 ปกติ และที่ได้รับ omeprazole ด้วย OGR1 inhibitor (Cu^{2+}), PI3K inhibitor (Wortmannin; Wort.), PLC inhibitor (U-73122) หรือ PKC inhibitor (Gö 6850) ก่อนการศึกษาอัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน (รูปที่ 6A) พบว่า Cu^{2+}, U-73122, และ Gö 6850 ลดอัตราการขนส่ง Mg^{2+} อย่างมีนัยสำคัญทางสถิติ บ่งชี้ว่า OGR-1 ซึ่งเป็น G-protein coupled receptor ชนิด G_q ทำงานผ่าน PLC และ PKC ในการกระตุ้นการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน

ผู้วิจัยจึงญยังได้ศึกษากลไกการส่งสัญญาณภายในเซลล์ในภาวะกรดด้านโพรงลำไส้ (pH 5.5) การทำงานของ ASIC1a จะกดการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานโดยบ่มแผ่นเซลล์เพาะเลี้ยง Caco-2 ปกติ และที่ได้รับ omeprazole ด้วย ASIC1a inhibitor (PcTx1), PI3K inhibitor (Wortmannin; Wort.), PKC inhibitor (Gö 6850), หรือ intracellular Ca^{2+} chelator (BAPTA-AM) ก่อนการศึกษาอัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน (รูปที่ 6B) พบว่า PcTx1 และ BAPTA-AM เพิ่มอัตราการขนส่ง Mg^{2+} อย่างมีนัยสำคัญทางสถิติ บ่งชี้ว่า ASIC1a ซึ่งเป็น Ca^{2+} permeable chhanel ทำงานผ่าน intracellular Ca^{2+} signaling ในการยับยั้งการขนส่ง Mg^{2+} แบบไม่ใช้พลังงาน



<u>รูปที่ 6</u> อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานผ่านแผ่นเซลล์เพาะเลี้ยง Caco-2. อัตราการขนส่ง Mg^{2+} แบบไม่ใช้พลังงานโดยมีความลาดเอียงความเข้มข้นของ Mg^{2+} ที่ 40 mmol/L โดยศึกษาในระดับค่า pH ด้านโพรงลำไส้ที่ 7.0 (A) หรือ 5.5 (B)

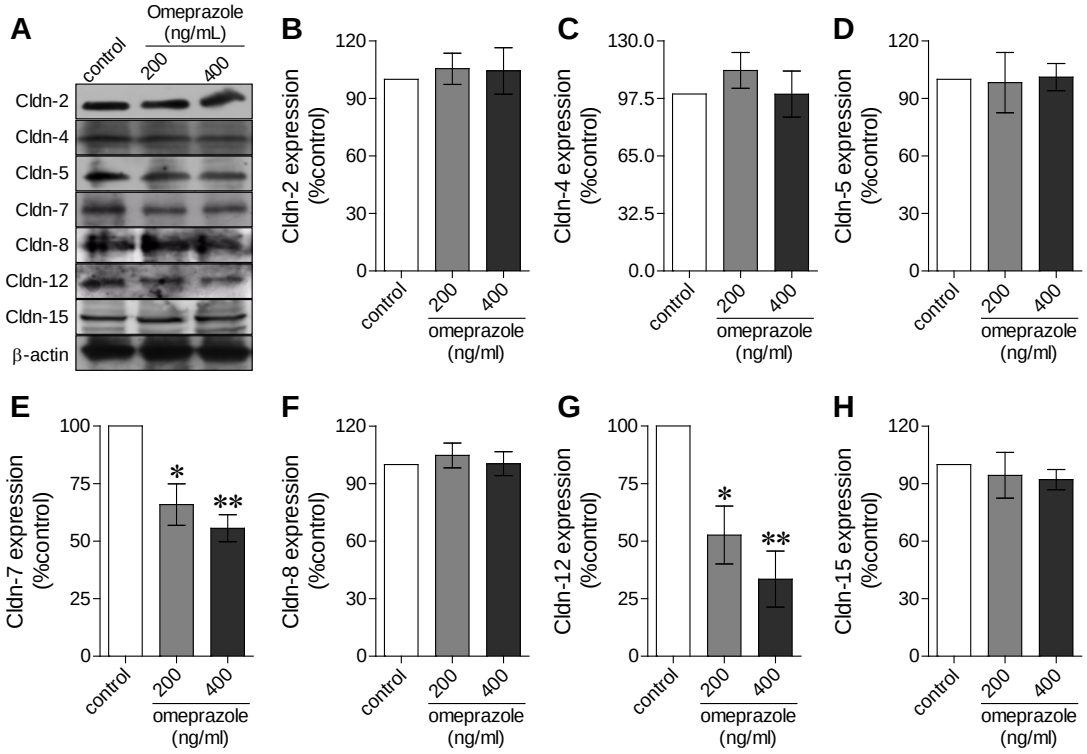
*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

4.4 ผลของ omeprazole ภาวะกรดด้านโพรงลำไส้ และ omeprazole ร่วมกับภาวะกรดด้านโพรงลำไส้ ต่อการแสดงออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ในแผ่นเซลล์เพาะเลี้ยง Caco-2

ดังแสดงในรูปที่ 7A, 7E, และ 7G พบว่า omeprazole ที่ความเข้มข้น 200 และ 400 ng/ml มีฤทธิ์กดการแสดงออกของ Cldn-7 และ Cldn-12 อย่างมีนัยสำคัญทางสถิติ แต่ไม่เปลี่ยนแปลงการแสดงออกของ Cldn-2, -4, -5, -8, และ -15 (รูปที่ 7A, 7B, 7C, 7D, 7F, และ 7H) ในแผ่นเซลล์เพาะเลี้ยง Caco-2

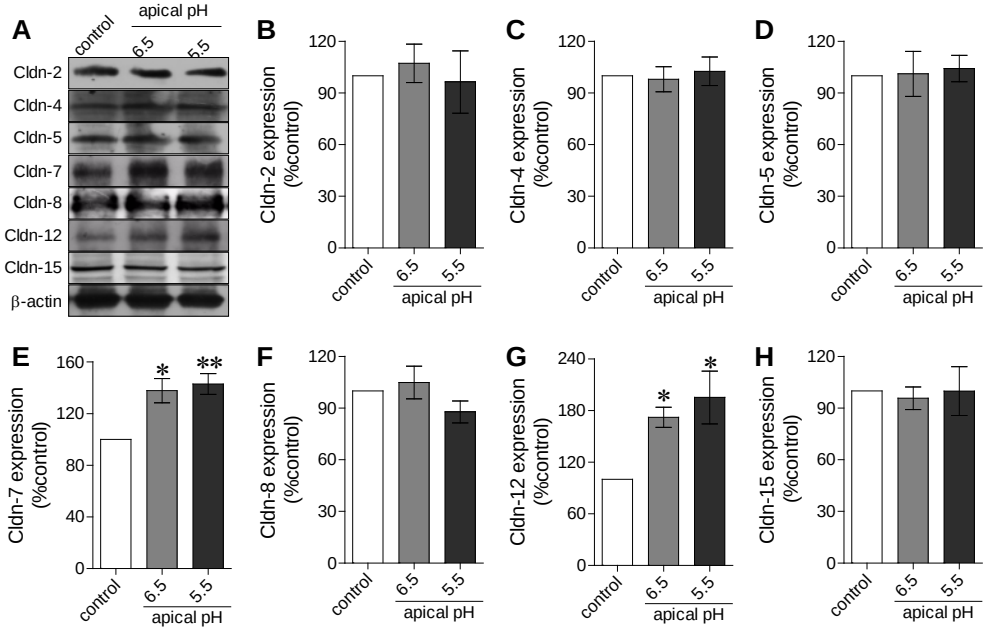
ภาวะกรดด้านโพรงลำไส้ (pH 6.5 และ 5.5) มีฤทธิ์เพิ่มการแสดงออกของ Cldn-7 และ Cldn-12 (รูปที่ 8A, 8E, และ 8H) อย่างมีนัยสำคัญทางสถิติ แต่ไม่เปลี่ยนแปลงการแสดงออกของ Cldn-2, -4, -5, -8, และ -15 (รูปที่ 8A, 8B, 8C, 8D, 8F, และ 8H) ในแผ่นเซลล์เพาะเลี้ยง Caco-2

ภาวะกรดด้านโพรงลำไส้ยังสามารถยับยั้งการออกฤทธิ์ของ omeprazole ในการกดการแสดงออกของ Cldn-7 และ -12 ในแผ่นเซลล์เพาะเลี้ยง Caco-2 ที่ถูกบ่มด้วย omeprazole ความเข้มข้น 400 ng/ml (รูปที่ 9)



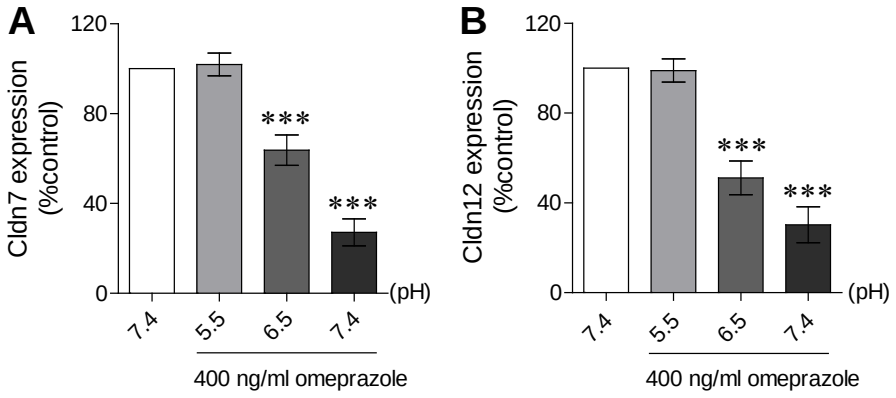
<u>รูปที่ 7</u> ผลของ omeprazole ต่อการแสดงออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ในแผ่นเซลล์เพาะเลี้ยง Caco-2. Representative immunobloting และ densitometric analysis ที่บ่งบอกระดับการแสดงออกของ Cldn-2 (A และ B), Cldn-4 (A และ C), Cldn-5 (A และ D), Cldn-7 (A และ E), Cldn-8 (A และ F), Cldn-12 (A และ G), และ Cldn-15 (A และ H) ในเซลล์เพาะเลี้ยง Caco-2 ปกติหรือได้รับ 200 หรือ 400 ng/mL omeprazole.

*P < 0.05, **P < 0.01 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).



<u>รูปที่ 8</u> ผลของ apical acidity ต่อการแสดงออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ในแผ่นเซลล์เพาะเลี้ยง Caco-2. Representative immunobloting และ densitometric analysis ที่บ่งบอกระดับการแสดงออกของ Cldn-2 (A และ B), Cldn-4 (A และ C), Cldn-5 (A และ D), Cldn-7 (A และ E), Cldn-8 (A และ F), Cldn-12 (A และ G), และ Cldn-15 (A และ H) ในเซลล์เพาะเลี้ยง Caco-2 ปกติหรือได้รับ 200 หรือ 400 ng/mL omeprazole.

*P < 0.05, **P < 0.01 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).



<u>รูปที่ 9</u> ผลของ omeprazole ร่วมกับ apical acidity ต่อการแสดงออกของ Cldn-7 และ -12 ในแผ่นเซลล์เพาะเลี้ยง Caco-2. Representative densitometric analysis ที่บ่งบอกระดับการแสดงออกของ Cldn-7 (A) และ Cldn-12 (B) ในเซลล์เพาะเลี้ยง Caco-2 ที่ได้รับอาหารเลี้ยงเซลล์ที่มีค่า pH 6.5 หรือ 5.5 ร่วมกับ omeprazole 400 ng/ml.

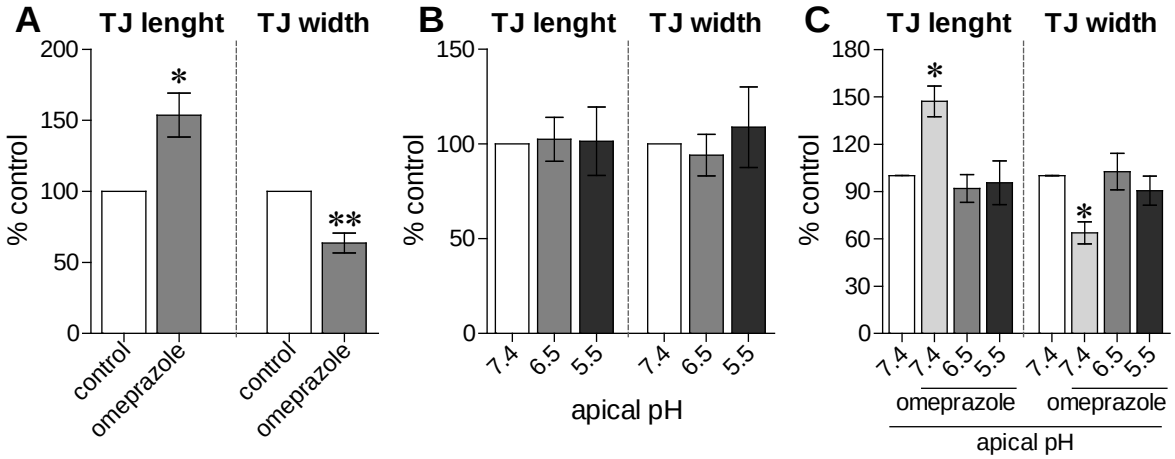
***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

4.5 อิทธิพลของ omeprazole และ apical acidity ต่อ tight junction

จากการศึกษาด้วย TEM ก่อนจะนำมาวิเคราะห์ความยาว และความกว้างของ tight junction ด้วยโปรแกม ImageJ พบว่า omeprazole ความเข้มข้น 400 ng/ml มีฤทธิ์เพิ่มความยาว และลดความกว้างของ

tight junction ในแผ่นเซลล์เพาะเลี้ยง Caco-2 อย่างสำคัญทางสถิติ (รูปที่ A) แต่ภาวะกรดด้านโพรงลำไส้ไม่มีผลต่อความยาว และลดความกว้างของ tight junction ในแผ่นเซลล์เพาะเลี้ยง Caco-2 (รูปที่ 10B)

อย่างไรก็ตามภาวะกรดด้านโพรงลำไส้สามารถต้านฤทธิ์ของ omeprazole ความยาว และลดความกว้างของ tight junction ในแผ่นเซลล์เพาะเลี้ยง Caco-2 ให้กลับเป็นปกติได้ (รูปที่ 10C)



<u>รูปที่ 10</u> ความยาวและความกว้างของ tight junction (TJ) ในแผ่นเซลล์เพาะเลี้ยง Caco-2. ความยาวและความกว้างของ tight junction (TJ) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ omeprazole 400 ng/ml (A) หรือ ได้รับอาหารเลี้ยงเซลล์ที่มีค่า pH 6.5 หรือ 5.5 (B) หรือได้รับอาหารเลี้ยงเซลล์ที่มีค่า pH 6.5 หรือ 5.5 ร่วมกับ omeprazole 400 ng/ml.
*$P < 0.05$ เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

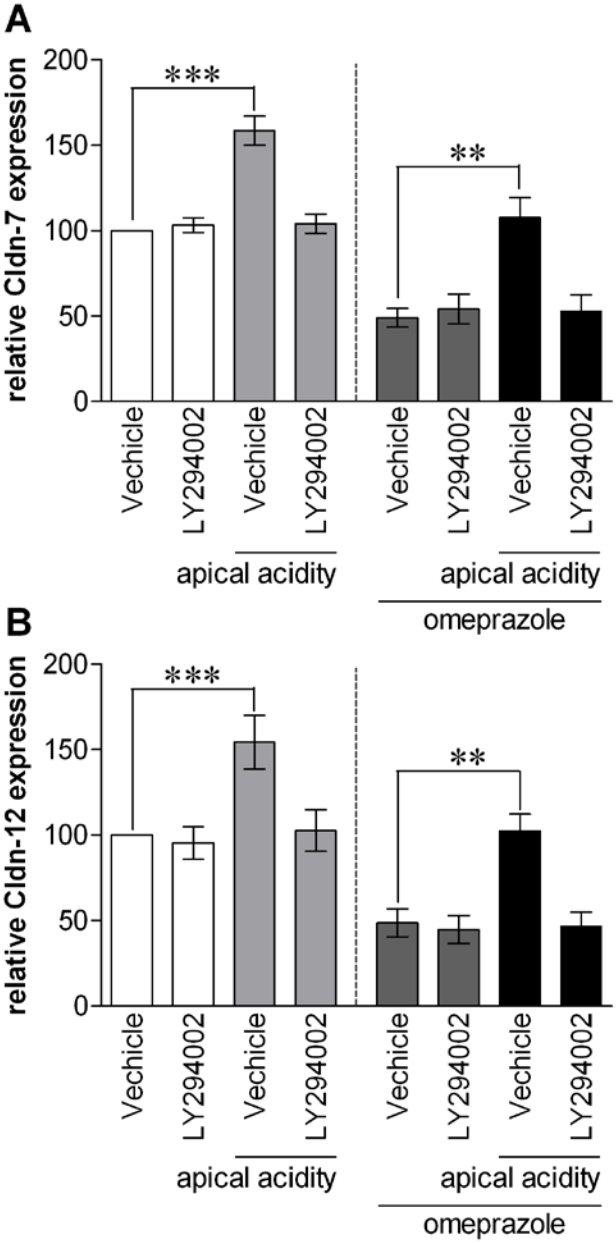
4.6 ตำแหน่งการแสดออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ใน tight junction ของแผ่นเซลล์เพาะเลี้ยง Caco-2

การศึกษาด้วยเทคนิค Immunoglod TEM เพื่อดูการกระจายตัวและระบุตำแหน่งการแสดงออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ใน tight junction โดยในแต่ละกลุ่มการทดลองผู้วิจัยทำการศึกษา 5 กลุ่มตัวอย่าง และศึกษาใต้กล้องจุลทรรศน์อิเลคตรอนจำนวน 10 สไลด์ต่อ 1 กลุ่มตัวอย่าง ผลการศึกษาพบว่า omeprazole ภาวะกรดด้านโพรงลำไส้ และ omeprazole ร่วมกับภาวะกรดด้านโพรงลำไส้ ไม่มีผลเปลี่ยนแปลงการกระจายตัวและตำแหน่งการแสดงออกของ Cldn-2, -4, -5, -7, -8, -12 และ -15 ในแผ่นเซลล์เพาะเลี้ยง ทั้งนี้อาจจะเนื่องมาจากปริมาณการแสดงออกของ claudin แต่ละชนิดใน tight junction มีน้อย เมื่อศึกษาตำแหน่งการแสดงออกของ Cldn ในแต่ tight junction จึงไม่พบความเปลี่ยนแปลง หรือเทคนิคที่เลือกใช้อาจจะไม่มีประสิทธิภาพพอที่จะแยกความแตกต่างของตำแหน่งการแสดงของ Cldn ใน tight junction ได้

4.7 Cellular signaling of apical acidity normalized omeprazole effect

จากการศึกษาอิทธิพลของ apical acidity ที่ pH 5.5 ต่อการแสดงออกของ Cldn-7 และ -12 ในแผ่นเซลล์เพาะเลี้ยง Caco-2 พบว่า apical acidity มีฤทธิ์เพิ่มการแสดงออกของ Cldn-7 และ -12 ในแผ่นเซลล์เพาะเลี้ยง Caco-2 ปกติ และที่ได้รับ omeprazole 400 ng/ml อย่างไรก็ตามอิทธิพลของ apical acidity ต่อการแสดงออกของ Cldn-7 และ -12 นั้นจะถูกยับยั้งเมื่อบ่มแผ่นเซลล์เพาะเลี้ยงด้วย LY294002 ซึ่งเป็นตัวยับยั้ง PI3K signaling (รูปที่ 11) บ่งชี้ว่าภาว apical acidity ควบคุมการแสดงออกของ Cldn-7 และ -12 ผ่านทางการกระตุ้น PI3K signaling pathway นอกจากนั้นผู้วิจัยยังพบว่า apical acidity ควบคุม

การแสดงออกของ ควบคุมการแสดงออกของ Cldn-7 และ -12 ผ่านทางการกระตุ้น cAMP respond element binding protein (CREB)



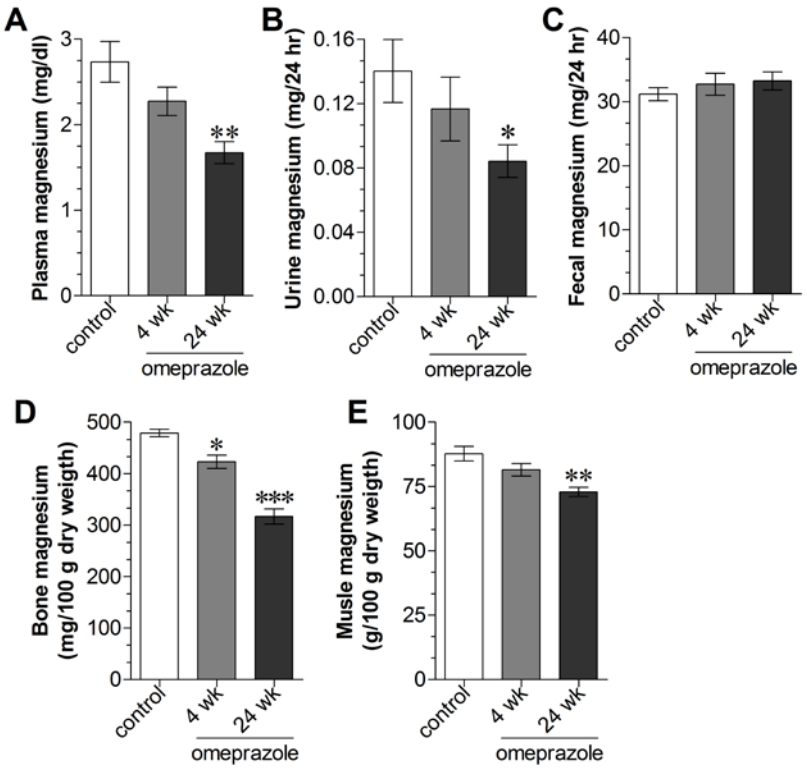
<u>รูปที่ 11</u> Cellular signaling ของภาวะ apical acidity ควบคุมการแสดงออกของ Cldn-7 และ -12 ในแผ่นเซลล์เพาะเลี้ยง Caco-2. Representative densitometric immunobloting analysis ที่บ่งบอกระดับการแสดงออกของ Cldn-7 (A) และ Cldn-12 (B) ในเซลล์เพาะเลี้ยง Caco-2 ปกติ หรือได้รับ 400 ng/mL omeprazole.

$^{**}P < 0.01$, $^{***}P < 0.001$ เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

4.7 ผลของ omeprazole ต่อ Mg^{2+} homeostasis ในหนูขาวเพศผู้

นอกจากนั้นผู้วิจัยยังได้ศึกษาต่อเนื่องถึงผลของ omeprazole ต่อการรักษาสมดุลในหนูขาวเพศผู้สายพันธ์ Sprague-Dawley โดยหนูขาวจะได้รับการฉีด omeprazole ความเข้มข้น 20 mg/kg เป็น

เวลา 4 หรือ 24 สัปดาห์ พบว่าหนูที่ได้รับ omrprazole เป็นเวลา 24 สัปดาห์ มีปริมาณ Mg^{2+} ในกระแสเลือด (รูปที่ 12 A) ในปัสสาวะ (รูปที่ 12 B) ในกระดูก (รูปที่ 12 D) และในกล้ามเนื้อ (รูปที่ 12 E) ต่ำลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม บ่งชี้ว่า omeprazole มีฤทธิ์ลดปริมาณ Mg^{2+} ในกระแสเลือดและแหล่งสะสมในร่างกายที่กระดูกและกล้ามเนื้อ ทั้งนี้ omeprazole ไม่ได้ออกฤทธิ์ที่ไตเพราะมีปริมาณการขับ Mg^{2+} ทางปัสสาวะน้อย แต่อาจจะออกฤทธิ์ที่ลำไส้โดยยับยั้งการดูดซึม Mg^{2+} ในลำไส้



<u>รูปที่ 12</u> ผลของ omeprazole ต่อ Mg^{2+} homeostasis ในหนูขาวเพศผู้สายพันธ์ Sprague-Dawley. ปริมาณ Mg^{2+} ในกระแสเลือด (A) ในปัสสาวะ (B) ในอุจจาระ (C) ในกระดูก (D) และในกล้ามเนื้อ (E) ของหนูขาวเพศผู้ที่ได้รับ omeprazole 20 mg/kg

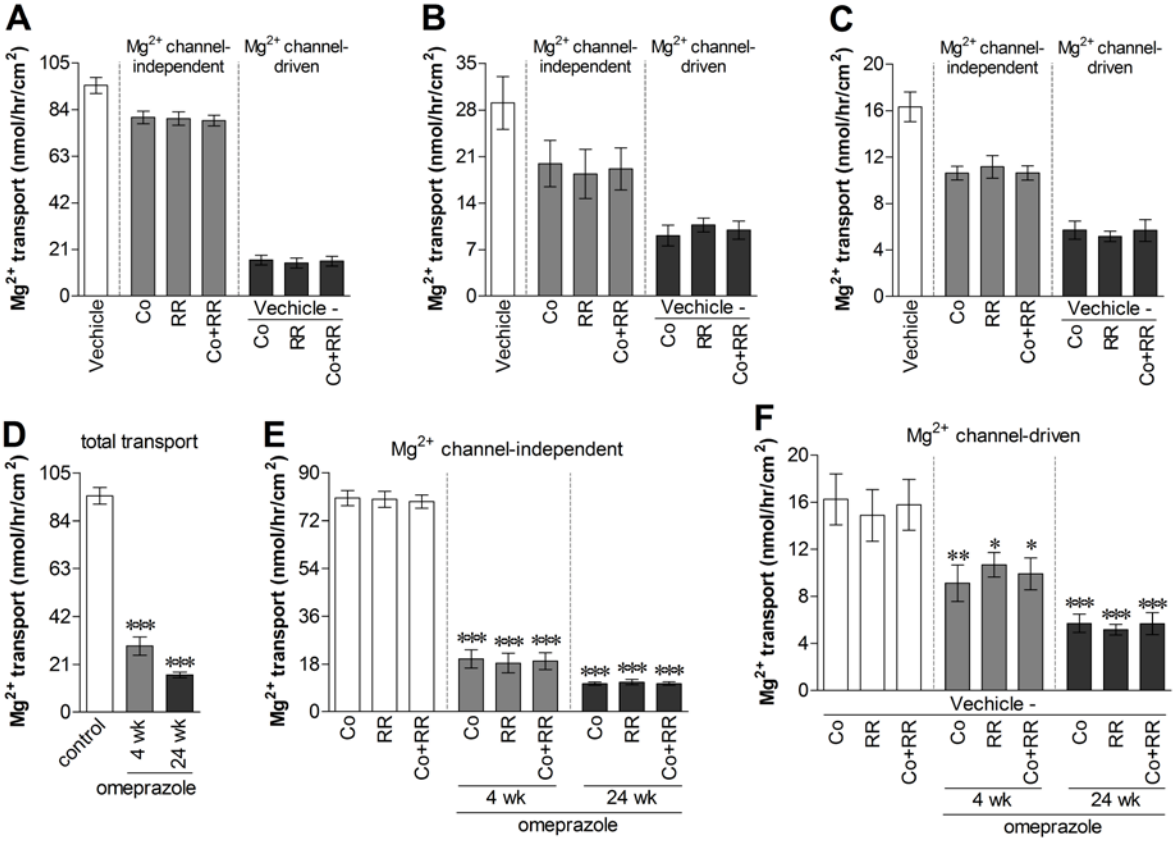
*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

4.8 ผลของ omeprazole ต่อการดูดซึม Mg^{2+} ในลำไส้เล็กของหนูขาวเพศผู้

ผู้วิจัยได้ทำการศึกษาต่อเนื่อง โดยวัดอัตราการดูซึม Mg^{2+} ในลำไส้เล็กส่วน duodenum ในการทดลองนี้ผู้วิจัยแบ่งลำไส้เล็กส่วน duodenum ออกเป็น 4 ส่วน และทำการศึกษาภายใต้ Ussing chamber setup 4 ชุดอิสระ ทำการศึกษาอัตราการขนส่ง Mg^{2+} ทั้งหมด (total transport) โดยให้มี concentration gradient ของ $MgCl_2$ ที่ 40 mmol/l ในเนื่อเยื่อ 1 ชิ้น เนื้อเยื่ออีก 3 ชิ้นที่เหลือ ทำการบ่มด้วย Co(III)hexamine (Co), rhuthenium red (RR), หรือ Co+RR ก่อนศึกษาอัตราการขนส่ง Mg^{2+} โดยให้มี concentration gradient ของ $MgCl_2$ ที่ 40 mmol/l เช่นกัน Co เป็น Mg^{2+} channel antagonist และ RR เป็น pan specific TRP channel antagonist การบ่มด้วยสารทั้ง 2 ชนิดนี้จะยับยั้งการทำงานของ Mg^{2+} channel ทั้งหมด และยับยั้งการขนส่ง Mg^{2+} แบบผ่านเซลล์ ทำให้ได้อัตราการขนส่งแบบผ่านช่องว่างระหว่างเซลล์เรียกว่า Mg^{2+} channel-independent Mg^{2+} transport จากนั้นเมื่อนำอัตราการขนส่งแบบ total

transport ลบด้วย Mg^{2+} channel-independent Mg^{2+} transport จะได้อัตราการขนส่งแบบผ่านเซลล์ เรียกว่า Mg^{2+} channel-driven Mg^{2+} transport

รูปที่ 13 แสดงผลการทดลองจากการศึกษาอัตราการขนส่ง Mg^{2+} ในลำไส้ส่วน duodenum ของหนูกลุ่มควบคุม (รูปที่ 13A) กลุ่มที่ได้รับ omeprazole 4 สัปดาห์ (รูปที่ 13B) และ กลุ่มที่ได้รับ omeprazole 24 สัปดาห์ (รูปที่ 13C) และเมื่อเทียบ total transport (รูปที่ 13D), Mg^{2+} channel-independent Mg^{2+} transport (รูปที่ 13E), และ Mg^{2+} channel-driven Mg^{2+} transport (รูปที่ 13F) พบว่า omeprazole มีฤทธิ์กดการดูดซึม Mg^{2+} ในสำไส้เล็กของหนูขาวอย่างมีนัยสำคัญทางสถิติ



<u>รูปที่ 13</u> ผลของ omeprazole ต่อการดูซึม Mg^{2+} ในลำไส้เล็กส่วน duodenum. อัตราการขนส่ง Mg^{2+} ในลำไส้ส่วน duodenum ของหนูกลุ่มควบคุม (**A**) กลุ่มที่ได้รับ omeprazole 4 สัปดาห์ (**B**) และ กลุ่มที่ได้รับ omeprazole 24 สัปดาห์ (**C**), อัตราการขนส่ง Mg^{2+} แบบ total transport (**D**), Mg^{2+} channel-independent Mg^{2+} transport (**E**), และ Mg^{2+} channel-driven Mg^{2+} transport (**F**) ในสำไส้เล็กของหนูขาวอย่างมีนัยสำคัญทางสถิติ

*P < 0.05, **P < 0.01, ***P < 0.001 เมื่อเปรียบเทียบกับกลุ่มควบคุมในแต่ละการทดลอง. (n = 5).

5. Output

ผลงานวิจัยจากงบประมาณโครงการวิจัยนี้ประกอบด้วย

5.1 **Thongon N**, Ketkeaw P, Nuekchob C. The roles of acid-sensing ion channel 1a and ovarian cancer G protein-coupled receptor 1 on passive Mg2+ transport across intestinal

epithelium-like Caco-2 monolayers. **Journal of Physiological Sciences 2014;** 64(2):129-39. doi: 10.1007/s12576-013-0301-8

*corresponding author; 90% contribution

5.2 **Thongon T***, Penguy J, Kulwong S, Khongmueang K, Thongma M. Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Sprague-Dawley rats. **Pflügers Archiv - European Journal of Physiology 2016 (revised)**

*corresponding author; 80% contribution

ภาคผนวก

1. **Thongon N**, Ketkeaw P, Nuekchob C. The roles of acid-sensing ion channel 1a and ovarian cancer G protein-coupled receptor 1 on passive Mg2+ transport across intestinal epithelium-like Caco-2 monolayers. **Journal of Physiological Sciences 2014;** 64(2):129-39. doi: 10.1007/s12576-013-0301-8

2. **Thongon T***, Penguy J, Kulwong S, Khongmueang K, Thongma M. Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Sprague-Dawley rats. **Pflügers Archiv - European Journal of Physiology 2016 (revised)**

J Physiol Sci (2014) 64:129–139
DOI 10.1007/s12576-013-0301-8

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-

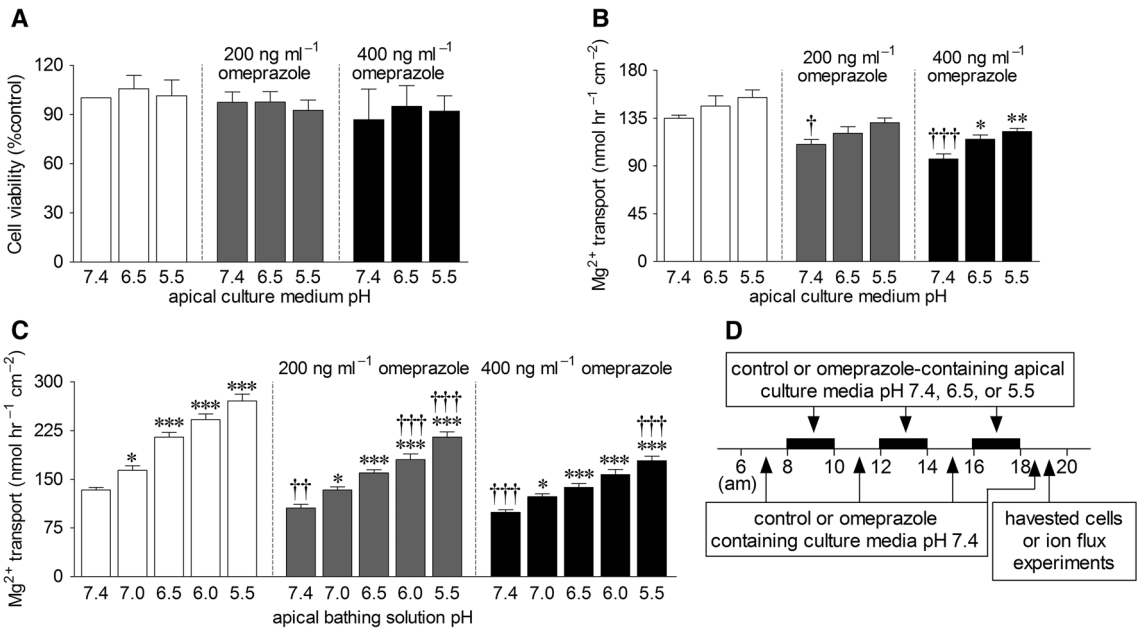

Fig. 1 Apical proton regulates intestinal passive Mg^{2+} transport. The relative cell viability of Caco-2 exposed to acidic apical culture medium, omeprazole, and omeprazole plus acidic was elucidated by MTT assay (**a**). Passive Mg^{2+} transport across Caco-2 monolayer was measured in the presence 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

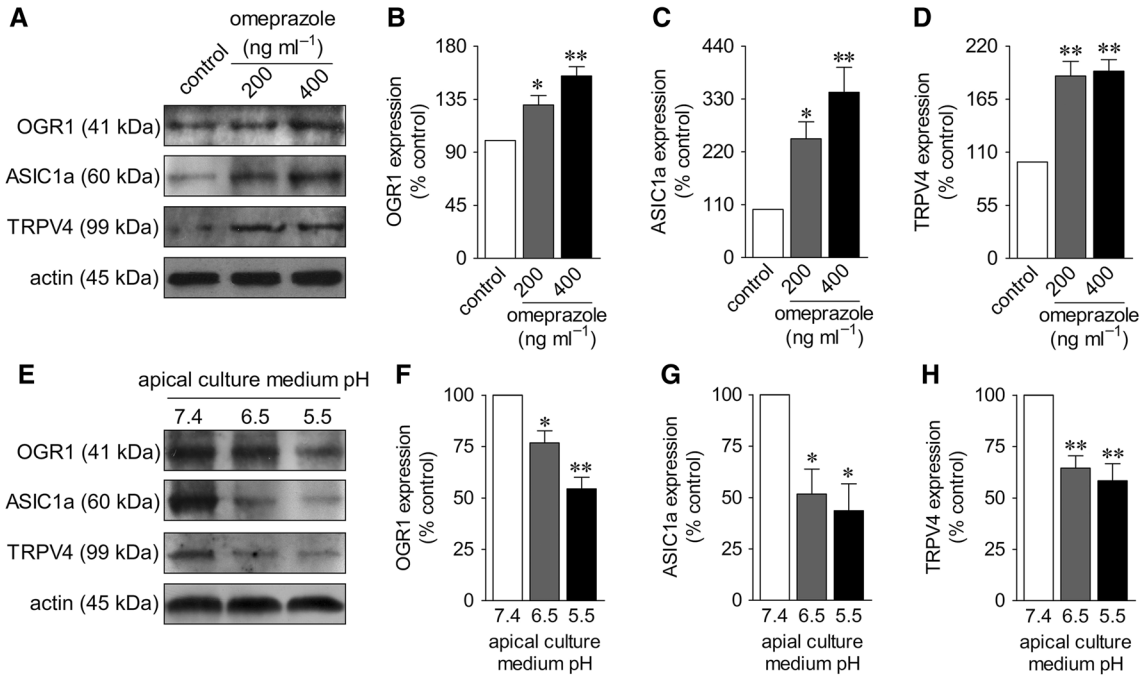

Fig. 2 Acidic apical culture medium and omeprazole regulate intestinal acid sensor expression. Representative immunoblotting and densitometric analysis of OGR1 (**a**, **b**, respectively), ASIC1a (**a**, **c**, respectively), and TRPV4 expressions (**a**, **d**, respectively) in Caco-2 cells exposed 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. ($n = 5$)

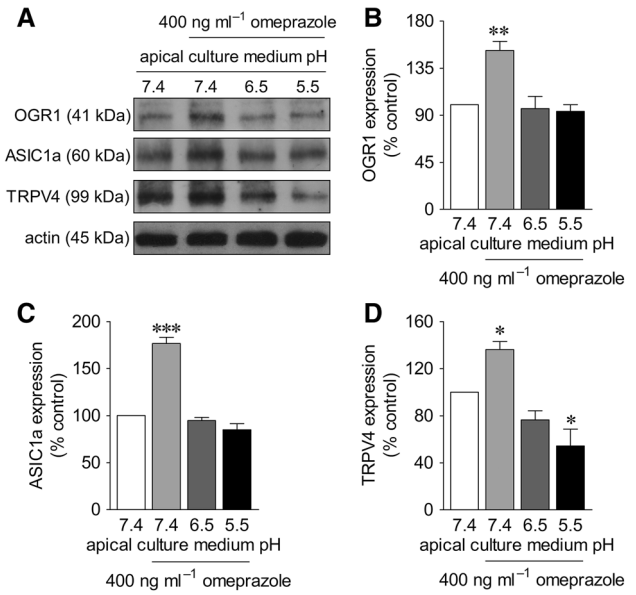

Fig. 3 Acidic apical culture medium abolishes omeprazole effect on acid sensor expression. Representative immunoblotting and densitometric analysis of OGR1 (**a**, **b**, respectively), ASIC1a (**a**, **c**, respectively), and TRPV4 expressions (**a**, **d**, respectively) in Caco-2 cells intermittently 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

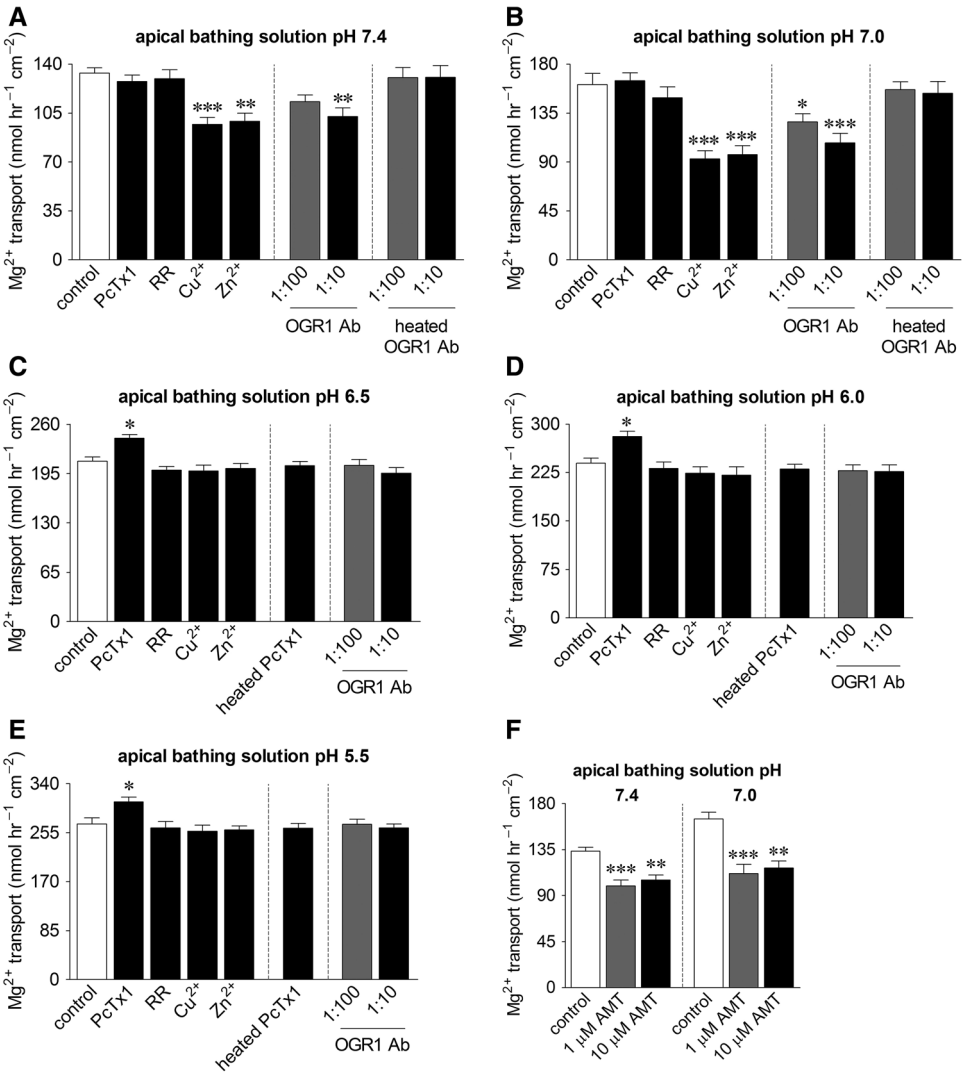

Fig. 4 OGR1 and ASIC1a regulate intestinal passive Mg^{2+} transport. In this prior of passive Mg^{2+} transport studies, Caco-2 monolayers were pre-incubated with 30 nM PcTx1, 10 μM RR, 10 μM Cu^{2+}, 10 μM Zn^{2+}, 1:10 or 1:100 OGR1 Ab, 100 °C-heated OGR1 Ab, or 100 °C-heated 30 nM PcTx1 before being 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

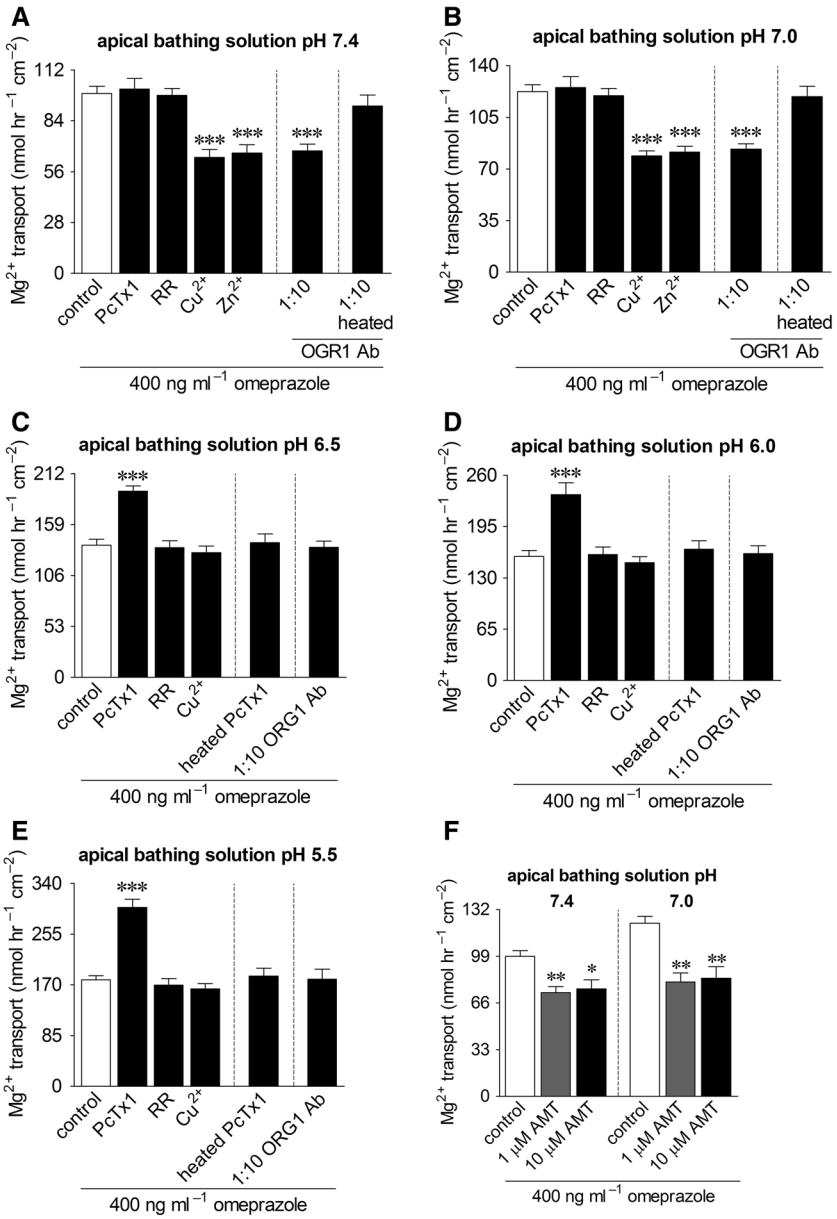

Fig. 5 OGR1 and ASIC1a regulate passive Mg^{2+} transport in omeprazole exposed epithelium. Passive Mg^{2+} transport by 400 ng/mL omeprazole-exposed Caco-2 monolayers that were pre-incubated with 30 nM PcTx1, 10 μM RR, 10 μM Cu^{2+}, 10 μM Zn^{2+}, 1:10 OGR1 Ab, 100 °C-heated OGR1 Ab, or 100 °C-heated 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

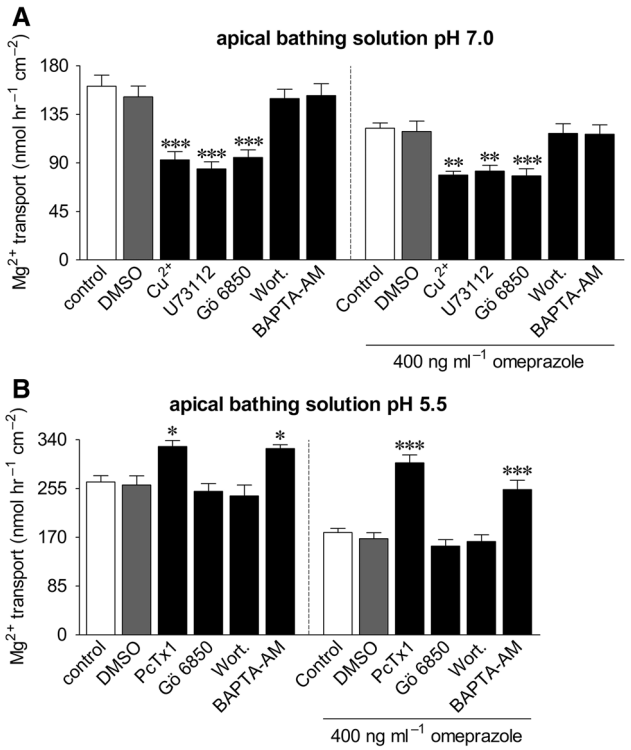

Fig. 6 Signaling pathways of OGR1 and ASIC1a regulate passive Mg^{2+} transport. Passive Mg^{2+} transport by using control or omeprazole-exposed Caco-2 monolayers that were pre-incubated with 30 nM PcTx1, 10 μM Cu^{2+}, or 10 μM Zn^{2+}, 10 μM U-73122, 1 μM Gö 6850, 200 nM Wort., or 50 μM BAPTA-AM in 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],

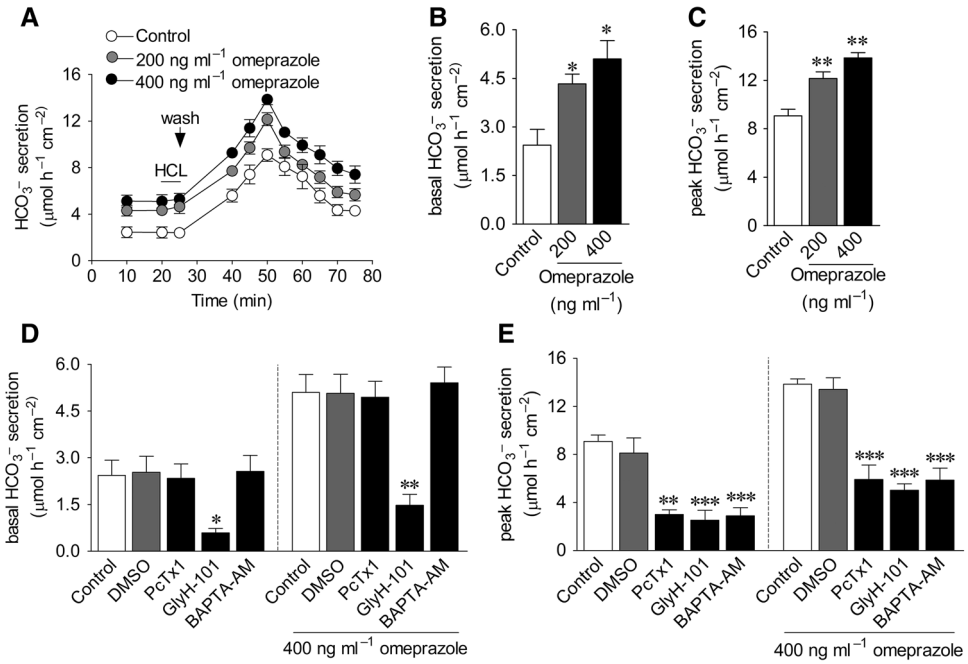

Fig. 7 ASIC1a mediates apical HCL stimulating HCO_3^- secretion. Time course of HCO_3^- secretion by control or omeprazole-exposed Caco-2 monolayers that was apically induced by 10 mM HCL (**a**). Basal (at 20 min; **b**, **d**) and peak acid-stimulated HCO_3^- secretion (at 50 min; **c**, **e**) by 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.

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Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Spraque-Dawley rats

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Abstract:	Hypomagnesemia is the most concerned side effect of proton pump inhibitors (PPIs) in chronic users. However, the mechanism of PPIs-induced systemic Mg2+ deficit is currently unclear. The present study aimed to elucidate the direct effect of short-term and long-term PPIs administrations on whole body Mg2+ homeostasis and duodenal Mg2+ absorption in rats. Mg2+ homeostasis was studied by determining the serum Mg2+ level, urine and fecal Mg2+ excretions, and bone and muscle Mg2+ contents. Duodenal Mg2+ absorption as well as paracellular charge selectivity were studied. Our result showed that gastric and duodenal pH markedly increased in omeprazole-treated rats. Omeprazole significantly suppressed plasma Mg2+ level, urinary Mg2+ excretion, bone and muscle Mg2+ content. Thus, omeprazole induced systemic Mg2+ deficiency. By using Ussing chamber techniques, it was shown that omeprazole markedly suppressed duodenal Mg2+ channel-driven and Mg2+ channel-independent Mg2+ absorptions and cation selectivity. Inhibitors of mucosal HCO3- secretion significantly increased duodenal Mg2+ absorption in omeprazole-treated rats. Transient receptor potential melastatin 6 (TRPM6)-driven transcellular and claudin (Cldn)-mediated paracellular Mg2+ absorptions are regulated by luminal proton. We therefore hypothesized that secreted HCO3- in duodenum decreased luminal proton, this impeded duodenal Mg2+ absorption. Higher plasma total 25-OH vitamin D, diuresis, and urine PO43− were also demonstrated in hypomagnesemic rats. As a compensatory mechanism for systemic Mg2+ deficiency, the expressions of duodenal TRPM6, cyclin M4 (CNNM4), Cldn-2, Cldn-7, Cldn-12, and Cldn-15 proteins were enhanced in omeprazole-treated rats. Our findings support the potential role of duodenum on the regulation of Mg2+ homeostasis and explain the pathophysiology of

	hypomagnesemia.
Response to Reviewers:	Correction 1.Name of author No.4 had changed from Kanyarat Khongmeang to Kanyanat Khongmueang. 2.The rate Mg2+ transport had changed from µmol/hr/cm2 to nmol/hr/cm2. 3.Grant No. form Burapha University in Acknowledgements had changed from AHS 2-1/2556 to AHS08/2558. Responses to the reviewers Reviewer#1: Comment 1. The authors stated that 'The present study aimed to elucidate the mechanism of prolonged PPIs administration on the induction of hypomagnesemia' (Abstract, line 19). To my view, Thongon et al. did not provide any specific mechanistic explanation for this phenomenon. The observation that chronic administrations of proton pump inhibitors results in a defect of intestinal magnesium intake in humans and rodent models is not novel and well described in the literature. The authors reported many phenotypic alterations indicating that omeprazole-treated animals developed systemic magnesium deficiency. These findings are not surprising and, more unimportant, have unclear relevance for the mechanistic understanding as how exactly omeprazole could suppress magnesium uptake in the intestine. Accordingly, I suggest a substantial revision of the text of the manuscript in order to exclude statements and speculations, which are not supported by the experimental data provided. The authors should outline clearly what are new findings in their work and how exactly these results advance the field. Respond: We thank the reviewer for these critical comments and suggestions. In compliance with the comments and suggestions, we have comprehensively revised our manuscript. We also re-examined and performed additional experiments to improve our data as followed; 1.(Abstract, Page 2) - We replaced with new sentences in line 1-3 -We added a new sentence in line 9 -We replaced with new sentences in line 10-12 -We added new sentences in line 12-17 -We added a new sentences in line 20-21 2.(Introduction, Page 3 - 5) -(paragraph 1, line 7-11) We replaced with new sentences. -(paragraph 2) we added or preplaced with new sentences in line 6-7, 9-14, and 15-17) -(paragraph 3) we added this new paragraph -(paragraph 4) we replaced with new sentences in line 5-7, and add a new sentence in line 8-9 3.(Materials and Methods) -(Magnesium flux measurement, Page 8, paragraph 1) We re-performed duodenal Mg2+ transport by pre-incubated duodenal tissue with Mg2+ channel blocker Co(III)hexaammine and noncompetitive pan specific TRP channel inhibitor ruthenium red. We added or preplaced with new sentences in line 1-2, 7-15, and 19-20. -(Magnesium flux measurement, Page 8, paragraph 2) We also performed additional experiment to elucidate the involvement of mucosal duodenal HCO3– secretion on omeprazole-affected duodenal Mg2+ transport by pre-incubated mucosal site of duodenal tissues with Cl–/HCO3– exchanger inhibitor DIDS and CFTR inhibitor GlyH-101. 4.(Results) -(Omeprazole suppressed duodenal Mg2+ absorption, Page 12-13, paragraph 1) We re-examined the expression of TRPM6 duodenal tissues. The results are illustrated in Fig. 3A. Because we have not enough duodenal tissues to re-study the expression of CNNM4 by immunohistochemistry analysis, however, we instead demonstrated the effect of omeprazole on duodenal CNNM4 expression by Western blot analysis (Fig. 3B). We replaced the sentences in line 9-11 of paragraph 1. -(Omeprazole suppressed duodenal Mg2+ absorption, Page 13, paragraph 2) We re-performed duodenal Mg2+ transport, as mention above. The results illustrated in Fig.

4. We also replaced the sentences in line 6-18 of paragraph 2.
-(Contribution of duodenal HCO3– secretion on omeprazole-suppressed Mg2+ transport, Page 14-15) We performed additional experiment to elucidate the involvement of mucosal duodenal HCO3– secretion on omeprazole-affected duodenal Mg2+ transport, as mention above. The results illustrated in Fig. 6. We also add this new text in page 14-15.
5.(Discussion)
-(paragraph 1, Page 16) we added a new sentence in line 11-12
-(paragraph 3, Page 16-17) we added a new sentence in line 1-17. We also added a new sentence in line 23-24.
-(paragraph 5, Page 18) we added this new paragraph to propose the possible explanations of why the over-expression of TRPM6 cannot counteract PPIH.
-(paragraph 6, Page 18-19) we added this new paragraph to propose the possible explanations of why the over-expression of claudins cannot counteract PPIH.
-(paragraph 8, Page 19) we added this new paragraph as conclusion.

Comment 2. Fig. 2C clearly shows that fecal magnesium excretion rate was normal in the omeprazole-treated rats. These results challenge the idea that omeprazole suppressed the intestinal uptake of magnesium in vivo. Furthermore, these observations are contradictory to the findings that many proteins involved in intestinal magnesium transport were up-regulated. The authors should provide a stronger experimental evidence for the suggested effect of omeprazole on intestinal magnesium intake in vivo.
Answer: Previous in vivo study of Hess and colleagues demonstrated that 20 mg/kg omeprazole treatment for 14 days induced hypomagnesemia with normal fecal Mg2+ excretion in C57BL/J6 mice [1]. Whether stimulation of colonic Mg2+ absorption or not, the over-expression of colonic TRPM6 could not counteract hypomagnesemia [1]. Our results agreed with Hess et al. [1] that PPIH rats had normal fecal Mg2+ excretion. The possible explanation was a very low fraction of duodenal Mg2+ uptake from ingested Mg2+. The average food intake of control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats were 23.99, 25.95, and 23.76 g/day, respectively. Since, pellet chow contained 0.23% wt/wt magnesium, thus, control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats ingested Mg about 5.52, 5.97, and 5.47 g/day, respectively. Since 1 gram Mg equal to 8.12 mEq Mg and 4.06 mmol Mg (http://www.mgwater.com/convert. shtml). The rats in 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated group ingested about 22.41, 24.24, and 22.21 mmol Mg/day, respectively. Duodenal Mg2+ uptake was 94.95, 29.397, and 16.33 nmol in control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats. Therefore, a very low fraction of duodenal Mg2+ uptake probably did not affected fecal magnesium excretion. Moreover, up-regulation TRPM6, CNNM4, and claudins proteins might counteracts omeprazole-impeded duodenal Mg2+ absorption. However, Bai et al. [2] demonstrated only 1% reduction of intestinal Mg2+ absorption for 1 year can deplete Mg2+ from its storage up to 80%, which can induce hypomagnesemia.
For another question “why the expression of TRPM6 and those cation selective Cldns could not counteract PPIH in our rat model” has been raised. We propose the possible explanation as follow, which is also presented in Discussion of our manuscript. TRPM6 function requires an interaction with membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP2), whose hydrolysis through activation of Gq-protein coupled recptor-phospholipase C (PLC) dependent pathway fully inactivates TRPM6 channels [3]. Since omeprazole enhanced Gq-protein coupled receptor-PLC activation in intestinal epithelium [4], it might induced PIP2 degradation and then inactivated TRPM6 channels in duodenum of PPIH rats. In addition, luminal proton stimulates TRPM6 activity. Decrement of extracellular pH from 7.4 to 4.0 triggered 3.8-fold increase in TRPM6-driven Mg2+ influx [5]. Since the pH of half stimulation of TRPM6 was 4.3 [5], an increased in duodenal pH to 7.50 indicated a lower TRPM6 stimulation in our PPIH rat model. Moreover, TRPM6 mutation suppressed small intestinal Mg2+ absorption and caused severe hypomagnesemia [6]. Although mutation of TRPM6 has not been reported in PPIH, this might be involved in development of hypomagnesemia in our rat model.
It is widely accepted that Cldn modulates paracellular ion permeability [7]. Tight junctions (TJ) are a series of anastomosing membrane strands that occluded the intercellular space between epithelium cells [7, 8]. Dynamic reorganization of TJ strands, i.e., breaking, resealing, and branching, enables paracellular transport without interfering the barrier integrity [8]. Previous in vitro study revealed that over-expression

of Cldn-8 or Cldn-15 markedly increased number of TJ strands and decreased paracellular permeability [9, 10]. Therefore, simultaneously over-expressions of Cldn-2, -7, -12, and -15 in PPIH rats probably led to large increase in number of tight junction strands in duodenal epithelium which might impeded tight junction dynamic and paracellular Mg2+ transport. In addition, elevation of extracellular pH was found to increase the sensitivity of Ca2+ sensing receptor (CaSR) [11]. The activation of epithelium-associated CaSR induced Cldn-16 trans-localization from TJ to cytosol, which then suppressed paracellular passive Mg2+ transport [12]. Although Cldn-16 was not detected in duodenum, hypersensitivity of duodenal-related CaSR might be involved in the inhibition of Cldn-dependent paracellular Mg2+ absorption in PPIH rats.

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Comment 3.The authors stated that TRPM6 (3A) and CNNM4 (3B) were abundantly expressed in ileum and colon (positive control), respectively (line 27). In fact, Fig. 3A shows no staining of TRPM6 in duodenal sections from control and omeprazole-treated animals. Yet, proper negative and positive controls are missing (how we can exclude that TRPM6 antibody stained the ileum unspecifically?). A similar problem applies to CNNM4 staining. Since the result shown on Fig. 3 have been used to justify in vitro measurements of magnesium transport in the duodenum, I suggest a thorough re-examination of TRPM6 and CNNM4 expression patterns.

Respond: We re-examined the expression of TRPM6 in duodenal tissues. TRPM6 (Fig. 3A) was expressed in the apical membrane of duodenal villous cells. The positive signals of TRPM6 expression was increased in omeprazole-treated duodenum. Because we have not enough duodenal tissues to re-study the expression of CNNM4 by immunohistochemistry analysis, however, we instead demonstrated the effect of omeprazole on duodenal CNNM4 expression by Western blot analysis (Fig. 3B).

Comment 4. Measurement using Ussing chamber were performed in a medium containing unphysiologically high 40 mM Mg2+. Are there changes at physiological levels of Mg (1-2 mM Mg2+)?

Answer: Previous in vivo study used stable 25Mg to determine proximal intestinal Mg2+ uptake. At low luminal Mg2+ concentration of 1.6 mM, concentration of 25Mg present in plasma was unchanged throughout the experimental period (Fig. 4A of Lameris et al., Am J Physiol Gastrointest Liver Physiol 2015;308(3):G206–G216). This

results indicates a very low duodenal Mg2+ absorption when luminal Mg2+ concentration was 1-2 mM. Therefore, by using colorimetric-based Xylidyl Blue method, our technique was not sensitive and suitable for detecting a very small duodenal Mg2+ absorption at low apical Mg2+ concentration of 1-2 mM.

Comment 5. Ruthenium red is a highly unspecific channel blocker and it is too speculative to claim that this compound blocks magnesium transport due to inactivation of TRPM6. Please use either a specific TRPM6 inhibitor or change interpretation of the results accordingly. See also Point 1.
Answer: We re-performed duodenal Mg2+ transport by pre-incubated mucosal site of duodenal tissue with Mg2+ channel blocker Co(III)hexaammine and noncompetitive pan specific TRP channel inhibitor ruthenium red. We also change our interpretation to determine Mg2+ transport to total, Mg2+ channel-independent, and Mg2+ channel-driven Mg2+ transport, as mentioned above (answer of comment 1).

Comment 6. Discussion section contains a lengthy description of published works without attempt to integrate the original data into a common model. For instance, I am confused how to link together (a) up-regulation of magnesium transporters to (b) unchanged fecal magnesium excretion rate in vivo and (c) substantially decreased para- and transcellular magnesium transport in in vitro.
Answer: We revised Discussion part of our manuscript, as mentioned above. The interpretation of (a) up-regulation of magnesium transporters to (b) unchanged fecal magnesium excretion rate in vivo and (c) substantially decreased para- and transcellular magnesium transport in in vitro are explained as mentioned above (answer of comment 2).

Reviewer #2: "Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Spraque-Dawley rats" is a rat study describing the effects of PPI injection on intestinal Mg2+ absorption. The authors convincingly show that chronic PPI use induces hypomagnesemia and disturbed duodenal Mg2+ absorption in rats. Their elegant study combines in vivo and ex vivo experiments to provide evidence at the expression, functional and whole-animal physiology levels. However, there are some minor points that need to be addressed.
Comment 1. The authors use 20mg/kg of omeprazole, which is comparable to previous studies. However, for the main part of the study the authors use subcutaneous injections. Given that the bioavailability of subcutaneous injections will be higher than the oral ingestion, 20mg/kg is quite a high dose. The length of the study (24 weeks) is also much longer than previous studies. Therefore, the dose may not be in the physiological range. Please comment.
Answer: Although there was no pharmacokinetic study, pharmacokinetic parameters of subcutaneous omeprazole administration should comparable to those of intravenous administration. Omeprazole is rapidly and completely metabolized in rats [1]. The plasma half-life of omeprazole is 16.6 and 24.3 min after intravenous (20 mg/kg) and oral administration, respectively [1]. Moreover, plasma concentration of omeprazole has decreased to 0.5 μg/ml after 45 and 180 min after intravenous and oral administration, respectively [1]. Therefore, plasma omeprazole concentration after intravenous administration is comparable to physiological concentration of omeprazole after orally intake [1]. Despite a relatively short half-life, the pharmacodynamic effect can be measured for 24-72 hrs after initial administration [2]. These characteristics make the omeprazole quite safe for repeated (chronic) administration and effective for prolonged proton pump inhibition [2]. Segawa et al. [3] study the effect of 5, 10, and 20 mg/kg omeprazole administration through intraperitoneally, perorally and intravenously routes on gastric secretion after 2 or 24 hr of administration. After 2 hr of administration, 20 mg/kg of omeprazole was the most potent in each administration group. Moreover, there was no significant difference among the groups given 20 mg/kg of omeprazole intraperitoneally, intravenously and perorally [3]. Therefore, 20 mg/kg intravenously omeprazole administration is a suitable dose to suppress gastric acid secretion in rats [1, 3]. Based on our study, 20 mg/kg subcutaneous omeprazole injection effectively suppressed gastric acid secretion. Moreover, general

characteristics and body weight gain throughout 24 wk of experiment of subcutaneous omeprazole injected group were similar to that of control group. Therefore, prolonged subcutaneous omeprazole injection is safe for our rat model.

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Comment 2) From a conceptual point of view, it is difficult to understand why the authors exclude the colon from their analyses and focus on the duodenum. Previous studies have shown that the colon is more important for Mg2+ absorption (e.g. Lameris et al. Am J Physiol Gastrointest Liver Physiol 308: G206-G216, 2015.). Although there may be some expression of TRPM6/CNNM4 in earlier parts of the intestinal tract, the highest expression is in colon. Are data on colonic Mg2+ absorption / expression levels available?

Answer: Different intestinal segments contribute unequally to Mg2+ absorption. As percentages of the total input, duodenum absorbs 11%, jejunum 22%, ileum 56%, and large intestine 11% [1]. Thus duodenum and colon absorb dietary Mg2+ in the same fraction. Larmeris et al. [2] demonstrated bulk of intestinal Mg2+ absorption occurs in small intestine. The colonic Mg2+ absorption was higher than small intestine Mg2+ absorption by about 2% when luminal Mg2+ concentration was 1.6 mM and 3-4% when luminal Mg2+ concentration was 10 mM (Fig. 4A and 4B at 30 min of Larmeris et al. [2]). Moreover, recent in vivo studies proposed that PPIs mainly affected colonic Mg2+ handling by inducing magnesiotropic genes expressions of mice colon [3, 4]. However, the effect of PPIs on Mg2+ homeostasis was controversial. Hess and colleagues [3] demonstrated that 20 mg/kg omeprazole treatment for 14 days suppressed serum Mg2+ level with normal urinary and fecal Mg2+ excretion in C57BL/J6 mice. On the other hand, Lameris et al. [4] reported that dietary Mg2+ restriction, but not 20 mg/kg omeprazole administration for 28 days, suppressed serum Mg2+ level in C57BL/J6 mice. Interestingly dietary inulin, which was stimulating colonic Mg2+ absorption [5], could not normalized plasma Mg2+ level in PPIH mice [3]. Therefore, large intestine may not be a suitable intestinal segment that should be modulated to counteract PPIH. On the other hand, previous in vitro studies proposed that PPIs impeded Mg2+ absorption in small intestine [6-8]. Luminal proton stimulated transient receptor potential melastatin 6 (TRPM6)-driven transcellular active and claudin (Cldn)-mediated paracellular passive Mg2+ absorptions [5, 7]. Mertz-Nielsen et al. [9] reported that omeprazole significantly enhanced duodenal HCO_3^- secretion in healthy subjects. Since omeprazole suppressed pancreatic secretion [10], thus, omeprazole specifically induced duodenal HCO_3^- secretion. Previous study reported that omeprazole markedly enhanced apical HCO_3^- secretion, decreased apical proton, and subsequently suppressed intestinal Mg2+ absorption [7, 8]. Therefore, in the present study we observe the effect of PPIs on duodenal Mg2+ absorption. In addition, we also performed additional experiment to elucidate the involvement of mucosal duodenal HCO_3^- secretion on omeprazole-affected duodenal Mg2+ transport by pre-incubated duodenal tissues with Cl^-/HCO_3^- exchanger inhibitor DIDS and CFTR inhibitor GlyH-101. Our study revealed the potential role of duodenal Mg2+ handling, which was usually ignored, on the regulation of Mg2+ homeostasis and pathophysiology of PPIH. Duodenal HCO_3^- secretion might be one of the critical factors of PPIs-impeded intestinal Mg2+ absorption.

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25th of April 2016
Dear Prof. Dr. Thomas Gudermann,

Please consider our revised manuscript, “Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Sprague-Dawley rats”, for publication in Pflügers Archiv - European Journal of Physiology.

We appreciate the interest that the reviewers have taken in our manuscript and the constructive criticisms they have given. We have addressed all of suggestions and comments of the reviewers. In accordance with the comments and suggestions, we have comprehensively revised the text throughout our manuscript. We re-examined the expression of TRPM6 and CMM4 in duodenal tissues. We re-performed duodenal Mg^{2+} transport by pre-incubating duodenal tissue with Mg^{2+} channel blocker Co(III)hexaammine and noncompetitive pan specific TRP channel inhibitor ruthenium red. We also performed additional experiment to elucidate the contribution of mucosal duodenal HCO_3^- secretion on omeprazole-affected duodenal Mg^{2+} transport by pre-incubated duodenal tissues with Cl^-/HCO_3^- exchanger inhibitor DIDS and CFTR inhibitor GlyH-101. We believe these changes have clearly improved our manuscript. We have also included point-by-point responses to the reviewers in addition to making the changes in the manuscript.

In addition, we have found typographical errors in name of author number 4, rate of Mg^{2+} flux, and Grant No. form Burapha University. We have been corrected these typographical errors in our revised manuscript. In addition, these typographical errors did not affect the results, data interpretation, discussion, or conclusion.

In the present study, we propose the potential role of duodenal Mg^{2+} handling, which has up to now been ignored, on the regulation of Mg^{2+} homeostasis and pathophysiology of proton pump inhibitors-induced hypomagnesemia (PPIH). Duodenal HCO_3^- secretion might be one of the critical factors of PPIs-impeded intestinal Mg^{2+} absorption. Reduction of duodenal Mg^{2+} absorption was shown in omeprazole-treated rats, whether hypomagnesemia was presented or not. Hypomagnesemia occurred only if Mg^{2+} storage pool was depleted in prolong omeprazole treated rats. These findings are of potential importance for the prevention of PPIH, and should be of interest to the readers of the Pflügers Archiv - European Journal of Physiology.

Thank you again for consideration of our revised manuscript.

Sincerely Yours,

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Correction

1. Name of author No.4 had changed from **Kanyarat Khongmeang** to **Kanyanat Khongmueang**.
2. The rate Mg^{2+} transport had changed from **µmol/hr/cm^2** to **nmol/hr/cm^2**.
3. Grant No. form Burapha University in Acknowledgements had changed from **AHS 2-1/2556** to **AHS08/2558**.

Responses to the reviewers

Reviewer#1:

Comment 1. The authors stated that 'The present study aimed to elucidate the mechanism of prolonged PPIs administration on the induction of hypomagnesemia' (Abstract, line 19). To my view, Thongon et al. did not provide any specific mechanistic explanation for this phenomenon. The observation that chronic administrations of proton pump inhibitors results in a defect of intestinal magnesium intake in humans and rodent models is not novel and well described in the literature. The authors reported many phenotypic alterations indicating that omeprazole-treated animals developed systemic magnesium deficiency. These findings are not surprising and, more unimportant, have unclear relevance for the mechanistic understanding as how exactly omeprazole could suppress magnesium uptake in the intestine. Accordingly, I suggest a substantial revision of the text of the manuscript in order to exclude statements and speculations, which are not supported by the experimental data provided. The authors should outline clearly what are new findings in their work and how exactly these results advance the field.

Respond: We thank the reviewer for these critical comments and suggestions. In compliance with the comments and suggestions, we have comprehensively revised our manuscript. We also re-examined and performed additional experiments to improve our data as followed;

1. (Abstract, Page 2)
   - We replaced with new sentences in line 1-3
   - We added a new sentence in line 9
   - We replaced with new sentences in line 10-12
   - We added new sentences in line 12-17
   - We added a new sentences in line 20-21
2. (Introduction, Page 3 - 5)
   - (paragraph 1, line 7-11) We replaced with new sentences.
   - (paragraph 2) we added or preplaced with new sentences in line 6-7, 9-14, and 15-17)
   - (paragraph 3) we added this new paragraph
   - (paragraph 4) we replaced with new sentences in line 5-7, and add a new sentence in line 8-9
3. (Materials and Methods)

- (Magnesium flux measurement, Page 8, paragraph 1) We re-performed duodenal Mg^{2+} transport by pre-incubated duodenal tissue with Mg^{2+} channel blocker Co(III)hexaammine and noncompetitive pan specific TRP channel inhibitor ruthenium red. We added or preplaced with new sentences in line 1-2, 7-15, and 19-20.
- (Magnesium flux measurement, Page 8, paragraph 2) We also performed additional experiment to elucidate the involvement of mucosal duodenal HCO_3^- secretion on omeprazole-affected duodenal Mg^{2+} transport by pre-incubated mucosal site of duodenal tissues with Cl^-/HCO_3^- exchanger inhibitor DIDS and CFTR inhibitor GlyH-101.

4. (Results)

- (Omeprazole suppressed duodenal Mg^{2+} absorption, Page 12-13, paragraph 1) We re-examined the expression of TRPM6 duodenal tissues. The results are illustrated in Fig. 3A. Because we have not enough duodenal tissues to re-study the expression of CNNM4 by immunohistochemistry analysis, however, we instead demonstrated the effect of omeprazole on duodenal CNNM4 expression by Western blot analysis (Fig. 3B). We replaced the sentences in line 9-11 of paragraph 1.
- (Omeprazole suppressed duodenal Mg^{2+} absorption, Page 13, paragraph 2) We re-performed duodenal Mg^{2+} transport, as mention above. The results illustrated in Fig. 4. We also replaced the sentences in line 6-18 of paragraph 2.
- (Contribution of duodenal HCO_3^- secretion on omeprazole-suppressed Mg^{2+} transport, Page 14-15) We performed additional experiment to elucidate the involvement of mucosal duodenal HCO_3^- secretion on omeprazole-affected duodenal Mg^{2+} transport, as mention above. The results illustrated in Fig. 6. We also add this new text in page 14-15.

5. (Discussion)

- (paragraph 1, Page 16) we added a new sentence in line 11-12
- (paragraph 3, Page 16-17) we added a new sentence in line 1-17. We also added a new sentence in line 23-24.
- (paragraph 5, Page 18) we added this new paragraph to propose the possible explanations of why the over-expression of TRPM6 cannot counteract PPIH.
- (paragraph 6, Page 18-19) we added this new paragraph to propose the possible explanations of why the over-expression of claudins cannot counteract PPIH.
- (paragraph 8, Page 19) we added this new paragraph as conclusion.

Comment 2. Fig. 2C clearly shows that fecal magnesium excretion rate was normal in the omeprazole-treated rats. These results challenge the idea that omeprazole suppressed the intestinal uptake of magnesium in vivo. Furthermore, these observations are contradictory to the findings that many proteins

involved in intestinal magnesium transport were up-regulated. The authors should provide a stronger experimental evidence for the suggested effect of omeprazole on intestinal magnesium intake in vivo.

Answer: Previous in vivo study of Hess and colleagues demonstrated that 20 mg/kg omeprazole treatment for 14 days induced hypomagnesemia with normal fecal Mg^{2+} excretion in C57BL/J6 mice [1]. Whether stimulation of colonic Mg^{2+} absorption or not, the over-expression of colonic TRPM6 could not counteract hypomagnesemia [1]. Our results agreed with Hess et al. [1] that PPIH rats had normal fecal Mg^{2+} excretion. The possible explanation was a very low fraction of duodenal Mg^{2+} uptake from ingested Mg^{2+}. The average food intake of control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats were 23.99, 25.95, and 23.76 g/day, respectively. Since, pellet chow contained 0.23% wt/wt magnesium, thus, control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats ingested Mg about 5.52, 5.97, and 5.47 g/day, respectively. Since 1 gram Mg equal to 8.12 mEq Mg and 4.06 mmol Mg (http://www.mgwater.com/convert.shtml). The rats in 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated group ingested about 22.41, 24.24, and 22.21 mmol Mg/day, respectively. Duodenal Mg^{2+} uptake was 94.95, 29.397, and 16.33 nmol in control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats. Therefore, a very low fraction of duodenal Mg^{2+} uptake probably did not affected fecal magnesium excretion. Moreover, up-regulation TRPM6, CNNM4, and claudins proteins might counteracts omeprazole-impeded duodenal Mg^{2+} absorption. However, Bai et al. [2] demonstrated only 1% reduction of intestinal Mg^{2+} absorption for 1 year can deplete Mg^{2+} from its storage up to 80%, which can induce hypomagnesemia.

For another question “why the expression of TRPM6 and those cation selective Cldns could not counteract PPIH in our rat model” has been raised. We propose the possible explanation as follow, which is also presented in Discussion of our manuscript. TRPM6 function requires an interaction with membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP2), whose hydrolysis through activation of Gq-protein coupled recptor-phospholipase C (PLC) dependent pathway fully inactivates TRPM6 channels [3]. Since omeprazole enhanced Gq-protein coupled receptor-PLC activation in intestinal epithelium [4], it might induced PIP2 degradation and then inactivated TRPM6 channels in duodenum of PPIH rats. In addition, luminal proton stimulates TRPM6 activity. Decrement of extracellular pH from 7.4 to 4.0 triggered 3.8-fold increase in TRPM6-driven Mg^{2+} influx [5]. Since the pH of half stimulation of TRPM6 was 4.3 [5], an increased in duodenal pH to 7.50 indicated a lower TRPM6 stimulation in our PPIH rat model. Moreover, TRPM6 mutation suppressed small intestinal Mg^{2+} absorption and caused severe hypomagnesemia [6]. Although mutation of TRPM6 has not been reported in PPIH, this might be involved in development of hypomagnesemia in our rat model.

It is widely accepted that Cldn modulates paracellular ion permeability [7]. Tight junctions (TJ) are a series of anastomosing membrane strands that occluded the intercellular space between epithelium cells [7, 8]. Dynamic reorganization of TJ strands, i.e., breaking, resealing, and branching, enables paracellular transport without interfering the barrier integrity [8]. Previous in vitro study revealed that over-expression of Cldn-8 or Cldn-15 markedly increased number of TJ strands and decreased paracellular permeability [9, 10]. Therefore, simultaneously over-expressions of Cldn-2, -7, -12, and -15 in PPIH rats probably led to large increase in number of tight junction strands in duodenal epithelium which might impeded tight junction dynamic and paracellular Mg^{2+} transport. In addition, elevation of extracellular pH was found to increase the sensitivity of Ca^{2+} sensing receptor (CaSR) [11]. The activation of epithelium-associated CaSR induced

Cldn-16 trans-localization from TJ to cytosol, which then suppressed paracellular passive Mg^{2+} transport [12]. Although Cldn-16 was not detected in duodenum, hypersensitivity of duodenal-related CaSR might be involved in the inhibition of Cldn-dependent paracellular Mg^{2+} absorption in PPIH rats.

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Comment 3. The authors stated that TRPM6 (3A) and CNNM4 (3B) were abundantly expressed in ileum and colon (positive control), respectively (line 27). In fact, Fig. 3A shows no staining of TRPM6 in duodenal sections from control and omeprazole-treated animals. Yet, proper negative and positive controls are missing (how we can exclude that TRPM6 antibody stained the ileum unspecifically?). A similar problem applies to CNNM4 staining. Since the result shown on Fig. 3 have been used to justify in vitro measurements of magnesium transport in the duodenum, I suggest a thorough re-examination of TRPM6 and CNNM4 expression patterns.

Respond: We re-examined the expression of TRPM6 in duodenal tissues. TRPM6 (Fig. 3A) was expressed in the apical membrane of duodenal villous cells. The positive signals of TRPM6 expression was increased in omeprazole-treated duodenum. Because we have not enough duodenal tissues to re-study the expression of CNNM4 by immunohistochemistry analysis, however, we instead demonstrated the effect of omeprazole on duodenal CNNM4 expression by Western blot analysis (Fig. 3B).

Comment 4. Measurement using Ussing chamber were performed in a medium containing unphysiologically high 40 mM Mg^{2+}. Are there changes at physiological levels of Mg (1-2 mM Mg^{2+})?

Answer: Previous in vivo study used stable ^{25}Mg to determine proximal intestinal Mg^{2+} uptake. At low luminal Mg^{2+} concentration of 1.6 mM, concentration of ^{25}Mg present in plasma was unchanged throughout the experimental period (Fig. 4A of Lameris et al., Am J Physiol Gastrointest Liver Physiol 2015;308(3):G206–G216). This results indicates a very low duodenal Mg^{2+} absorption when luminal Mg^{2+} concentration was 1-2 mM. Therefore, by using colorimetric-based Xylidyl Blue method, our technique was not sensitive and suitable for detecting a very small duodenal Mg^{2+} absorption at low apical Mg^{2+} concentration of 1-2 mM.

Comment 5. Ruthenium red is a highly unspecific channel blocker and it is too speculative to claim that this compound blocks magnesium transport due to inactivation of TRPM6. Please use either a specific TRPM6 inhibitor or change interpretation of the results accordingly. See also Point 1.

Answer: We re-performed duodenal Mg^{2+} transport by pre-incubated mucosal site of duodenal tissue with Mg^{2+} channel blocker Co(III)hexaammine and noncompetitive pan specific TRP channel inhibitor ruthenium red. We also change our interpretation to determine Mg^{2+} transport to total, Mg^{2+} channel-independent, and Mg^{2+} channel-driven Mg^{2+} transport, as mentioned above (answer of comment 1).

Comment 6. Discussion section contains a lengthy description of published works without attempt to integrate the original data into a common model. For instance, I am confused how to link together (a) up-regulation of magnesium transporters to (b) unchanged fecal magnesium excretion rate in vivo and (c) substantially decreased para- and transcellular magnesium transport in in vitro.

Answer: We revised Discussion part of our manuscript, as mentioned above. The interpretation of (a) up-regulation of magnesium transporters to (b) unchanged fecal magnesium excretion rate in vivo and (c) substantially decreased para- and transcellular magnesium transport in in vitro are explained as mentioned above (answer of comment 2).

Reviewer #2: "Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Spraque-Dawley rats" is a rat study describing the effects of PPI injection on intestinal Mg^{2+} absorption. The authors convincingly show that chronic PPI use induces hypomagnesemia and disturbed duodenal Mg^{2+} absorption in rats. Their elegant study combines in vivo and ex vivo experiments to provide evidence at the expression, functional and whole-animal physiology levels. However, there are some minor points that need to be addressed.

Comment 1. The authors use 20mg/kg of omeprazole, which is comparable to previous studies. However, for the main part of the study the authors use subcutaneous injections. Given that the bioavailability of subcutaneous injections will be higher than the oral ingestion, 20mg/kg is quite a high dose. The length of the study (24 weeks) is also much longer than previous studies. Therefore, the dose may not be in the physiological range. Please comment.

Answer: Although there was no pharmacokinetic study, pharmacokinetic parameters of subcutaneous omeprazole administration should comparable to those of intravenous administration. Omeprazole is rapidly and completely metabolized in rats [1]. The plasma half-life of omeprazole is 16.6 and 24.3 min after intravenous (20 mg/kg) and oral administration, respectively [1]. Moreover, plasma concentration of omeprazole has decreased to 0.5 μg/ml after 45 and 180 min after intravenous and oral administration, respectively [1]. Therefore, plasma omeprazole concentration after intravenous administration is comparable to physiological concentration of omeprazole after orally intake [1]. Despite a relatively short half-life, the pharmacodynamic effect can be measured for 24-72 hrs after initial administration [2]. These characteristics make the omeprazole quite safe for repeated (chronic) administration and effective for prolonged proton pump inhibition [2]. Segawa et al. [3] study the effect of 5, 10, and 20 mg/kg omeprazole administration through intraperitoneally, perorally and intravenously routes on gastric secretion after 2 or 24 hr of administration. After 2 hr of administration, 20 mg/kg of omeprazole was the most potent in each administration group. Moreover, there was no significant difference among the groups given 20 mg/kg of omeprazole intraperitoneally, intravenously and perorally [3]. Therefore, 20 mg/kg intravenously omeprazole administration is a suitable dose to suppress gastric acid secretion in rats [1, 3]. Based on our study, 20 mg/kg subcutaneous omeprazole injection effectively suppressed gastric acid secretion. Moreover, general characteristics and body weight gain throughout 24 wk of experiment of subcutaneous omeprazole injected group were similar to that of control group. Therefore, prolonged subcutaneous omeprazole injection is safe for our rat model.

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Comment 2) From a conceptual point of view, it is difficult to understand why the authors exclude the colon from their analyses and focus on the duodenum. Previous studies have shown that the colon is more important for Mg^{2+} absorption (e.g. Lameris et al. Am J Physiol Gastrointest Liver Physiol 308: G206-G216, 2015.). Although there may be some expression of TRPM6/CNNM4 in earlier parts of the intestinal tract, the highest expression is in colon. Are data on colonic Mg^{2+} absorption / expression levels available?

Answer: Different intestinal segments contribute unequally to Mg^{2+} absorption. As percentages of the total input, duodenum absorbs 11%, jejunum 22%, ileum 56%, and large intestine 11% [1]. Thus duodenum and colon absorb dietary Mg^{2+} in the same fraction. Larmeris et al. [2] demonstrated bulk of intestinal Mg^{2+} absorption occurs in small intestine. The colonic Mg^{2+} absorption was higher than small intestine Mg^{2+} absorption by about 2% when luminal Mg^{2+} concentration was 1.6 mM and 3-4% when luminal Mg^{2+} concentration was 10 mM (Fig. 4A and 4B at 30 min of Larmeris et al. [2]). Moreover, recent in vivo studies proposed that PPIs mainly affected colonic Mg^{2+} handling by inducing magnesiotropic genes expressions of mice colon [3, 4]. However, the effect of PPIs on Mg^{2+} homeostasis was controversial. Hess and colleagues [3] demonstrated that 20 mg/kg omeprazole treatment for 14 days suppressed serum Mg^{2+} level with normal urinary and fecal Mg^{2+} excretion in C57BL/J6 mice. On the other hand, Lameris et al. [4] reported that dietary Mg^{2+} restriction, but not 20 mg/kg omeprazole administration for 28 days, suppressed serum Mg^{2+} level in C57BL/J6 mice. Interestingly dietary inulin, which was stimulating colonic Mg^{2+} absorption [5], could not normalized plasma Mg^{2+} level in PPIH mice [3]. Therefore, large intestine may not be a suitable intestinal segment that should be modulated to counteract PPIH. On the other hand, previous in vitro studies proposed that PPIs impeded Mg^{2+} absorption in small intestine [6-8]. Luminal proton stimulated transient receptor potential melastatin 6 (TRPM6)-driven transcellular active and claudin (Cldn)-mediated paracellular passive Mg^{2+} absorptions [5, 7]. Mertz-Nielsen et al. [9] reported that omeprazole significantly enhanced duodenal HCO_3^- secretion in healthy subjects. Since omeprazole suppressed pancreatic secretion [10], thus, omeprazole specifically induced duodenal HCO_3^- secretion. Previous study reported that omeprazole markedly enhanced apical HCO_3^- secretion, decreased apical proton, and subsequently suppressed intestinal Mg^{2+} absorption [7, 8]. Therefore, in the present study we observe the effect of PPIs on duodenal Mg^{2+} absorption. In addition, we also performed additional experiment to elucidate the involvement of mucosal duodenal HCO_3^- secretion on omeprazole-affected duodenal Mg^{2+} transport by pre-incubated duodenal tissues with Cl^-/HCO_3^- exchanger inhibitor DIDS and CFTR inhibitor GlyH-101. Our study revealed the potential role of duodenal Mg^{2+} handling, which was usually ignored, on the regulation of Mg^{2+} homeostasis and pathophysiology of PPIH. Duodenal HCO_3^- secretion might be one of the critical factors of PPIs-impeded intestinal Mg^{2+} absorption.

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Comment 3. How do the authors explain the low expression of TRPM6 and CNNM4 in their control rats (fig 3)?

Respond: We re-examined the expression of TRPM6 in duodenal tissues. TRPM6 (Fig. 3A) was expressed in the apical membrane of duodenal villous cells. The positive signals of TRPM6 expression was increased in omeprazole-treated duodenum. Because we have not enough duodenal tissues to re-study the expression of CNNM4 by immunohistochemistry analysis, we instead demonstrated the effect of omeprazole on duodenal CNNM4 expression by western blot analysis (Fig. 3B).

Comment 4. The blots in figure 6 are over-exposed and cannot be used for quantification. Especially actin should be repeated, to make sure that there are no differences in basal protein levels.

Answer: We thank the reviewer for this valuable comment. However, in Western blot analysis, every membrane was re-probed with anti-β-actin monoclonal antibodies to confirmed basal protein level and that equal amount of protein loaded. The relative expression of each claudin was expressed as %control using its corresponding is being control group.

Respond: We added a sentence “Membranes were also reprobed with 1:5,000 anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology).” in Western blot analysis, Line 11-12, Page 9.

Comment 5. Please correct spelling of Sprague Dawley rats throughout the manuscript.

Respond: We added a sentence “Membranes were also reprobed with 1:5,000 anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology).” in Western blot analysis, Line 11-12, Page 9.

Omeprazole suppressed plasma magnesium level and duodenal magnesium absorption in male Sprague-Dawley rats

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Author contributions: Thongon N designed and performed experiments, analyzed and interpreted the results, wrote and edited the manuscript. Penguy J, Kulwong S, Khongmueang K, and Thongma M performed experiments.

Conflict of interest statement: The authors declare no conflicts of interest.

Abstract

Hypomagnesemia is the most concerned side effect of proton pump inhibitors (PPIs) in chronic users. However, the mechanism of PPIs-induced systemic Mg^{2+} deficit is currently unclear. The present study aimed to elucidate the direct effect of short-term and long-term PPIs administrations on whole body Mg^{2+} homeostasis and duodenal Mg^{2+} absorption in rats. Mg^{2+} homeostasis was studied by determining the serum Mg^{2+} level, urine and fecal Mg^{2+} excretions, and bone and muscle Mg^{2+} contents. Duodenal Mg^{2+} absorption as well as paracellular charge selectivity were studied. Our result showed that gastric and duodenal pH markedly increased in omeprazole-treated rats. Omeprazole significantly suppressed plasma Mg^{2+} level, urinary Mg^{2+} excretion, bone and muscle Mg^{2+} content. Thus, omeprazole induced systemic Mg^{2+} deficiency. By using Ussing chamber techniques, it was shown that omeprazole markedly suppressed duodenal Mg^{2+} channel-driven and Mg^{2+} channel-independent Mg^{2+} absorptions and cation selectivity. Inhibitors of mucosal HCO_3^- secretion significantly increased duodenal Mg^{2+} absorption in omeprazole-treated rats. Transient receptor potential melastatin 6 (TRPM6)-driven transcellular and claudin (Cldn)-mediated paracellular Mg^{2+} absorptions are regulated by luminal proton. We therefore hypothesized that secreted HCO_3^- in duodenum decreased luminal proton, this impeded duodenal Mg^{2+} absorption. Higher plasma total 25-OH vitamin D, diuresis, and urine PO_4^{3-} were also demonstrated in hypomagnesemic rats. As a compensatory mechanism for systemic Mg^{2+} deficiency, the expressions of duodenal TRPM6, cyclin M4 (CNNM4), Cldn-2, Cldn-7, Cldn-12, and Cldn-15 proteins were enhanced in omeprazole-treated rats. Our findings support the potential role of duodenum on the regulation of Mg^{2+} homeostasis and explain the pathophysiology of hypomagnesemia.

Keywords: hypomagnesemia, intestinal Mg^{2+}absorption, Mg^{2+} homeostasis, proton pump inhibitors, Ussing chamber

Introduction

Magnesium (Mg^{2+}) is an essential co-factor or activator of at least 800 enzymes which are involved in numerous cellular functions, i.e., energy metabolism, cell cycle, and membrane transport. Mg^{2+} deficiency has been implicated in several diseases, e.g., Pakinson's disease, asthma, hypertension, and osteoperosis [9, 41]. Therefore, plasma Mg^{2+} level is tightly regulated within a narrow range by collaborative actions of the intestinal absorption, renal excretion, bone and muscle storage. Parathyroid hormone (PTH) and vitamin D had been reported to regulate plasma Mg^{2+} level [25, 46]. The bulk of intestinal Mg^{2+} absorption, approximately 90%, occurs through paracellular passive mechanism [33], whereas transcellular active Mg^{2+} absorption plays an important role during low dietary Mg^{2+} intake [33.]. It has been previously proposed that small intestine absorbs Mg^{2+} exclusively through paracellular route, but transcellular Mg^{2+} uptake exists exclusively in colon [9, 24]. While renal tubular Mg^{2+} handling is well documented [9, 46], cellular mechanism and regulatory factor of intestinal Mg^{2+}absorption are largely unknown.

Acid peptic disorders are the result from either excessive gastric acid secretion or diminished mucosal defense that affects millions people worldwide [29]. The most effective therapeutic agents for these disorders is proton pump inhibitors (PPIs), which are the fifth best-selling drug that has been taken by millions of chronic users worldwide [29, 32]. However, since 2006, there is a growing body of evidence indicating that PPIs-induced hypomagnesemia (PPIH) is a serious side effect of PPIs in chronic users [6, 7, 11, 27, 40]. The mechanism of PPIs induced systemic Mg^{2+} deficit is currently unclear. Previous reports suggested that PPIH might be due to chronic suppression of intestinal Mg^{2+} absorption and severe depletion of body Mg^{2+} storage pool [6, 7, 11, 40]. While oral Mg^{2+} failed to normalized plasma Mg^{2+} level, intravenous Mg^{2+} supplement rapidly cured hypomagnesemia [6, 11, 40]. In addition, hypomagnesemia was rapidly resolved when PPIs was discontinued, and then recurred again within 1–2 wk if PPIs was re-prescribed [6, 11]. These data suggested PPIs rapidly suppressed intestinal Mg^{2+}

absorption. However, short-term omeprazole administration did not affect intestinal Mg^{2+} absorption in human [37]. There was no evidence of urinary Mg^{2+} wasting in those PPIH patients [6, 7, 11, 27, 40]. On the other hand, a large-scale clinical investigation reported that PPIH was restricted to patients taking diuretics, i.e., loop and thiazide diuretics [8]. Since these diuretics could suppress Mg^{2+} reabsorption [3], renal Mg^{2+} wasting probably involved in the development of PPIH.

Recent in vivo studies proposed that PPIs mainly affected colonic Mg^{2+} handling by inducing magnesiotropic genes expressions in mice colon [17, 24]. However, the effect of PPIs on Mg^{2+} homeostasis was still controversial. Hess and colleagues [17] demonstrated that 20 mg/kg omeprazole treatment for 14 days suppressed serum Mg^{2+} level with normal urinary and fecal Mg^{2+} excretions in C57BL/J6 mice. On the other hand, Lameris et al. [24] reported that dietary Mg^{2+} restriction, but not 20 mg/kg omeprazole administration for 28 days, suppressed serum Mg^{2+} level in C57BL/J6 mice. Interestingly, dietary inulin, which stimulated colonic Mg^{2+} absorption [35], could not normalized plasma Mg^{2+} level in PPIH mice [17]. Therefore, large intestine may not be a suitable intestinal segment that should be modulated to counteract PPIH. On the other hand, previous in vitro studies proposed that PPIs impeded Mg^{2+} absorption in small intestine [43–45]. Luminal proton stimulated transient receptor potential melastatin 6 (TRPM6)-driven transcellular active and claudin (Cldn)-mediated paracellular passive Mg^{2+} absorptions [18, 20, 35, 44]. Mertz-Nielsen et al. [30] reported that omeprazole significantly enhanced duodenal HCO_3^- secretion in healthy subjects. Since omeprazole suppressed pancreatic secretion [48], thus it specifically induced duodenal HCO_3^- secretion. Previous study reported that omeprazole markedly enhanced apical HCO_3^- secretion, decreased apical proton, and subsequently suppressed intestinal Mg^{2+} absorption [44, 45]. However, the effect of PPIs on duodenal Mg^{2+} absorption remains unknown.

In the present study, we aimed to elucidate the direct effect of short-term (4 wk) and long-term (24 wk) omeprazole-treatments on whole-body Mg^{2+} homeostasis in male Sprague-

Dawley rats by determining serum Mg^{2+} level, urine and fecal Mg^{2+} excretions, and bone and muscle Mg^{2+} contents. Plasma Ca^{2+}, PO_4^{3-}, PTH, and total 25-OH vitamin D, as well as urine Ca^{2+} and PO_4^{3-} were also determined. Duodenal total, Mg^{2+} channel-driven transcellular, and Mg^{2+} channel-independent paracellular Mg^{2+} absorptions, as well as paracellular charge selectivity, were studied. The involvement of mucosal HCO_3^- secretion on omeprazole-impeded duodenal Mg^{2+} absorption was also examined. The expressions of duodenal TRPM6, cyclin M4 (CNNM4), Cldn-2, -7, -12, and -15 of omeprazole-treated rats were also elucidated. The ultrastructure of duodenum and head of femurs were observed.

Materials and methods

Animals

This study was performed in strict compliance with the Animal for Scientific Purposes Act of Thailand and in accordance with Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes, National Research Council of Thailand. All experimental procedures were approved by the Ethics Committee on Animal Experiment of Burapha University, Thailand. Male Sprague-Dawley rats (9 wk old, weighting 250-350 g) were purchased from the National Laboratory Animal Centre, Mahidol University, Thailand. The animals were randomly allocated into three experimental groups, i.e., control, 4 wk-omeprazole treatment, and 24 wk-omeprazole tratement. They were acclimatized for 7 days before starting of the experiments. They were housed in a temperature-, humidity-, and light-controlled room with standard pellet chow containing 0.23% wt/wt magnesium, 1.0% wt/wt calcium, 0.9% phosphorus, and 4,000 IU/kg vitamin D (CP, Bangkok, Thailand) and reverse osmosis water given ad libitum. The health, body weight, and food intake were monitored and recorded daily.

Experimental design

In the present study we decided to use subcutaneous omeprazole injection that safely and effectively inhibited gastric acid secretion in rat [21] and human [2]. The first series of experiment aimed to elucidate the efficacy of subcutaneous omeprazole (20 mg/kg: Ocid® IV; Zydus Cadila, India) and oral gavage omeprazole (20 mg/kg: Losec®; AstraZeneca, Thailand) administrations on gastric acid suppression. The pellet chow were removed 4 hr before and then retrieved 30 min after oral gavage or subcutaneous omeprazole administration. At 2 hr and 24 hr after administration, stomach and duodenum were removed under thiopental anesthesia (70 mg/kg; Anesthal, Jagsonpal Pharmaceuticals ltd, India). Stomach and duodenum pH were determined using diagnostic test strips (MColorpHast™ pH-Indicator Strips, Merck-Millipore, German).

The second series of experiment aimed to study the effect of short-term and long-term omeprazole-treatments on Mg^{2+} homeostasis in the rats. Control and 24 wk-omeprazole-treated rats were respectively received daily subcutaneous sham or subcutaneous omeprazole (20 mg/kg) injection for 24 wk. In the 4 wk-omeprazole-treatment group, rats received subcutaneous sham injection daily for 20 wk and subsequently followed by subcutaneous omeprazole injection for 4 wk. For urine and feces collections, rats were housed in metabolic cages for 24 hr. The health of all rats were checked daily throughout 24 wk of injection. At the experiment end point, the rats were anesthetized with thiopental, blood were collected from left ventricle, and the rats were subsequently sacrificed. Duodenum, left and right femurs, and left soleus muscle were collected.

Analytical procedures

Plasma and urine Mg^{2+}, Ca^{2+}, and PO_4^{3-} concentrations were respectively determined by xylidyl blue II, asenazo III, and phosphomolybdate method, and analyzed by an automate clinical chemistry analyzer (ILab Taurus; Instrumentation Laboratory, Bedford, MA, USA). Total serum 25-OH vitamin D level was determined by Tosoh™ Bioscience ST AIA-PACK 25-

OH vitamin D and an automate Tosoh AIA-900 analyzer (Tosoh Bioscience, Inc., South San Francisco, CA, USA). Plasma PTH level was determined by ARCHITECT Intact PTH and ARCHITECT i2000sr automatic immunoassay analyzer (Abbott Diagnostics, Abbott Park, IL, USA). Soleus muscles were chopped and digested with nitric acid (Sigma, St. Louis, MO, USA). Left femurs and feces were dried, ashed, and subsequently extracted with nitric acid (Sigma-Aldrich). Muscle, bone, and fecal Mg^{2+} content were determined by an atomic absorption spectrophotometer (Shimadzu, Tokyo, Japan).

Epithelial electrical parameter measurement and dilution potential experiment

Rat duodenum was cut longitudinally, rinsed gently, mounted in a Ussing chamber (World Precision Instrument, Sarasota, FL, USA), and bathed on both sides with normal bathing solution containing (in mmol/mL) 118 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 1.25 $CaCl_2$, 23 $NaHCO_3$, 12 D-glucose, 2.5 L-glutamine, and 2 D-mannitol. The solution was maintained at 37 °C, pH of 7.4, osmolality of 290-295 mmol/kg H_2O, and continuously gassed with 5% CO_2 in 95% O_2. Transepithelial potential difference (PD) and short-circuits current (*I*sc) were determined by Ag/AgCl electrodes and an epithelial voltage/current clamp apparatus (model ECV-4000; World Precision Instrument) as previously described [42]. Transepithelial resistance (TER) was calculated from PD and *I*sc by Ohm's law.

To determine paracellular charge selectivity by measuring absolute sodium permeability (P_{Na}) and chloride permeability P_{Cl} and relative P_{Na}/P_{Cl} [16], dilution potential experiment was performed by modified method of Thongon et al. [42]. In brief, duodenal tissue was equilibrated for 10 min within Ussing chamber in a normal bathing solution containing 145 mmol/l NaCl before the apical solution was replaced with 72.5 mmol/l NaCl-containing solution. Difference between the PD before and after fluid replacement (i.e., dilution potential) were recorded. The P_{Na}/P_{Cl} was calculated by using the Goldman-Hodgkin-Katz equation, whereas P_{Na} and P_{Cl} were calculated by using Kimizuka-Koketsu equations.

Magnesium flux measurement

The duodenum of each rat was dissected into 4 pieces, which then were mounted onto 4 individual modified Ussing chamber setups. The tissues were equilibrated for 10 min as mentioned above. To study total Mg^{2+} flux the apical solution of one Ussing chamber setup was substituted with Mg-bathing solution containing (in mmol/l) 40 $MgCl_2$, 2.5 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 115 mannitol, and 10 HEPES pH 7.4. While, the basolateral solution was substituted with Mg-free bathing solution containing (in mmol/l) 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 250 D-mannitol, and 10 HEPES pH 7.4. To investigate the Mg^{2+} channel-independent Mg^{2+} transport, mucosal sites of duodenal tissues in other setups were pre-incubated for 10 min with Co(III)hexaammine (1 mmol/l; Sigma), ruthenium red (20 µmol/l; Sigma), or Co(III)hexaammine+ruthenium red. The Mg^{2+} channel blocker Co(III)hexaammine, which is competing the Mg^{2+}-hexadydrate molecules, had been reported to completely inhibit TRPM6-driven Mg^{2+} influx [49]**.** The noncompetitive pan specific TRP channel inhibitor ruthenium red also suppressed TRPM6-dependent Mg^{2+} influx [47]. Suppression of mucosal Mg^{2+} channel-driven Mg^{2+} influx in enterocyte epithelium should impeded transcellular active Mg^{2+} absorption. After pretreatment the apical and basolateral solutions were substituted with Mg-bathing solution and Mg-free bathing solution, respectively. At 30, 60, and 90 min after solution replacements, 100 µl solution was collected from the basolateral side, as well as from apical side. The Mg^{2+} concentration and the rate Mg^{2+} flux were determined by the method of Thongon and Krishmanra [43]. The difference between the rate of total Mg^{2+} transport and Mg^{2+} channel-independent Mg^{2+} transport was calculated to be the rate of Mg^{2+} channel-driven Mg^{2+} transport.

To study the involvement of basal duodenal HCO_3^- secretion on omeprazole-affected Mg^{2+} transport, mucosal site of duodenal tissues were pre-incubated for 10 min with 500 µmol/l 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; Sigma) or 50 µmol/l N-(2-Naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide (GlyH-101;

Calbiochem, San Diego, CA, USA). After inbibitor pre-incubations, Mg^{2+} flux study was performed as mention above.

Western blot analysis

The duodenal segment was cut longitudinally to expose the mucosa. Duodenal epithelial cells were collected by scraping the mucosal surface with an ice-cold glass slide, and lysed in Piece® Ripa Buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) with 10% v/v protease inhibitor cocktail (Sigma). The lysates were sonicated, centrifuged at 12,000 g for 15 min, and then heated for 5 min at 95°C. Proteins (50 μg) or Cruz Marker™ Molecular Weight Standards (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were loaded and separated on SDS-PAGE gel, then transferred to a polyvinylidene difluoride membrane (PVDF; Amersham, Buckinghamshire,UK). Membranes were blocked with 5% nonfat milk overnight at 4°C and probed overnight at 4°C with 1:1,000 primary antibodies (Santa Cruz Biotechnology) raised against CNNM4 (sc-68437), Cldn-2 (sc-55617), Cldn-7 (sc-33532), Cldn-12 (sc-98608), and Cldn-15 (sc-25712). Membranes were also reprobed with 1:5,000 anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology). Subsequently, membrane were incubated with 1:10,000 HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 2 hr at 25°C, visualized by Thermo Scientific SuperSignal® West Pico Substrate (Thermo Fisher Scientific Inc.) and captured on CL-XPosure Film (Thermo Fisher Scientific Inc.). Densitometric analysis was performed using ImageJ for Mac Os X.

Haematoxylin and eosin (H&E) staining and immunohistochemistry analysis

Mouse duodenal tissues were dissected and preserved overnight at 4°C in 4% wt/vol paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich). After being dehydrated and cleared by graded ethanol and xylene, respectively, they were embedded in paraffin, then cut cross-sectionally into 3-μm thick section. The deparaffinized sections were stained with

haematoxylin and eosin and examined under a light microscope (model BX51; Olympus, Tokyo, Japan).

For immunohistochemistry analysis, deparaffinized sections were incubated at 37°C for 15 min in Dako proteinase K reagent (Dako North America, CA, USA). Nonspecific bindings were blocked by 10 min Dako Real™ peroxidase blocking reagent (Dako North America). The sections were incubated at 4°C overnight with 1:50 diluted primary antibody against TRPM6 (sc-98695, Santa Cruz Biotechnology). Subsequently, the sections were incubated for 25 min at room temperature with 1:300 HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), followed by a 60-min incubation with EnVision™ Flex DAB + chromogen (Dako North America). For negative controls, the sections were incubated with blocking solution in the absence of primary antibodies. Finally, all sections were counterstained with hematoxylin and examined under a light microscope (Olympus).

Scanning electron microscope (SEM)

Femurs were dried at 85°C for 72 hr in an incubator. Fractured head of femur was coated with an ultra-thin gold layer by a sputter coater (Polaron SC7620; Quorum Technologies Ltd, Kent, UK). Surface structure of the trabeculae of femur was captured using SEM (LEO1450 VP; LEO Electron Microscopy Ltd, Clifton Road, UK).

Statistical analysis

Results were expressed as means ± SE. Two sets of data were compared using unpaired Student's t-test. One-way analysis of variance (ANOVA) with Dunnett's posttest was used for comparison of multiple sets of data. All data were analyzed by GraphPad Prism for Mac Os (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of omeprazole administration on gastric and duodenal pH

These experiments were performed to demonstrate the effect of 20 mg/kg oral gavage and subcutaneous omeprazole administrations on gastric acid secretion by means of gastric and duodenal pH measurements. After 2 hr of omeprazole administration gastric and duodenal pH of omeprazole-treated rats were significantly higher than that of sham-treated control rats (Fig. 1A, and 1B). Gastric and duodenal pH of omeprazole-treated rats remained higher after 24 hr of the last dose when compared with control rats. These results suggested that omeprazole administration (20 mg/kg, daily) effectively inhibited gastric acid secretion. Therefore, we chose subcutaneous omeprazole injection to treat the rats throughout 24 wk of experiment.

Effect of omeprazole on Mg^{2+} homeostasis

These experiments aimed to observe the effect of short-term and long-term omeprazole treatment on Mg^{2+} homeostasis in the rats. Throughout 24 wk of experiment all rats were healthy and showed equal increase of body weight (Fig. 1C). The mean body weight, food and water intake, and fecal output at last week of experiment of omeprazole-treated groups were not different from those of sham-treated control group (Table 1). However, 24 wk-omeprazole-treated groups showed statistically higher diuresis than control goup.

Plasma Mg^{2+} concentration (in mg/dl) of 24 wk-omeprazole-treated group (1.67 ± 0.13), but not 4 wk-omeprazole-treated group (2.27 ± 0.17), was significantly decreased in comparison to sham-treated group (2.73 ± 0.24) (Fig. 2A). The 24 wk-omeprazole-treated rats also had significantly lower 24 hr urinary excretion compared to control rats (Fig. 2B). Fecal Mg^{2+} excretion of all experimental groups did not differ (Fig. 2C). Bone Mg^{2+} contents of 4 wk- and 24 wk-omeprazole-treated groups (423.02 ± 12.66 and 316.76 ± 14.86 mg/100 g dry weight, respectively) were significantly decreased in comparison to control group (479.02 ± 7.34 mg/100 g dry weight) (Fig. 2D). Muscle Mg^{2+} content of 24 wk-omeprazole-treated group, but

not 4 wk-omeprazole-treated group, was significantly lower than that of control group (Fig. E). These results indicated hypomagnesemia and depletion of Mg^{2+} storage in prolonged omeprazole-treated rats.

Effect of omeprazole on plasma total 25-OH vitamin D, PTH, Ca^{2+}, and PO_4^{3-}, as well as urine Ca^{2+} and PO_4^{3-}, levels

Since PTH and vitamin D had been reported to regulate plasma Mg^{2+} level [25, 41, 46], plasma 25-OH vitamin D and PTH levels in PPIH rats were determined. As demonstrated in Table 2, total plasma 25-OH vitamin D of 24 wk-omeprazole-treated rats, but not 4 wk-omeprazole-treated rats, was significantly higher than that of control rats. However, plasma PTH level of all experimental groups was not statistically different.

Plasma Ca^{2+} level of 24 wk-omeprazole-treated rats was significantly lower than that of control rats. Plasma phosphate and urine Ca^{2+} levels were not different among all experimental groups. Urinary phosphate excretion of 24 wk-omeprazole-treated rats significantly higher than that of control rats.

Omeprazole suppressed duodenal Mg^{2+} absorption

Intestinal epithelium absorbs Mg^{2+} through transcellular active and paracellular passive mechanisms. Transcellular active Mg^{2+} absorption depends on the activity of apical TRPM6 and basolateral CNNM4 proteins [9]. Paracellular passive Mg^{2+} uptake was modulated by the expression and function of tight junction-associated Cldns [9, 16, 20, 44]. It has been previously proposed that small intestine absorbs Mg^{2+} through paracellular, but not transcellular mechanism [9]. However, TRPM6 was abundantly expressed in brush-border membranes of duodenum [47]. CNNM4 had also been identified in small intestine [50]. In the present study we performed immunohistochemical analysis to visualize the expression of TRPM6 in duodenal slices. As illustrated in Fig. 3A, TRPM6 was expressed in the apical membrane of duodenal villous cells.

The positive signals of TRPM6 expression was increased in omeprazole-treated duodenum. We also observed the expression of duodenal CNNM4 protein (Fig. 3B) by western blot analysis. As expected, the expressions of CNNM4 was significantly increased in omeprazole-treated groups compared to control group (Fig. 3B). Therefore, rat duodenum might absorbed Mg^{2+} through both paracellular and transcellular mechanisms.

We previously reported that omeprazole directly suppressed paracellular Mg^{2+} transport and cation selectivity in intestinal-like Caco-2 monolayers [43]. While another group suggested that omeprazole might enhanced transcellular active Mg^{2+} absorption in colon [24]. These experiments aimed to observe the direct effect of short-term and long-term omeprazole treatments on duodenal Mg^{2+} channel-independent and Mg^{2+} channel-driven Mg^{2+} absorptions. Figure 4 demonstrated the rates of Mg^{2+} absorptions, i.e., total, Mg^{2+} channel-independent, and Mg^{2+} channel-driven Mg^{2+} transport, of sham-treated control (Fig. 4A), 4 wk- (Fig. 4B), and 24 wk-omeprazole-treated groups (Fig. 4C). In the same experimental group Mg^{2+} channel-independent Mg^{2+} transports, as well as Mg^{2+} channel-driven Mg^{2+} transport, upon various inhibitor pretreatments were not statistically different (Fig. 4A–4C). Both 4 wk- and 24 wk-omeprazole-treated groups had statistically lower total Mg^{2+} absorption than the control group (Fig. 4D). The Mg^{2+} channel-independent Mg^{2+} transport, which was referred to paracellular Mg^{2+} transport, of 4 wk- and 24 wk-omeprazole-treated groups were statistically lower than that of Co(III)hexaammine-treated control group (Fig. 4E). In addition, Mg^{2+} channel-driven Mg^{2+} transport, which was referred to transcellular Mg^{2+} transport, of 4 wk- and 24 wk-omeprazole-treated groups were also statistically lower than that of Co(III)hexaammine-treated control group (Fig. 4E). These results suggested that omeprazole suppressed duodenal paracellular and transcellular Mg^{2+} absorptions.

Omeprazole had no effect on duodenal PD of all groups (Table 3). The *I*sc of 24 wk-, but not 4 wk-, omeprazole-treated group was significantly decreased in comparison to control group.

On the other hand, 24 wk-omeprazole-treated group had higher TER compared to control group. These results indicated lower net ionic movement across omeprazole-treated duodenum.

Omeprazole suppressed duodenal paracellular cation selectivity

Since omeprazole impeded duodenal paracellular Mg^{2+} absorption, then, we observed epithelial charge selectivity which modulated paracellular permeability [16, 42, 43]. By using the dilution potential technique, we observed P_{Na}, P_{Cl}, and P_{Na}/P_{Cl}. The results showed that 4 wk- and 24 wk-omeprazole-treated rats had significantly lower P_{Na}/P_{Cl} and P_{Na} compared to sham-treated control group (Table 3). The P_{Cl} was equal in all experimental groups. Therefore, omeprazole suppressed duodenal paracellular cation selectivity.

Omeprazole altered ultrastructure of duodenum

From the observation of 108 slides (9 slides per rat, 4 rats per group), we found blunted and shortened duodenal villi in 24 wk-omeprazole-treated rats (Fig 5A–5C). Moreover, villous to crypt (V:C) ratio of 24 wk-omeprazole-treated rats was significantly lower than that of control groups (Fig 5D). Suggested shortened of villi or crypt hyperplasia [39] which was indicated lower absorption or higher secretion.

Contribution of duodenal HCO_3^- secretion on omeprazole-suppressed Mg^{2+} transport

Mertz-Nielsen et al. [30] reported that omeprazole promoted human duodenal HCO_3^- secretion. Previous in vitro study showed that omeprazole enhanced apical basal and HCl-stimulated HCO_3^- secretion which led to a lower Mg^{2+} transport across intestinal-liked Caco-2 monolayers [45]. The Cl^-/HCO_3^- exchanger and cystic fibrosis transmembrane conductance regulator (CFTR) in the brush-border membranes of duodenum provide important routes for duodenal HCO_3^- secretion [4]. In this experiment we pre-incubated mucosal site of duodenal tissue with Cl^-/HCO_3^- exchanger inhibitor DIDS and CFTR inhibitor GlyH-101 prior to perform

Mg^{2+} transport study in Ussing chamber setups. As demonstrated in Figure 6, DIDS and GlyH-101 had no effect on Mg^{2+} transport in control duodenum. However, both DIDS and GlyH-101 significantly increased Mg^{2+} transport in 4 wk- and 24-wk omeprazole treated groups when compared to its corresponding vehicle-treated group. These results indicated that omeprazole impeded duodenal Mg^{2+} absorption due partly to mucosal HCO_3^- secretion.

Omeprazole enhanced duodenal Cldn-2, Cldn-7, Cldn-12, and Cldn-15 expressions

It is widely accepted that tight junction-associated Cldn protein modulates epithelial paracelullar permeability and charge selectivity [16]. Since omeprazole suppressed duodenal paracellular Mg^{2+} absorption and cation selectivity, we further observed the expression of Cldn proteins. Cldn-16 and -19 had been proposed as paracellular channels for Mg^{2+} in kidney [19]. However Cldn-16 and -19 were not detected along small intestine [13], suggesting that other Cldns might be involved in paracellular intestinal Mg^{2+} absorption. In the present study we observed the expressions of cation selective Cldn, i.e., Cldn-2, -7, -12, and -15, which were expressed in small intestine [13, 14]. Unexpectedly, the expressions of Cldn-2, -7, -12, and -15 were significantly increased in omeprazole-treated groups compared to control group (Fig. 7). These results demonstrated compensatory responds of duodenal epithelium for systemic Mg^{2+} deficit.

Omeprazole altered structure of trabeculae bone

In human, chronic PPIs administration led to increased risk of fracture and significant suppression of the trabecular bone density [1, 28]. By using SEM, we investigated the structure of trabeculae bone in the head of femurs of omeprazole-treated rats. The 24 wk-omeprazole-treated rats had thinner and longer trabeculae compared to sham-treated control group (Fig 8). Wider spaces between trabeculae were also observed in omeprazole-treated rats.

Discussion

There is a growing body of evidence suggesting that severe hypomagnesemia is a side effect of PPIs in chronic users [6–8, 11, 27]. About 18%, 29%, and 61% of PPIH cases, respectively, had PPIs prescription for at least 2, 10, and 5 years [7]. In the present study we found that 24 wk-omeprazole-treated rats had hypomagnesemia. Comparing to human's age, approximately 16.7 rat days equal to 1 human year [34], thus, 168 d omeprazole administration in rats equal to 10 years in human. On the other hand, 28 d omeprazole administration in mice [24] and rats, which is less than 2 human years, had no effect on the plasma Mg^{2+} level. In addition, Denziger et al. [8] reported an association of PPIH with loop and thiazide diuretics using, which agreed with our results that PPIH rats had higher diuresis. Previous studies reported that 1,25-OH vitamin D promoted renal Mg^{2+} excretion [23, 25]. In the present study, the level of total 25-OH vitamin D markedly increased in omeprazole-treated rats. In addition, loop and thiazide diuretics suppresses renal tubular Mg^{2} reabsorption [3]. Thus, higher renal Mg^{2+} excretion is probably involved in development of hypomagnsemia in chronic PPIs users. However, lower urinary Mg^{2+} had been reported in PPIH in human [6, 11, 40] and our rat model, suggesting that hypomagnesemia should be of concern in person who continuously using PPIs for more than 2 years with diuretic administration.

Previous reports suggested that suppression of intestinal Mg^{2+} absorption and depletion of Mg^{2+} storage could be involved in the pathophysiology of PPIH [6–8, 11, 27, 40]. Mg^{2+} retention test demonstrated severe Mg^{2+} storage depletion in PPIH patients [6]. Intravenous Mg^{2+} but not high dose oral Mg^{2+} supplement rapidly cured hypomagnesemia [11, 40], indicating that PPIs impeded intestinal Mg^{2+} absorption [6, 27]. Our results supported these case reports that long-term omeprazole administration suppressed plasma Mg^{2+} level, duodenal Mg^{2+} absorption, bone and muscle Mg^{2+} levels in PPIH rat model. The proposed pathophysiology of PPIH in prolonged users is that PPIs continuously suppresses small intestinal Mg^{2+} absorption

which subsequently stimulates Mg^{2+} releasing from its storage pool. The later development of hypomagnesemia is due to the time required for depletion of Mg^{2+} from its storage [6].

The exact mechanism of PPIs suppressed intestinal Mg^{2+} absorption is currently under debate. Previous in vivo studies indicated that PPIs mainly affected an important colonic Mg^{2+} handling and induced magnesiotropic genes expressions in mouse colon [17, 24]. However, stimulation of colonic Mg^{2+} absorption failed to normalized plasma Mg^{2+} level in PPIH mice [17]. On the other hand, our previous in vitro studies indicated that PPIs affected Mg^{2+} absorption in small intestine [43–45]. Previous in vivo study reported that omeprazole enhanced human duodenal HCO_3^- secretion [30], which probably suppressed small intestinal Mg^{2+} absorption [45]. In the present study, the data suggested that omeprazole enhanced basal duodenal HCO_3^- secretion and suppressed duodenal Mg^{2+} channel-driven transcellular and Mg^{2+} channel-independent paracellular Mg^{2+} absorption. Moreover, after feeding duodenal HCO_3^- secretion markedly increased by several activating factors, e.g., gastric acid, CO_2, and neurohumeral factors [4]. Secreted HCO_3^- suppressed luminal proton and subsequently increased intra-duodenal pH. Since luminal proton enhanced transcellular and paracellular Mg^{2+} absorptions [18, 24, 26, 44,], this impeded large amount Mg^{2+} uptake by small intestine. In addition, in human small intestine, luminal acidic environment varied between pH 5.5–7.0 [31] which is necessary for mineral absorption by stabilizing their solubility [12]. Elevation of luminal pH led to a lower soluble Mg^{2+}, which decreased from 79.61 % at pH 4.4–5.15 to 8.71% at pH 7.8–8.15 [5], that affected intestinal Mg^{2+} absorption. Our recent results agreed with previous study that omeprazole induced duodenal HCO_3^- secretion [30, 45] and effectively increased duodenal pH from 5.38 to 7.50. Therefore, PPIs suppressed small intestinal uptake is due partly to less soluble Mg^{2+} in small intestine.

As reported previously, omeprazole enhanced TRPM6 mRNA expression in mice colon [24], and in this study, it enhanced TPRM6, CNNM4, Cldn-2, -7, -12, and -15 protein expressions in rat duodenum. Although plasma 25-hydroxyvitamin D level increased in

omeprazole-treated rats, the regulation of TRPM6 expression was vitamin D-independent mechanism [25]. Alternatively, vitamin D enhanced small intestinal Cldn-2 and -12 but not Cldn-7 and -15 expressions [14]. Thus, omeprazole probably enhanced duodenal Cldn-2 and -12 expressions through vitamin D dependent mechanism. Previous in vitro study revealed that omeprazole suppressed Cldn-7 expression in Caco-2 cells [44]. However, our recent study demonstrated that omeprazole enhanced Cldn-7 expression, which probably due to the difference in humoral factors. However, the regulatory factors and mechanism of how omeprazole affects intestinal TPRM6, CNNM4, Cldn-2, -7, -12, and -15 protein expressions required further study.

Based on our results a critical question "*why the expression of TRPM6 and those cation selective Cldns could not counteract PPIH in our rat model*" has been raised. TRPM6 function required an interaction with membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP2), whose hydrolysis through activation of G_q-protein coupled recptor-phospholipase C (PLC) dependent pathway fully inactivated TRPM6 channels [22]. Since omeprazole enhanced G_q-protein coupled receptor-PLC activation in intestinal epithelium [45], it might induced PIP2 degradation and then inactivated TRPM6 channels in duodenum of PPIH rats. In addition, luminal proton stimulates TRPM6 activity. Decrement of extracellular pH from 7.4 to 4.0 triggered 3.8-fold increase in TRPM6-driven Mg^{2+} influx [26]. Since the pH of half stimulation of TRPM6 was 4.3 [26], an increased in duodenal pH to 7.50 indicated a lower TRPM6 stimulation in our PPIH rat model. Moreover, TRPM6 mutation suppressed small intestinal Mg^{2+} absorption and caused severe hypomagnesemia [3]. Although mutation of TRPM6 has not been reported in PPIH, this might be involved in development of hypomagnesemia in our rat model.

It is widely accepted that Cldn modulates paracellular ion permeability [16]. Tight junctions (TJ) are a series of anastomosing membrane strands that occluded the intercellular space between epithelium cells [15, 16]. Dynamic reorganization of TJ strands, i.e., breaking,

resealing, and branching, enables paracellular transport without interfering the barrier integrity [15]. Previous in vitro study revealed that over-expression of Cldn-8 or Cldn-15 markedly increased number of TJ strands and decreased paracellular permeability [38, 51]. Therefore, simultaneously over-expressions of Cldn-2, -7, -12, and -15 in PPIH rats probably led to large increase in number of tight junction strands in duodenal epithelium which impeded tight junction dynamic and paracellular Mg^{2+} transport. In addition, elevation of extracellular pH was found to increase the sensitivity of Ca^{2+} sensing receptor (CaSR) [10]. The activation of epithelium-associated CaSR induced Cldn-16 trans-localization from TJ to cytosol, which then suppressed paracellular passive Mg^{2+} transport [20]. Although Cldn-16 was not detected in duodenum [13], hypersensitivity of duodenal-related CaSR might be involved in the inhibition of Cldn-dependent paracellular Mg^{2+} absorption in PPIH rats.

As major Mg^{2+} storage pool, during Mg^{2+} depletion bone Mg^{2+} content gradually declined due to activation of osteoclastic bone resorption and suppression of osteoblastic bone formation [36]. Chronic omeprazole user had lower plasma Mg^{2+} level that led to increased risk of fractures [1]. Maggio et al., [28] reported a suppression of trabecular bone density in prolonged PPIs users, which agreed with our results that thinner and longer trabeculae had been observed in PPIH rats. However, the effect of prolonged PPIs administration on bone physiology remains unknown.

In conclusion, the present study revealed the potential role of duodenum in handling Mg^{2+} and regulating of Mg^{2+} homeostasis and pathophysiology of PPIH. Duodenal HCO_3^- secretion might be one of the critical factors of PPIs-impeded intestinal Mg^{2+} absorption. Reduction of duodenal Mg^{2+} absorption was shown in omeprazole-treated rats, whether hypomagnesemia was presented or not. Hypomagnesemia occurred only if Mg^{2+} storage pool was depleted in prolonged omeprazole-treated rats. Therefore, stimulation of intestinal Mg^{2+} absorption and/or Mg^{2+} supplement should be consider to avoid PPIH in person who continuously using PPIs.

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Figure legends

Figure 1. Effect of oral gavage (OG) or subcutaneous injection (SC) of omeprazole on rat gastric and duodenal pH. Gastric **(A)** and duodenal **(B)** pH were measured by using test strips after 2 or 24 hr after omeprazole administration. Body weight of control (*white circles*), 4 wk-omeprazole-treated (*gray circles*), and 24 wk-omeprazole-treated (*black circles*) throughout 24 wk of experiment **(C)**. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. ($n = 6$).

Figure 2. Effect of omeprazole on Mg^{2+} homeostasis in male Sprague-Dawley rats. Plasma Mg^{2+} level **(A)**, 24 hr urinary Mg^{2+} excretion **(B),** 24 hr fecal Mg^{2+} excretion **(C)**, bone Mg^{2+} content **(D)**, and muscle Mg^{2+} content **(E)** of control (*white bars*), 4 wk-omeprazole-treated (*gray bars*), and 24 wk-omeprazole-treated (*black bars*) group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. ($n = 6$).

Figure 3. Effect of omeprazole on duodenal TRPM and CNM4 expressions. The expression of TRPM6 **(A)** in duodenal villi (brownish signals, arrows) from negative control, control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated groups by immunohistochemical technique (scale bars, 100 μm). Neg.; negative, Cont.; control, Ome; omeprazole. The quantitative immunobloting and representative densitometric analysis of duodenal CNNM4 in control and omeprazole-treated groups **(B)**. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control group. ($n = 5$)

Figure 4. Effect of omeprazole on rat duodenal Mg^{2+} absorption. Rate of total (total: *white bars*), Mg^{2+} channel-independent (para: *gray bars*), and Mg^{2+} channel-driven (trans: *black bars*) Mg^{2+} transport in control **(A)**, 4 wk-omeprazole-treated **(B)**, and 24 wk-omeprazole-treated **(C)** groups. Comparison of total **(D)**, Mg^{2+} channel-independent **(E)**, and Mg^{2+} channel-driven (**F**) Mg^{2+} transport of control (*white bars*), 4 wk-omeprazole-treated (*gray bars*), and 24 wk-omeprazole-treated (*black bars*) groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the

Co(III)hexaammine-treated control group. (n = 5). Co; Co(III)hexaammine, RR; ruthenium red. (n = 5).

Figure 5. Effect of omeprazole on rat duodenum. Representative H&E stained sections of duodenum in control **(A)**, 4 wk-omeprazole-treated **(B)**, and 24 wk-omeprazole-treated **(C)** groups. Villous to crypt (V:C) ratio of control (*white bars*), 4 wk-omeprazole-treated (*gray bars*), and 24 wk-omeprazole-treated (*black bars*) groups. *P < 0.05 compared with the control group.

Figure 6. Contribution of mucosal HCO_3^- secretion on omeprazole-supressed duodenal Mg^{2+} absorption. The total Mg^{2+} transport of control (*white bars*), 4 wk-omeprazole-treated (*gray bars*), and 24 wk-omeprazole-treated (*black bars*) groups. **P < 0.01, ***P < 0.001 compared with its corresponding vehicle-treated group. (n = 5).

Figure 7. Effect of omeprazole on rat duodenal Cldn-2, -7, -12, and -15 expressions. The quantitative immunobloting analysis of duodenal Cldn-2, -7, -12, and -15 expressions in control and omeprazole-treated groups **(A)**. Representative densitometric analysis of Cldn-2, -7, -12, and -15 **(B)** expression in control (*white bars*), 4 wk-omeprazole-treated (*gray bars*), and 24 wk-omeprazole-treated (*black bars*) groups. ***P < 0.001 compared with the control group. (n = 5).

Figure 8. Effect of omeprazole on trabeculae structure of rat bone. SEM images revealed structure of the head of femurs of control **(A–C)** and and 24 wk-omeprazole-treated (**D–F**) groups. EP; epiphyseal.

Table legends

Table 1. Metabolic characteristics of control, 4 wk-omeprazole-treated, and 24 wk-omeprazole-treated rats. *$P < 0.05$ compared with the control group. ($n = 6$).

Table 2. The effect of omeprazole on plasma hormones and electrolytes. PTH, parathyroid hormone. *$P < 0.05$, ***$P < 0.001$ compared with the control group. ($n = 6$).

Table 3. The effect of omeprazole on electrical parameters and charge selectivity. PD, transepithelial potential difference; *I*sc, short-circuits current; TER, transepithelial resistance; P_{Na}, sodium permeability; P_{Cl}, chloride permeability; P_{Na}/P_{Cl} relative sodium to chloride permeability. ***$P < 0.001$ compared with the control group. ($n = 6$).

Figure1

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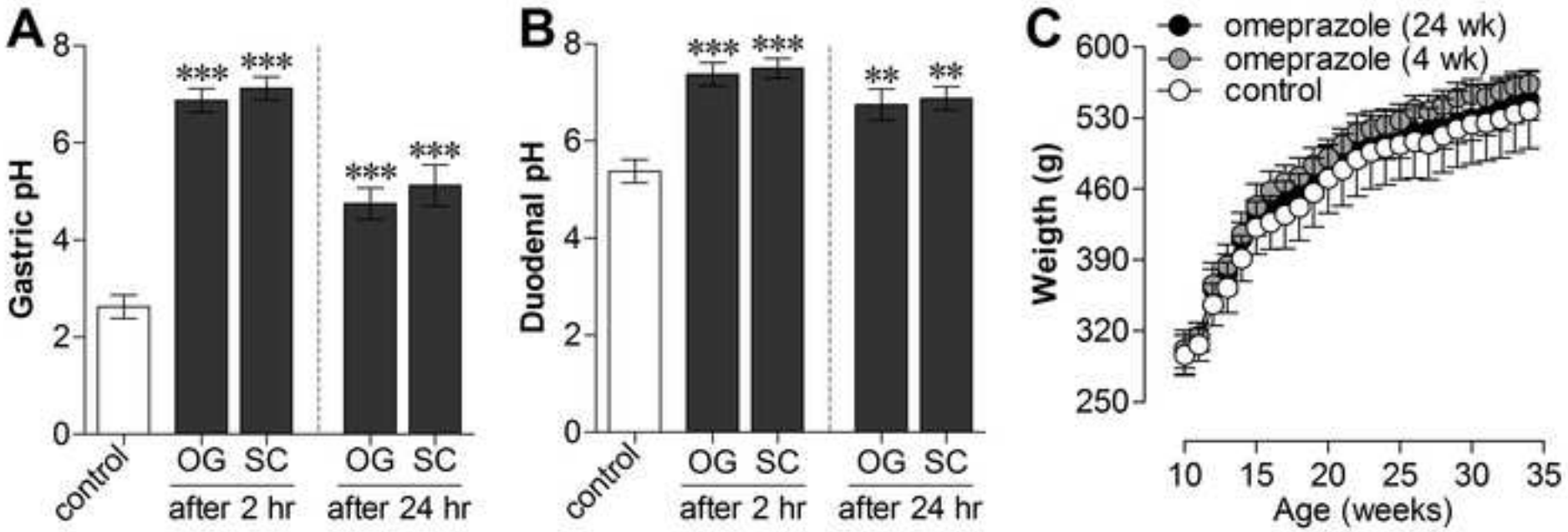

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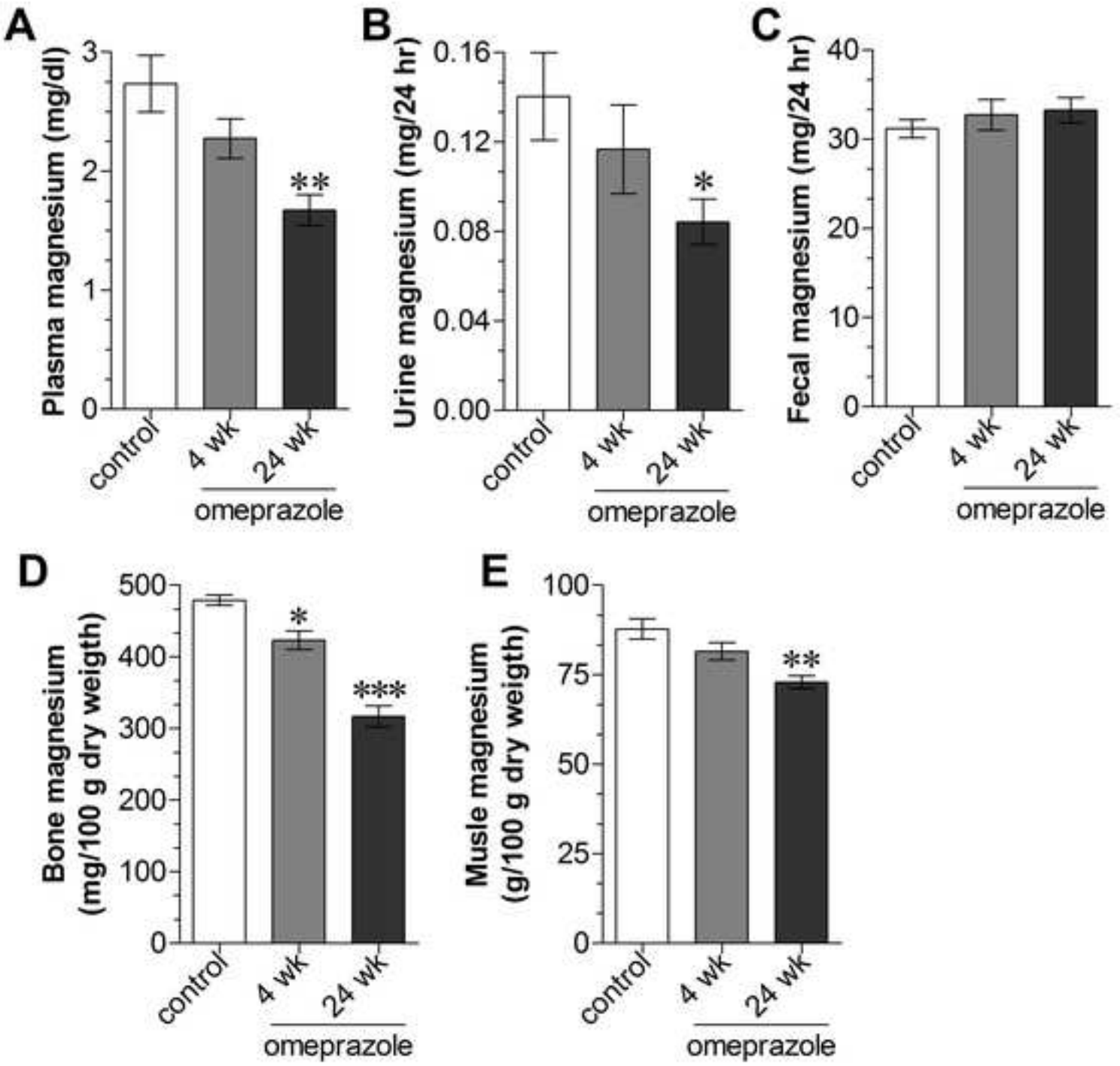

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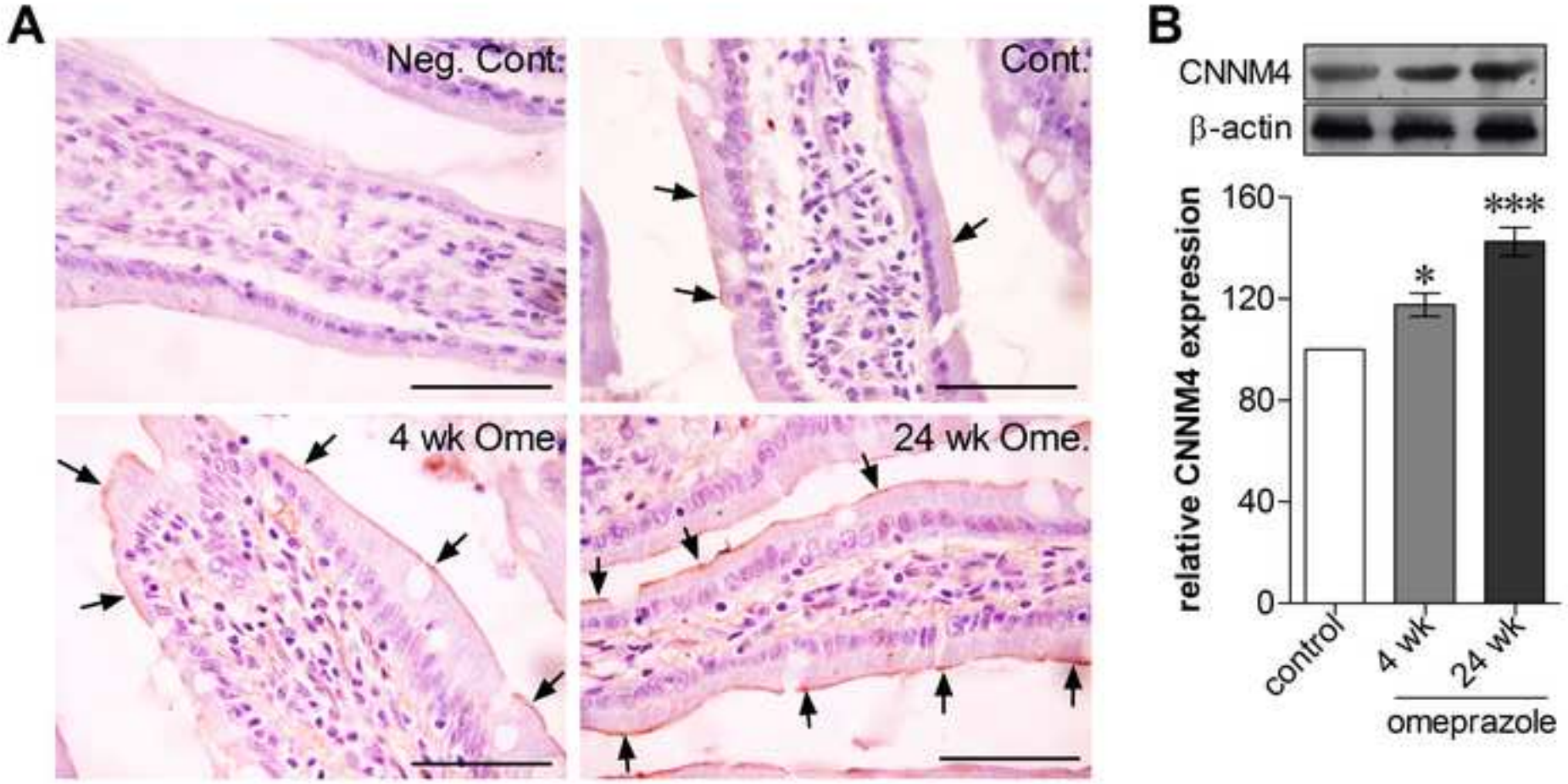

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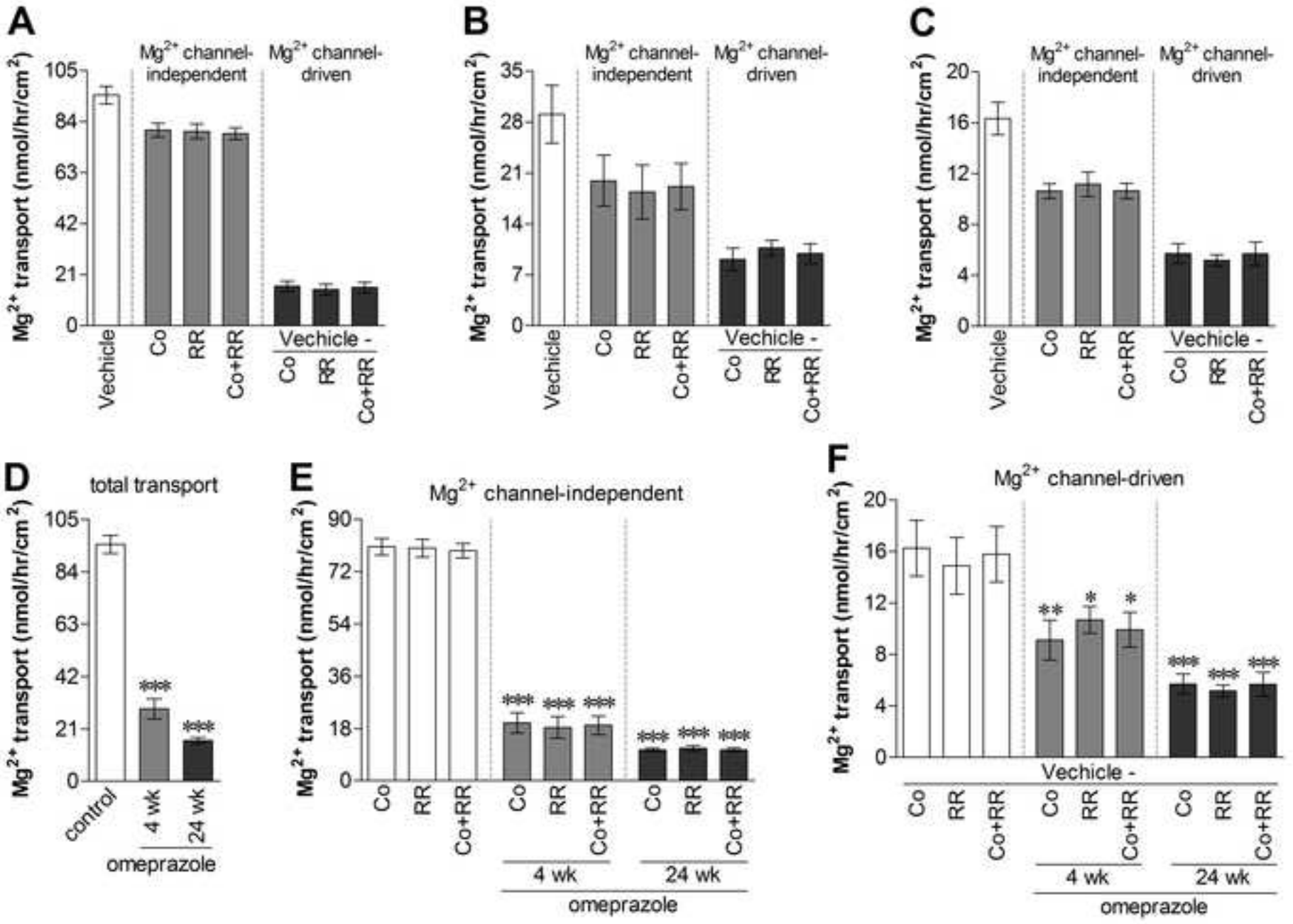

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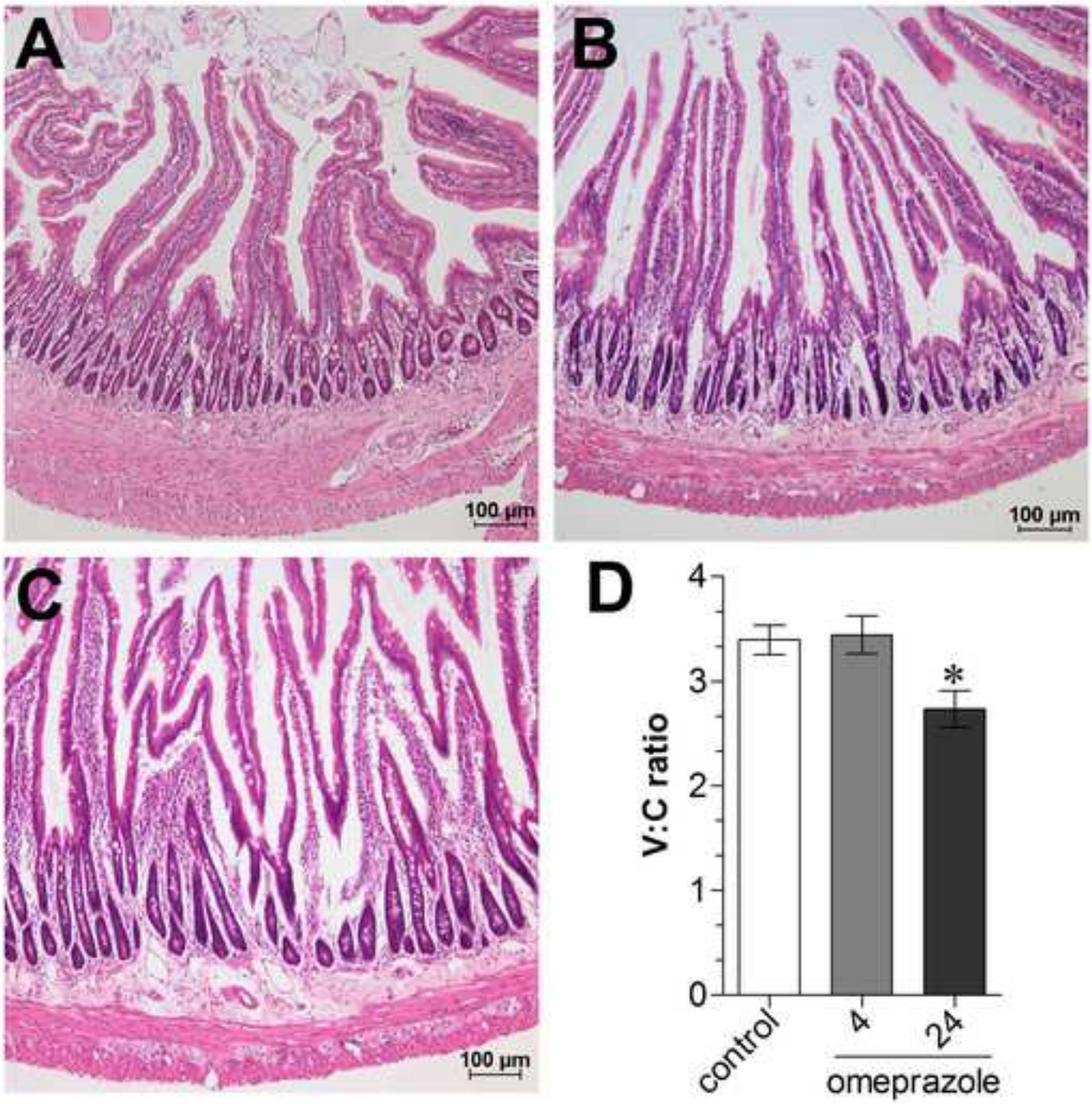

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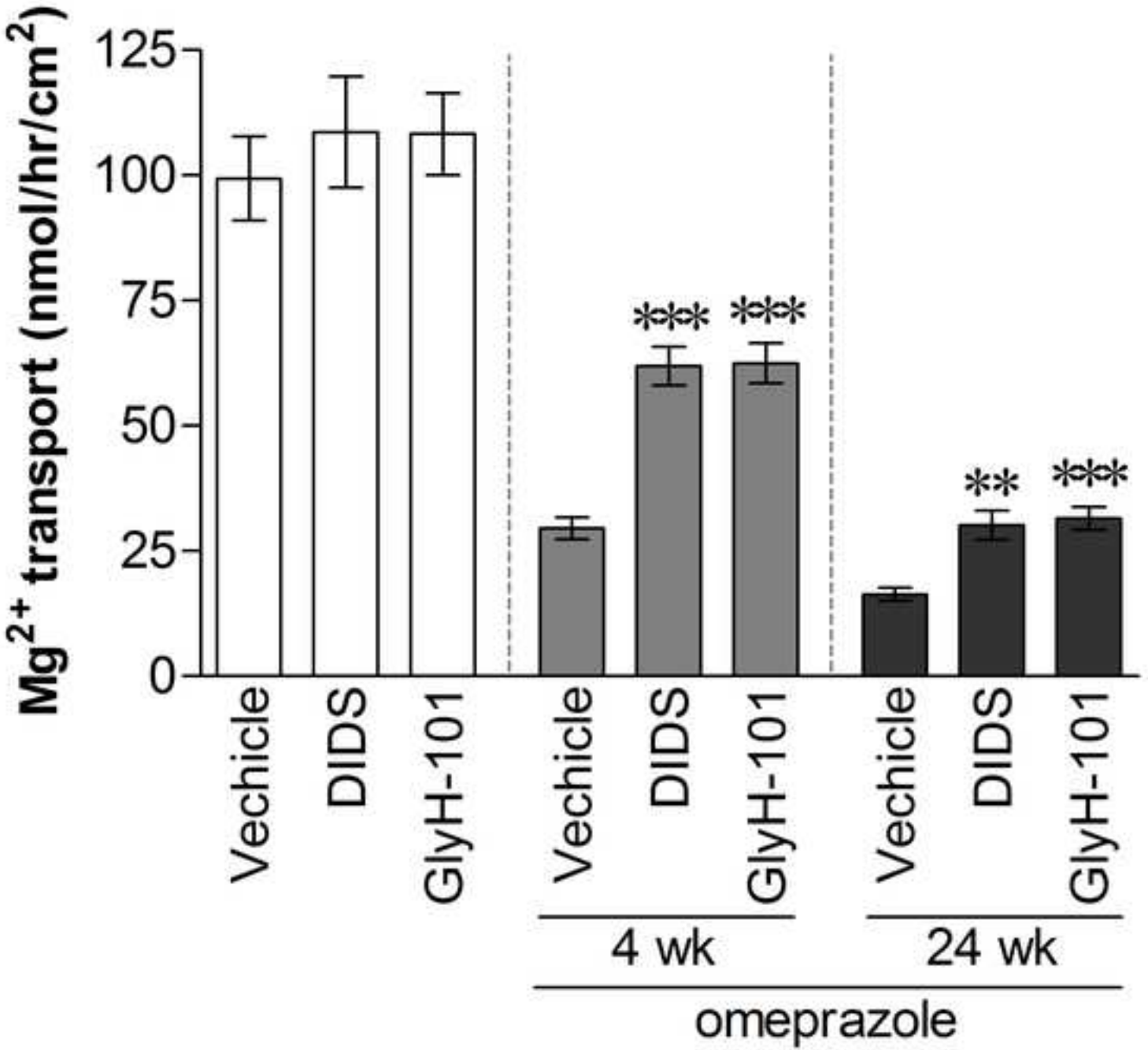

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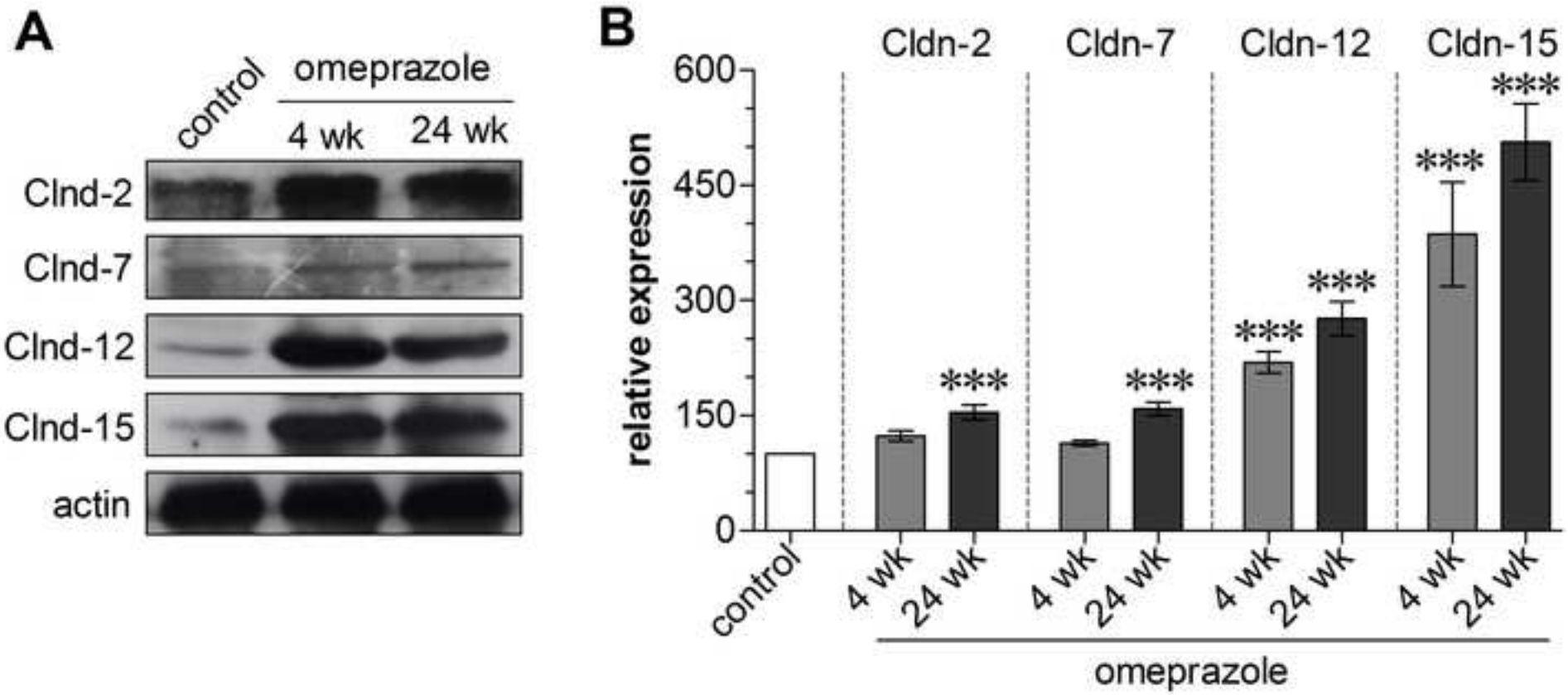

Figure8

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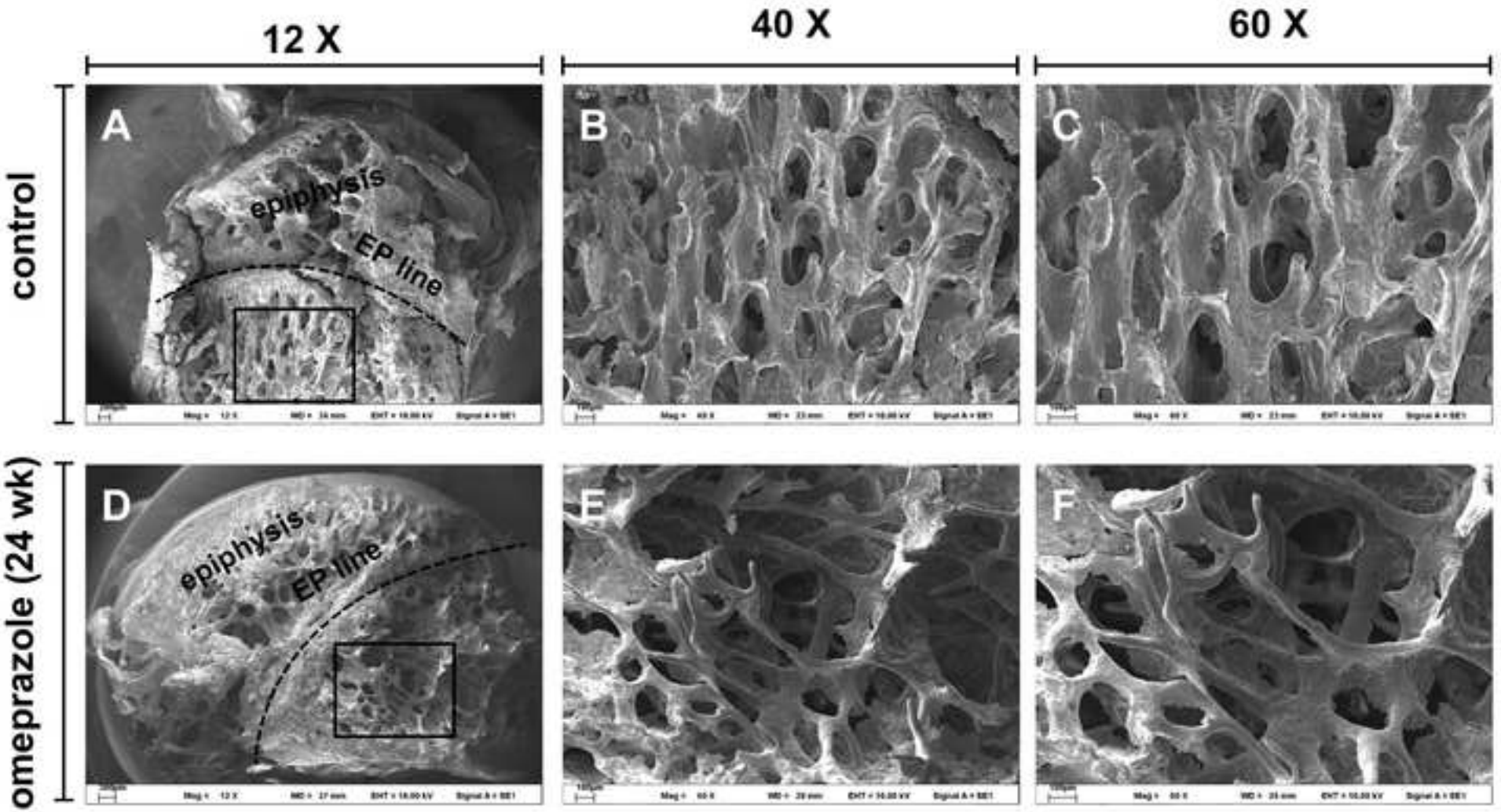

Table 1

		omeprazole (20 mg/kg)	
	control	4 wk	24 wk
Body weight (g)	537.78 ± 37.88	563.50 ± 13.51	548.38 ± 20.07
Food intake (g/day)	23.99 ± 0.67	25.95 ± 0.53	23.76 ± 0.52
Water intake (ml/day)	47.05 ± 1.46	49.06 ± 1.74	51.24 ± 1.49
Diuresis (ml/day)	6.28 ± 1.14	7.43 ± 1.11	10.25 ± 1.05*
Fecal dry weight (g/day)	8.80 ± 0.45	8.93 ± 0.50	8.97 ± 0.57

Table 2

		omeprazole (20 mg/kg)	
	control	4 wk	24 wk
25-OH Vitamin D (ng/ml)	16.68 ± 1.17	16.14 ± 1.47	31.90 ± 1.84***
PTH (pg/ml)	7.06 ± 1.85	5.76 ± 1.29	9.92 ± 2.01
plasma calcium (mg/dl)	10.64 ± 0.46	9.76 ± 0.52	9.04 ± 0.52*
plasma phosphate (mg/dl)	6.24 ± 0.38	6.20 ± 0.41	5.94 ± 0.48
urine calcium (mg/24 hr)	1.91 ± 0.33	1.60 ± 0.38	1.14 ± 0.37
urine phosphate (mg/24 hr)	27.35 ± 6.04	36.07 ± 6.44	45.52 ± 4.92*

Table 3

		omeprazole (20 mg/kg)	
	control	4 wk	24 wk
Electrical parameters			
PD (mV)	5.18 ± 0.42	4.82 ± 0.36	4.62 ± 0.28
*I*sc (μA/cm^2)	37.44 ± 2.23	32.89 ± 1.76	23.55 ± 2.28***
TER (Ω·cm^2)	137.84 ± 6.39	145.58 ± 5.98	201.93 ± 9.49***
Dilution potential experiment			
P_{Na}/P_{Cl}	2.03 ± 0.09	1.64 ± 0.05**	1.25 ± 0.03***
P_{Na} (10^{-6}·cm^2/s)	19.97 ± 0.73	16.51 ± 0.84**	10.52 ± 0.57***
P_{Cl} (10^{-6}·cm^2/s)	9.93 ± 0.57	10.07 ± 0.41	8.39 ± 0.39