Apical acidity decreases inhibitory effect of omeprazole on Mg^{2+} absorption and claudin-7 and -12 expression in Caco-2 monolayers

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Abstract

Clinical studies reported hypomagnesaemia in long-term omeprazole usage that was probably due to intestinal Mg^{2+} wasting. Our previous report demonstrated the inhibitory effect of omeprazole on passive Mg^{2+} transport across Caco-2 monolayers. The present study aimed to identify the underlying mechanism of omeprazole suppression of passive Mg^{2+} absorption. By using Caco-2 monolayers, we demonstrated a potent inhibitory effect of omeprazole on passive Mg^{2+}, but not Ca^{2+}, transport across Caco-2 monolayers. Omeprazole shifted the %maximum passive Mg^{2+} transport-Mg^{2+} concentration curves to the right, and increased the half maximal effective concentration of those dose-response curves, indicating a lower Mg^{2+} affinity of the paracellular channel. By continually monitoring the apical pH, we showed that omeprazole suppressed apical acid accumulation. Neomycin and spermine had no effect on passive Mg^{2+} transport of either control or omeprazole treated monolayers, indicating that omeprazole suppressed passive Mg^{2+} transport in a calcium sensing receptor (CaSR)-independent manner. The results of western blot analysis showed that omeprazole significantly suppressed claudin (Cldn)-7 and -12, but not Cldn-2, expression in Caco-2 cells. By using apical solution of pH 5.5, 6.0, 6.5, and 7.0, we found that apical acidity markedly increased passive Mg^{2+} transport, Mg^{2+} affinity of the paracellular channel, and Cldn-7 and -12 expression in Caco-2 monolayers. Apical acidity abolished the inhibitory effect of omeprazole on passive Mg^{2+} transport and Cldn-7 and -12 expression. Our results provided the evidence for the regulation of intestinal passive Mg^{2+} absorption by luminal acidity-induced increase in Cldn-7 and -12 expression.

Keywords: claudins; intestines; magnesium; omeprazole; proton pump inhibitors; tight junctions

Introduction

Magnesium (Mg^{2+}) is an essential co-factor of numerous enzymatic reactions in the human body. Normal Mg^{2+} homeostasis is tightly regulated by the harmonious function of the intestinal absorption and renal excretion (Konrad *et al.*, 2004; Quamme, 2008). Since dietary intake is the sole source of Mg^{2+} in humans, intestinal absorption plays a vital role in the regulation of normal Mg^{2+} balance. Different intestinal segments contribute unequally to Mg^{2+} absorption. In percentage of the total input, duodenum absorbs 11%, jejunum 22%, ileum 56%, and large intestine 11% (McCarthy and Kumar, 1999). Paracellular passive Mg^{2+} transport is the major mechanism responsible for approximately 90% of total intestinal Mg^{2+} absorption (Quamme, 2008).

There have previously been a number of case reports of severe hypomagnesemia in patients with long-term use of proton pump inhibitor (PPI) that probably resulted from intestinal Mg^{2+} wasting

(Epstein *et al.*, 2006; Cundy and Dissanayake, 2008; Shabajee *et al.*, 2008; Broeren *et al.*, 2009; Kuipers *et al.*, 2009; Hoorn *et al.*, 2010; Mackay and Bladon, 2010). Recently, we reported an inhibitory effect of the popular PPI omeprazole on the paracellular passive, but not active, Mg^{2+} transport in human enterocyte-like Caco-2 monolayers (Thongon and Krishnamra, 2011). However the underlying cellular mechanism of this omeprazole action on paracellular channel still had not been elucidated.

The acidic environment of the intestinal lumen is known to have effects on the secretory and absorption activities of the intestine. The luminal acidity along the entire human small bowel varied between pH 5.5-7.0 (Nugent *et al.*, 2001). Heijnen and coworkers (1993) reported that luminal acidic pH increased Mg^{2+} absorption in the ileum. Recently, elevation of extracellular pH was found to increase the sensitivity of CaSR (Quinn *et al.*, 2004; Doroszewicz *et al.*, 2005), and the activation CaSR in turn, suppressed paracellular passive Mg^{2+} transport (Ikari *et al.*, 2008). Thus, omeprazole suppressed paracellular passive Mg^{2+} absorption at least in part by modulating the luminal acidity and CaSR sensitivity (Thongon and Krishnamra, 2011).

Claudins (Cldn) are the integral membrane proteins of the tight junction with ~23 members which were expressed in a tissue specific manner (Furuse and Tsukita, 2006). Monomeric and heteromeric combination of Cldn in each epithelium created unique paracellular channel for paracellular ion transport (Tang and Goodenough, 2003; Krause *et al.*, 2008). Cldn-2 and -12 were reported to act as paracellular channels for divalent cation i.e., Ca^{2+} (Fujita *et al.*, 2008). In addition, paracellular Cldn-7 channel was highly permeable to Na^+, but not Cl^- (Alexandre *et al.*, 2005). Our previous finding reported that omeprazole inhibited paracellular Mg^{2+} and Na^+ channels (Thongon and Krishnamra, 2011). Therefore, omeprazole probably disturbed the expression and/or function of Cldn-2, -7, and -12, which were highly expressed in the jejunum, ileum, and Caco-2 cells (Fujita *et al.*, 2006, 2008). The present study, therefore, aimed to identify the underlying mechanism of the omeprazole-suppressed intestinal paracellular Mg^{2+} absorption. We hypothesized that omeprazole disturbed luminal acidity and increased CaSR activity. The activation of CaSR suppressed of paracellular Mg^{2+} channel that formed by Cldn in intestinal epithelium. Experiments were performed in Caco-2 monolayer, which was accepted as a suitable intestinal model for studying Mg^{2+} transport (Ekmekcioglu *et al.*, 2000; Thongon and Krishnamra, 2011).
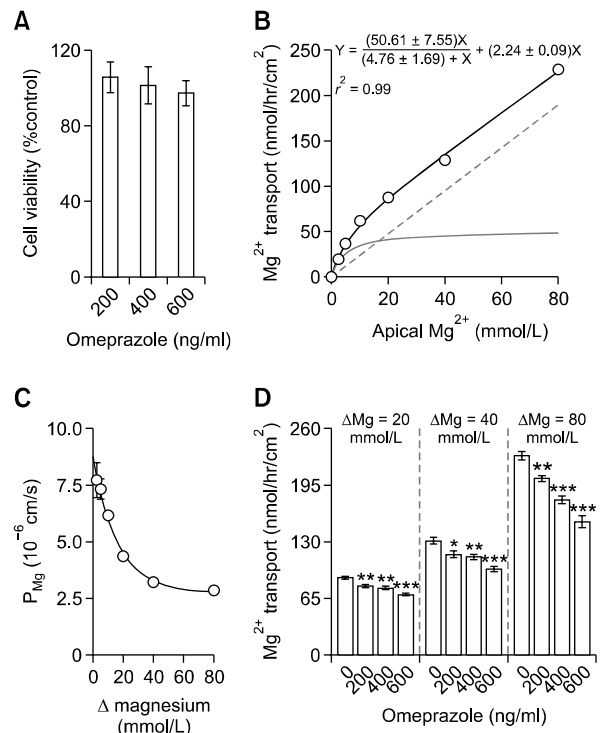

Figure 1. Omeprazole suppressed passive Mg^{2+} transport across Caco-2 monolayers. Relative cell viability of omeprazole - treated cells (A). Apical to basolateral Mg^{2+} transport of control monolayers (B). Light solid line represent the saturable active component. Dash line represent the non-saturable passive component. Paracellular P_{Mg} of control monolayers (C). Δ represent Mg^{2+} concentration gradient of apical and basolateral solution. Paracellular passive Mg^{2+} transport under 20, 40, or 80 (D) mmol/L Mg^{2+} concentration gradient. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control group. For each data point, $n = 6$.

Results

Omeprazole decreased passive Mg^{2+} transport across Caco-2 monolayers

Since omeprazole was reported to have a potent apoptotic effect on human polymorphonuclear leucocytes (Capodicasa *et al.*, 2008), cell viability assay was performed by incubating Caco-2 cells with the omeprazole at concentrations resembling those found in the human plasma of, i.e., 200 and 400 ng/ml (Macek *et al.*, 2007), as well as a supra-physiological concentration of 600 ng/ml. As demonstrated in Figure 1A, neither 200, 400, nor 600 ng/ml omeprazole had cytotoxic effect on Caco-2 cells. Figures 1B and 1C depicted the apical to basolateral Mg^{2+} transport and absolute Mg^{2+} permeability (P_{Mg}) of Caco-2 monolayers (Figures 1B and 1C) in the present transwell transporting setup which were comparable to those of previous modified Ussing chamber set up (Thongon and Krishmanra, 2011). Omeprazole significantly sup-
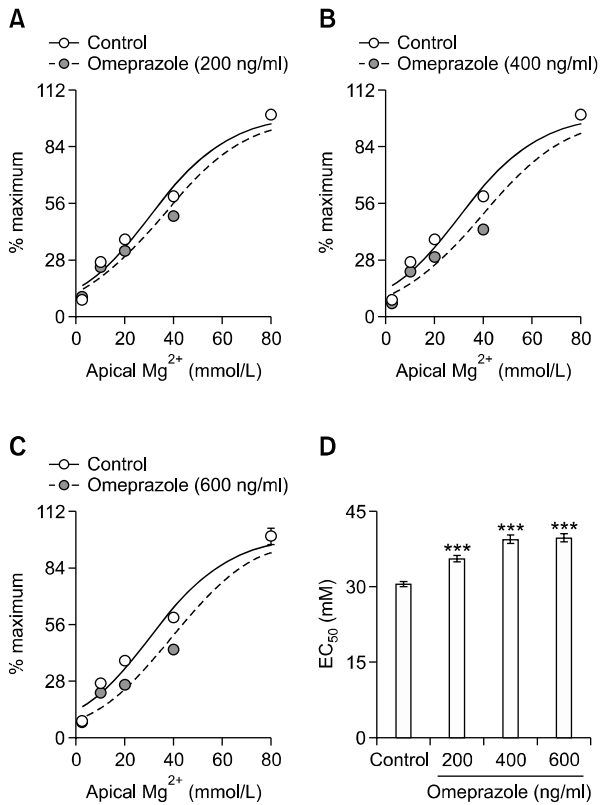
Figure 2. Omeprazole decreased Mg^{2+} affinity of the paracellular channels of Caco-2 monolayers. %maximum passive Mg^{2+} transport - Mg^{2+} concentration curve of control and with omeprazole at 200 (A), 400 (B), or 600 (C) ng/ml. EC_{50} of the dose - response curve of control and omeprazole trated monolayers (D). ***$P < 0.001$ vs control group. For each data point, $n = 6$.

Figure 3. Omeprazole had less effect on the paracellular passive Ca^{2+} transport across Caco-2 monolayers. Passive Ca^{2+} transport of control monolayers (A). Paracellular passive Ca^{2+} transport under 20, 40, or 80 (B) mmol/L Ca^{2+} concentration gradients. ΔCa represent Ca^{2+} concentration gradient of apical and basolateral solution. *$P < 0.05$ vs control group. For each data point, $n = 10$.

Figure 4. Omeprazole disturbed apical acidity of Caco-2 monolayers. Accumulative apical pH values at various time points after replacing media with fresh one for 14d control cells (A). Apical pH of the media of control and omeprazole exposed Caco-2 cells at 16 h, 20 h, and 24 h (B) after media change. Passive Mg^{2+} transport (40 mmol/L concentration gradient) of control and omeprazole-treated groups that were incubated with CaSR agonists neomycin or spermine (C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control group. For each data point, $n = 6$.

pressed the non-saturable passive Mg^{2+} transport in the presence of 20, 40, and 80 mmol/L (Figure 1D) Mg^{2+} concentration gradients. Therefore, the Mg^{2+} flux setup used in the present study was suitable for studying intestinal Mg^{2+} absorption.

The relationships of percent change of passive Mg^{2+} transport and apical Mg^{2+} concentration were compared among control and omeprazole treated groups (Figures 2A-2C). Omeprazole shifted the %change - apical Mg^{2+} concentration curves to the right. Omeprazole at 200, 400, and 600 ng/ml significantly increased the EC_{50} from 30.54 ± 1.13 to 35.54 ± 1.41, 39.39 ± 1.83, and 39.74 ± 1.84 mmol/L, respectively (Figure 2D). These results indicated that omeprazole decreased affinity of paracellular channel for Mg^{2+}.

Since passive transport of Mg^{2+} and Ca^{2+} probably involved similar tight junction-associated proteins, the effect of omeprazole on passive Ca^{2+} transport across Caco-2 monolayers was also examined. The apical Ca^{2+} concentration - Ca^{2+} transport showed a liner relationship ($Y = (3.16 \pm 0.14)X + (8.47 \pm 5.34)$,
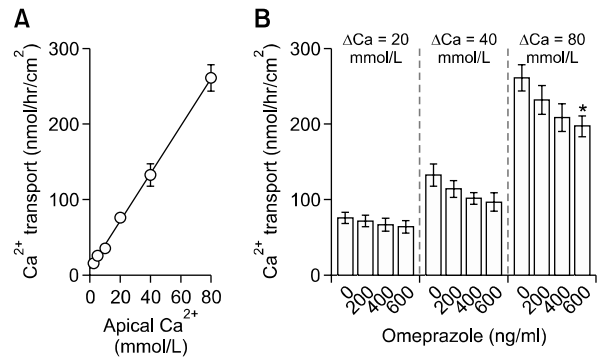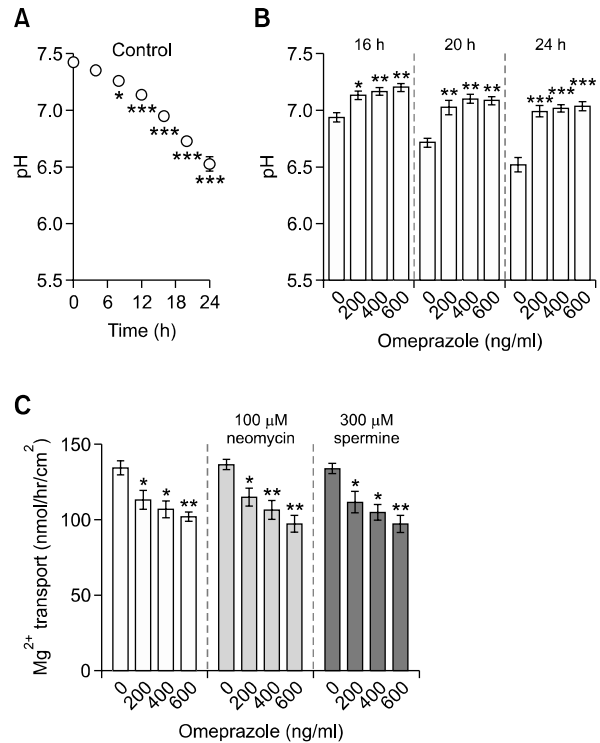
$r^2 = 0.94$) (Figure 3A). In the presence of 20 and 40 mmol/L Ca^{2+} concentration gradients (Figure 3B), omeprazole showed the tendency to decrease Ca^{2+}
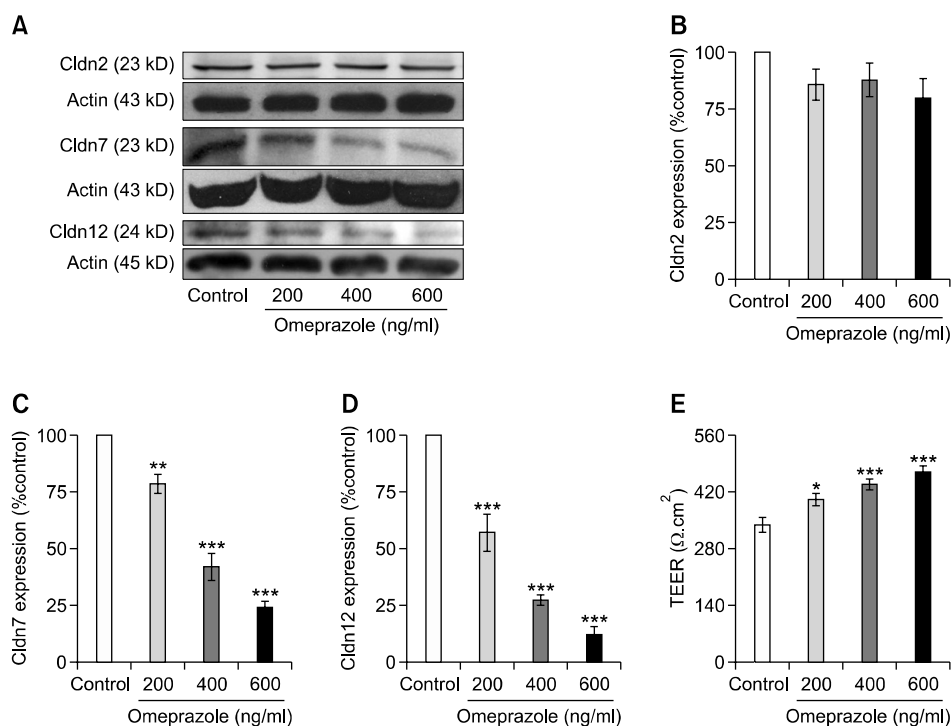
Figure 5. Omeprazole decreased Cldn-7 and -12 expression. The quantitative immunobloting analysis of Cldn-2, -7, and -12 in control and omeprazole-exposed Caco-2 cells (A). Representative densitometric analysis of Cldn-2 (B), -7 (C), and -12 (D) expression in control and omeprazole exposed cells. Trans-epithelial electrical resistance of control and omeprazole-exposed Caco-2 monolayers (E). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs control group. For each data point, $n = 5$.

transport, but the reductions were not statistically significant. Only at Ca^{2+} concentration gradient of 80 mmol/L with the concentration of 600 ng/ml that omeprazole significantly decreased passive Ca^{2+} transport. These results indicated that omeprazole had less inhibitory effect on passive Ca^{2+} transport than on passive Mg^{2+} absorption.

Omeprazole disturbed apical acidity of Caco-2 monolayers

Since luminal pH could modulate intestinal Mg^{2+} absorption (Heijnen *et al*., 1993), the pH of apical culture media of control and omeprazole-treated monolayers were continually measured for 24 h after culture media change. In the control condition, apical pH significantly decreased from 8 h to 24 h after media change (Figure 4A). These results indicated the apical acid secretion and accumulation that was probably due to the activity of the proton pump in Caco-2 monolayers (Abrahamse *et al*., 1992). Omeprazole at 200, 400, and 600 ng/ml clearly raised the apical pH at 16 h, 20 h, and 24 h (Figure 4B), suggesting that a potent PPI omeprazole could suppress apical acid secretion by Caco-2 monolayers.

Omeprazole suppressed passive Mg^{2+} transport by a CaSR-independent mechanism

Since an increase in the extracellular pH could enhance CaSR sensitivity (Quinn *et al*., 2004; Doroszewicz *et al*., 2005), and activation of CaSR in turn, decreased paracellular Mg^{2+} transport (Ikari *et al*., 2008), possible involvement of CaSR in passive Mg^{2+} transport was studied by using the polycationic CaSR agonists neomycin and spermine (Sigma) (Ye *et al*., 1997). As demonstrated in Figure 4C, neither 100 μM neomycin nor 300 μM spermine affected passive Mg^{2+} transport in control and omeprazole-treated monolayers. These results indicated that omeprazole-suppressed passive Mg^{2+} transport was CaSR - independent.

Omeprazole suppressed Cldn-7 and -12 expression

It was widely accepted that paracellular channel Cldn regulated paracellular passive ion transport across the epithelium (Tang and Goodenough, 2003; Krause *et al*., 2008). Therefore, the expressions of Cldn-2, -7, and -12 that was highly expressed in the jejunum, ileum, and Caco-2 cells (Fujita *et al*., 2006, 2008) were studied. The results of immunoblot and densitometric analysis demonstrated that omeprazole significantly reduced the expression of Cldn-7 (Figures 5A and 5C) and -12 (Figures 5A and 5D), but not of Cldn-2 (Figures 5A and 5B), in a concentration-dependent manner. Since the trans-
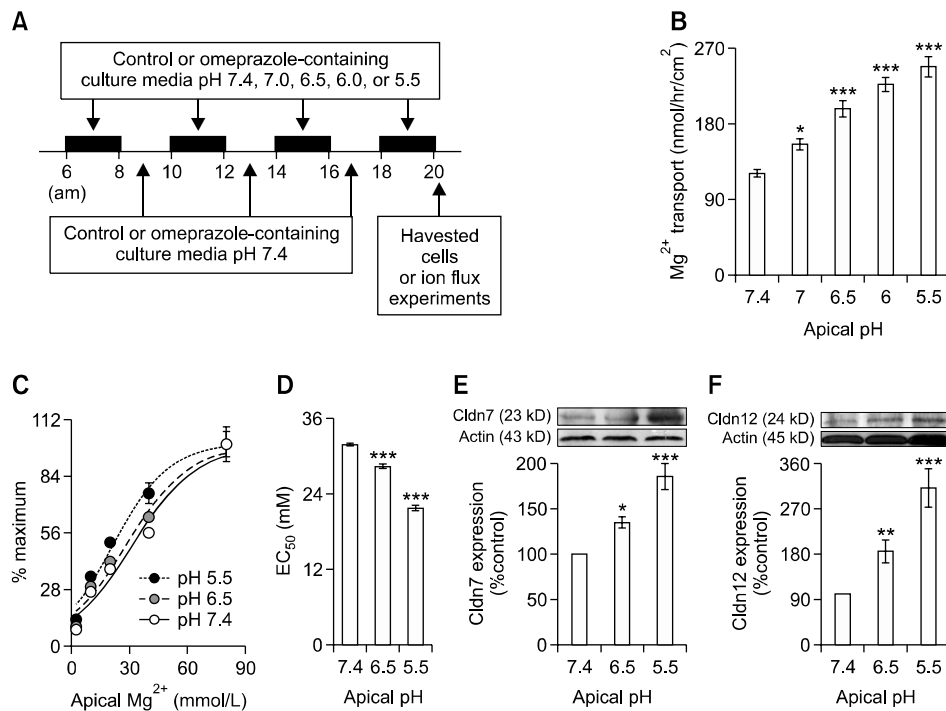
A

B

C

D

E

F

Figure 6. Apical acidity enhanced passive Mg^{2+} absorption and Cld-7 and -12 expression. Representative time line for apical acidic media treatment (A). Passive Mg^{2+} transport (40 mmol/L concentration gradient) of control monolayers (B). %maximum passive Mg^{2+} transport - Mg^{2+} concentration curve of control monolayers at pH 7.4, 6.5, and 5.5 (C). EC_{50} of the dose - response curve of control monolayers at pH 7.4, 6.5, and 5.5 (D). For each data point, n = 6. Immunoblotting and densitometric analysis of Cldn-7 (E) and -12 (F). For each data point, n = 5. *$P <$ 0.05, **$P <$ 0.01, ***$P <$ 0.001 vs control group (n = 5).

epithelial electrical resistance (TEER) inversely related to the paracellular cation conductances (Tang and Goodenough, 2003; Thongon and Krishnamra, 2011), TEER of control and omeprazole-exposed monolayers were observed. As demonstrated in Figure 5E, omeprazole significantly increased TEER of Caco-2 monolayers.

Apical acidity enhanced passive Mg^{2+} transport and Cldn-7 and -12 expression

To study the effect of apical acidity on the intestinal Mg^{2+} absorption and Cldn expression, Caco-2 monolayers were exposed for 2 h, 4 times a day to culture media with pH 7.4, 7.0, 6.5, 6.0, or 5.5 (Figure 6A) from day 7 to day 14 after seeding. In the control condition, apical acidity significantly increased passive Mg^{2+} transport at 40 mmol/L Mg^{2+} concentration gradient (Figure 6B). The relationship of percent change of passive Mg^{2+} transport and apical Mg^{2+} concentration in control condition at various apical pH was shown (Figure 6C). Apical acidic pH of 6.5 and 5.5 markedly shifted the relationship curve to the left (Figure 6C). Apical acidity of 6.5 and 5.5 decreased EC_{50} from 31.78 ± 1.11 at pH 7.4 to 28.32 ± 1.55 and 21.69 ± 1.80 mmol/L, respectively (Figure 6D). In addition, apical acidity significantly increased Cldn-7 (Figure 6E) and -12 (Figure 6F) expression in Caco-2 cells.

Apical acidity restored omeprazole effect on Caco-2 monolayers

Since apical acidity enhanced passive Mg^{2+} transport as well as the expression of Cldn-7 and -12 in control Caco-2 monolayers (Figure 6), this experiment aimed to elucidate the effect of low apical pH on Mg^{2+} transport and Cldn expression in omeprazole-exposed monolayers. Similar to the control condition, apical acidity markedly increased the paracellular passive Mg^{2+} transport (40 mmol/L concentration gradient) of both 200 (Figure 7A) and 400 ng/ml omeprazole treated monolayers (Figure 7D). In the presence of 40 mmol/L Mg^{2+} concentration gradient, the rate of passive Mg^{2+} transport of 200 ng/ml omeprazole-treated monolayers at pH 7.0 (118 ± 7.25, Figure 7A), of 400 ng/ml omeprazole-treated monolayers pH 7.0 (116.60 ± 5.12, Figure 7D), and pH 6.5 (132.30 ± 6.01, Figure 7D) were not significantly different from that of control monolayers at pH 7.4 (132.20 ± 3.96, Figure 1E). These results indicated that apical acidity could restore the inhibitory effect of omeprazole on passive Mg^{2+} absorption. Apical acidity also shifted the dose-response curve of 200 (Figure 7B) and 400 ng/ml omeprazole (Figure 7E) treated group to the left. The EC_{50} of of 200 (Figure 7C) and 400 ng/ml (Figure 7F) omeprazole treated monolayers also decreased with the increase in acidity. At apical pH 6.5, EC_{50} of 200 (30.99 ± 1.67 mmol/L, Figure 7B) and 400 ng/ml omeprazole (33.30 ± 1.99 mmol/L,
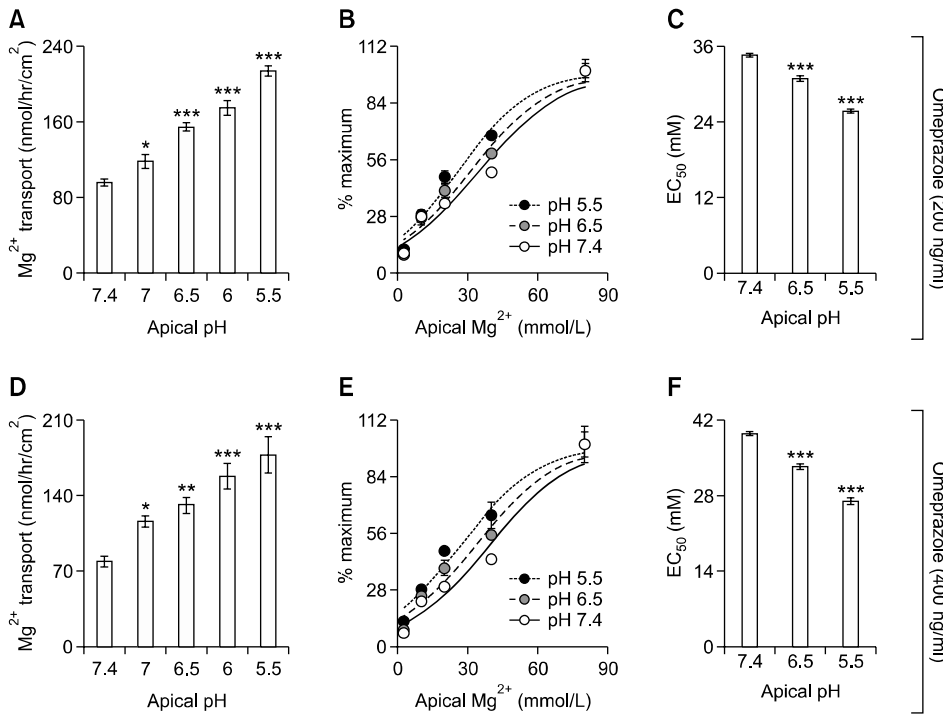
Figure 7. Apical acidity decreases omeprazole effect on passive Mg^{2+} absorption. Passive Mg^{2+} transport (40 mmol/L concentration gradient) of 200 (A) or 400 ng/ml omeprazole exposed monolayers at various apical pH (D). %maximum passive Mg^{2+} transport - Mg^{2+} concentration curve of 200 (B) or 400 ng/ml omeprazole exposed monolayers at pH 7.4, 6.5, and 5.5 (E). EC_{50} of the dose - response curve of 200 (C) or 400 ng/ml omeprazole exposed monolayers at pH 7.4, 6.5, and 5.5 (F). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control group. For each data point, $n = 6$.
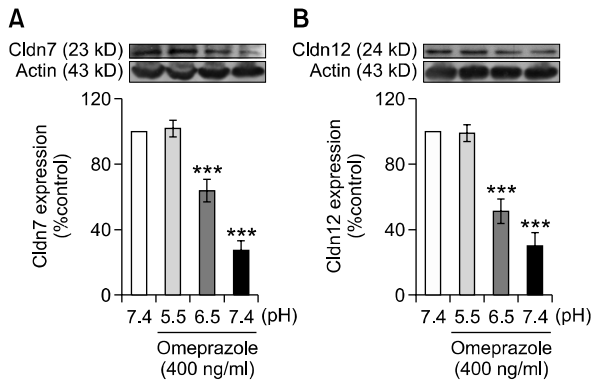

Figure 8. Apical acidity restored the inhibitory effect of omeprazole effect on Cldn-7 and -12 expression. Immunobloting and densitometric analysis of Cldn-7 (A) and -12 (B) of control and 400 ng/ml omeprazole exposed cells. ***$P < 0.001$ vs control group, $n = 5$.

Discussion

Proton pump inhibitors (PPI) have been as therapeutics for acid-related gastrointestinal diseases. PPI binds and inhibits acid secretion from parietal cells of the gastric mucosa, thus allows mucosal ulcers and inflammation to heal. However, prolonged PPI usage in chronic acid-gastric diseases led to hypocalcaemia and hypomagnesaemia were probably caused by intestinal wasting (Epstein *et al.*, 2006; Cundy and Dissanayake, 2008; Shabajee *et al.*, 2008; Broeren *et al.*, 2009; Kuipers *et al.*, 2009; Hoorn *et al.*, 2010; Mackay and Bladon, 2010). While hypocalcaemia could be remedied by elemental Ca^{2+} supplement, hypomagnesaemia could be treated only by withdrawal of PPI (Epstein *et al.*, 2006; Shabajee *et al.*, 2008). On the other hand, short-term PPI administration had no effect on intestinal Ca^{2+} and Mg^{2+} absorption (Serfaty-Lacrosniere *et al.*, 1995; Hansen *et al.*, 2010). In the present study, we clearly showed that omeprazole had an inhibitory effect on the passive Mg^{2+} absorption (Figure 1), but with much less effect on passive Ca^{2+} absorption (Figure 3). Therefore, omeprazole possibly inhibited the function of paracellular Mg^{2+} channels that were proposed to be created by the tight junction associated claudins (Cldn) (Simon *et al.*, 1999; Tang and Goodenough, 2003). However the effect of omeprazole on the active Ca^{2+} transport could not be excluded by the present study.

Figure 7E) were similar to that of control monolayers at pH 7.4 (Figure 6D). In addition to the stimulatory effect on Mg^{2+} flux, apical acidity also abolished the effect of omeprazole and normalized Cldn-7 and -12 expression in 400 ng/ml omeprazole treated monolayers (Figure 8). These results indicated that apical acidity abolished the inhibitory effect of omeprazole on passive Mg^{2+} transport and Cldn expression.

Among the biological cations, Mg^{2+} is the most charge dense cation with high hydration energy of about -1,922 kj/mol versus -1,592 kj/mol of Ca^{2+}. Therefore, Mg^{2+} tightly coordinates with 6-7 H_2O molecules and has the highest hydration radius (Wolf and Cittadini, 2003; Wolf *et al.*, 2003). In order to permeate the transporting epithelium, Mg^{2+} must partially or completely be stripped of the bound hydration shell by its transporting protein (Moomaw and Maguire, 2008). The relationship between %maximum passive Mg^{2+} transport and Mg^{2+} concentration showed that omeprazole shifted the curve to the right and increased EC_{50} (Figure 2), indicating a lower affinity of the paracellular channel for Mg^{2+}. In addition, the electrical field within the paracellular pore could mediate the dehydration process and determine the charge selectivity (Yu *et al.*, 2009). Omeprazole was previously found to decrease the negative electric field strength and cation selectivity of the paracellular cation channels in Caco-2 monolayers (Thongon and Krishnamra, 2011). Moreover, the Arrhenius plot showed a higher activation energy for passive Mg^{2+} transport of the omeprazole-exposed monolayers (Thongon and Krishnamra, 2011). Therefore, it appeared that omeprazole inhibited the function of paracellular Mg^{2+} channels in the intestinal epithelium by decreasing Mg^{2+} affinity and disturbing the dehydration process.

Electrostatic interactions in the paracellular pore can determine conductivity of ion transport through the pore (Tang and Goodenough, 2003; Yu *et al.*, 2009). Therefore, changing the electrostatic interaction for instance, by lower apical pH markedly increases the paracellular ion permeability (Tang and Goodenough, 2003). In the gastrointestinal tract, luminal acidity present along the entire small intestine (Nugent *et al.*, 2001) was required for passive Mg^{2+} absorption (Heijnen *et al.*, 1993). In the present study, %maximum passive Mg^{2+} transport - Mg^{2+} concentration curves were shifted to the left (Figure 6C) and EC_{50} was decreased (Figure 6D) by apical acidic condition (pH 7.0-5.5), indicating enhanced paracellular passive Mg^{2+} absorption (Figure 6B). On the other hand, inhibition of proton pumps in Caco-2 cells (Abrahamse *et al.*, 1992) by omeprazole decreased apical acidity (Figure 4) and paracellular passive Mg^{2+} transport (Figure 1) across the Caco-2 monolayers. Therefore, omeprazole, by inhibiting gastric acid secretion, disturbed the luminal acidity and suppressed the intestinal Mg^{2+} absorption. In long-term omeprazole usage, continuous wasting of intestinal Mg^{2+} gradually depleted Mg^{2+} store in the human body, which then led to hypomagnesaemia. However, the inhibitory effect of omeprazole on the paracelluar passive Mg^{2+} transport, as well as Mg^{2+} affinity of the paracellular

channel, was normalized by physiological luminal acidic condition (Figure 7). Therefore, oral Mg^{2+} supplement using acid-containing capsule that released Mg^{2+} and acid in the distal small intestine probably resolved hypomagnesaemia in long-term omeprazole usage.

Extracellular CaSR were expressed along the entire length of the gastrointestinal tract (Geibel and Hebert, 2009) and also in Caco-2 monolayers (Davies *et al.*, 2008). CaSR played a role in gastric acid secretion, intestinal fluid absorption and secretion, and colonic fluid secretion (Geibel and Hebert, 2009). In the renal epithelium, CaSR activation suppressed the paracellular passive Mg^{2+} transport by controlling Cldn-16 function in the tight junction (Ikari *et al.*, 2008). In the present study, CaSR agonists neomycin and apermine were found to have no effect on passive Mg^{2+} transport in both control and omeprazole treated monolayers (Figure 4C). Our results agreed with a previous study that showed regulation of paracellular passive Ca^{2+} transport across Caco-2 monolayers by extracellular Ca^{2+} in a CaSR-independent mechanism (Davies *et al.*, 2008). Therefore, omeprazole was likely to inhibit the intestinal passive Mg^{2+} absorption in a CaSR-independent manner.

Among the 23 members of claudins, Cldn-16 had been proposed as the paracellular channel for Mg^{2+} in kidney (Simon *et al.*, 1999). However Cldn-16 was not detected along the intestinal tract (Fujita *et al.*, 2006), suggesting that other Cldn might be involved in passive Mg^{2+} absorption. Since, omeprazole suppressed paracellular passive Mg^{2+} transport as well as the expression of Cldn-7 and -12 in Caco-2 monolayers (Figure 5), thus, monomeric or heteromeric combination of Cldn-7 and -12 possibly contributed to the intestinal paracellular Mg^{2+} absorption. Cldn contained 4 trans-membrane domains, 1 intracellular loop, and 2 extracellular loops (Krause *et al.*, 2008). Paracellular channels were thought to be formed by the extracellular loop 1 (ECL1), negative or positive amino acid residues of which determined the charge selectivity (Yu *et al.*, 2009). In ECL1, Cldn-7 contained two negatively charged residues (positions 38, and 53) and Cldn12 contained four negatively charged residues (positions 62, 66, 71 and 74), thus, both were likely to act as paracellular cation transporting proteins (Alexandre *et al.*, 2005: Fujita *et al.*, 2006, 2008).

It is known that Cldn expression can be modulated by acid. In the esophageal reflux model, marked apical acidity (pH 2) was shown to suppress Cldn-4 expression in the squamous epithelial (Oshima *et al.*, 2012). Moreover, apical gastric acid exposure down-regulated Cldn-3 expression in the esophageal epithelium (Oguro *et al.*, 2011). Our

laboratory previously showed that chronic metabolic acidosis up-regulated mRNA expression of Cldn-2, -3, -6, -8, -11, -12, -14, -19 and -22 in the duodenal enterocytes (Charoenphandhu *et al.*, 2007). In bone lining cells, chronic metabolic acidosis also suppressed Cldn-14 expression but increased Cldn-15 and -16 expression (Wongdee *et al.*, 2010). In addition, chronic metabolic acidosis suppressed Cldn-2 expression in renal epithelium (Balkovetz *et al.*, 2009). Thus, it seemed that acid modulated Cldn expression in a tissue specific manner. In the present study, apical acidity within the physiological level i.e., pH 6.5 and 5.5 up-regulated Cldn-7 and -12 expression in Caco-2 cells (Figures 6E and 6F). Apical acidity also normalized Cld-7 and -12 expression in omeprazole-exposed Caco-2 cells (Figure 8). Since Cldn-7 and -12 were cation-permeable proteins (Alexandre *et al.*, 2005: Fujita *et al.*, 2008), an increased in paracellular passive Mg^{2+} absorption (Figures 6 and 7) possibly resulted from the up-regulation of Cldn-7 and -12 in apical acid-exposed epithelium. Therefore, luminal acidity probably exerted its effect on normal intestinal uptake of Mg^{2+} in Cldn-7 and -12 dependent mechanism.

Methods

Cell culture

Caco-2 cells (ATCC No. HTB-37) were grown as previously described (Thongon *et al.*, 2008). For ion flux studies, the Caco-2 monolayers were developed by seeding cells (1.0×10^6 cells/cm^2) onto the permeable polyester Transwell-clear inserts (Corning, Corning, NY) and maintained for 14 days. For western blot analysis, cells were plated (5.0×10^5 cells/well) on 6-well plate and maintained for 14 days. For MTT reduction assay, cells were seeded (1.0×10^4 cells/well) on 96-well plate (Corning) and maintained for the same period of time. In the omeprazole-treated group, the monolayers were grown in media containing 200, 400, or 600, ng/ml omeprazole (Calbiochem, San Diego, CA).

Measurements of Ca^{2+} and Mg^{2+} fluxes

Apical to basolateral paracellular Ca^{2+} and Mg^{2+} fluxes studies were performed at a humidified atmosphere with 5% CO_2. After removal of the culture media, the apical and basolateral side of Caco-2 monolayers on transwell were added with apical and basolateral solutions, respectively. At 1 and 2 h, 500 μl solution was collected from the basolateral side for measurement of Ca^{2+} or Mg^{2+} concentration. Fluxes and permeabilities of Ca^{2+} and Mg^{2+} across the Caco-2 monolayers were calculated as previously described (Thongon and Krishnamra, 2011). The relationship of Mg^{2+} transport and apical Mg^{2+} concentration was fitted with modified Michaelis-Menten equation plus linear component (Thongon and Krishnamra, 2011).

Bathing solution

For Ca^{2+} transport studies, the basolateral solution contained (in mM) 1 MgCl$_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 250 mannitol, and 10 HEPES pH 7.4; whereas, the apical solution contained (in mM) 1 MgCl$_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine and 10 HEPES pH 7.4, with either 2.5 CaCl$_2$ and 242 mannitol, 5 CaCl$_2$ and 235 mannitol, 10 CaCl$_2$ and 230 mannitol, 20 CaCl$_2$ and 200 mannitol, 40 CaCl$_2$ and 115 mannitol, or 80 CaCl$_2$ and 90 mannitol.

For Mg^{2+} transport studies, the basolateral solution was (in mM) 1.25 CaCl$_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 250 D-mannitol, and 10 HEPES pH 7.4; the apical solution was (in mM) 2.5 CaCl$_2$, 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.

All solutions with an osmolality of 290-295 mmol/kg H$_2$O were gassed with 5% CO_2 in 95% O_2 30 min prior to use and maintained at 37°C. In the pH dependent ion flux studies, HEPES of pH 7.4 in apical solution was 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).

Measurements of Ca^{2+} and Mg^{2+} concentrations

Mg^{2+} concentration was determined by Xylidyl Blue colorimetric assay (pH 10.5) as previously described (Thongon and Krishnamra, 2011). Ca^{2+} concentration was determined by Arsenazo III colorimetric assay (pH 6.5) according to the method of Tang and Goodenough (2003). All chemicals were purchased from Sigma.

MTT reduction assay

Caco-2 cells were washed with PBS and treated with 1 mg/ml MTT solution (sigma) for 3 h 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).

Trans-epithelial electrical resistance

Trans-epithelial electrical resistance (TEER) was determined as previously described (Thongon *et al.*, 2008).

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 rabbit

polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) raised against human claudin-2, -7, -12. Membranes were also reprobed with 1:5,000 anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology). After 2 h 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, IL) and captured on Hyperfilm[TM] (Amersham). Densitometric analysis was performed using ImageJ for Mac Os X (Rasband, 1997-2011).

Statistical analysis

Results were expressed as means $\pm$ 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. Linear regression was performed to obtain the passive Ca^{2+} transport - apical Ca^{2+} concentration relationship. 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.).

Acknowledgements

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