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ORIGINAL PAPER

The inhibitory role of purinergic P2Y receptor on Mg^{2+} transport across intestinal epithelium-like Caco-2 monolayer

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Abstract

The mechanism of proton pump inhibitors (PPIs) suppressing intestinal Mg^{2+} uptake is unknown. The present study aimed to investigate the role of purinergic P2Y receptors in the regulation of Mg^{2+} absorption in normal and omeprazole-treated intestinal epithelium-like Caco-2 monolayers. Omeprazole suppressed Mg^{2+} transport across Caco-2 monolayers. An agonist of the $P2Y_2$ receptor, but not the $P2Y_4$ or $P2Y_6$ receptor, suppressed Mg^{2+} transport across control and omeprazole-treated monolayers. Omeprazole enhanced $P2Y_2$ receptor expression in Caco-2 cells. Forskolin and $P2Y_2$ receptor agonist markedly enhanced apical HCO_3^- secretion by control and omeprazole-treated monolayers. The $P2Y_2$ receptor agonist suppressed Mg^{2+} transport and stimulated apical HCO_3^- secretion through the G_q-protein coupled-phospholipase C (PLC) dependent pathway. Antagonists of cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+-HCO_3^- cotransporter-1 (NBCe1) could nullify the inhibitory effect of $P2Y_2$ receptor agonist on Mg^{2+} transport across control and omeprazole-treated Caco-2 monolayers. Our results propose an inhibitory role of $P2Y_2$ on intestinal Mg^{2+} absorption.

Keywords Caco-2 monolayers · Intestinal HCO_3^- secretion · Mg^{2+} absorption · Proton pump inhibitor · $P2Y_2$ receptor

Introduction

Although there is an abundant amount of magnesium (Mg^{2+}) within human cells, and it has vital roles in numerous biological functions [1], knowledge regarding regulatory mechanisms of Mg^{2+} homeostasis is still minimal. Theoretically, plasma Mg^{2+} level is regulated within a narrow range by the synergistic actions of intestinal absorption, bone and soft tissue storage, and renal excretion [1]. Since dietary intake is the only source of Mg^{2+}, intestinal absorption is vital for normal Mg^{2+} homeostasis. However, the understanding of the regulatory mechanism of intestinal Mg^{2+} absorption is still elusive. Principally, enterocyte epithelium absorbs Mg^{2+} via both saturable transcellular and non-saturable paracellular transport [1]. Approximately 90% of total intestinal Mg^{2+} uptake is processed through a Mg^{2+} channel-independent paracellular passive mechanism which exclusively occurs in the small intestine [1–3]. The Mg^{2+} channel-dependent transcellular active Mg^{2+} uptake plays an important role during low dietary Mg^{2+} intake [3].

In the small intestine, enterocyte epithelial cells are equipped with acid sensors, e.g., acid-sensing ion channel (ASIC), ovarian cancer G protein-coupled receptor 1 (OGR1), and transient receptor potential vanilloid (TRPV) that are implicated in mucosal defense by detecting mucosal protons and stimulating mucosal HCO_3^- secretion [4–9]. In addition to this mucosal defense, intestinal acid sensors also regulate ion transport across the enterocyte epithelium. Reiter et al. [10] reported that TRPV4 enhanced transcellular K^+ transport and paracellular permeability through Ca^{2+} signaling in HC11 epithelial monolayers. OGR1 enhanced Mg^{2+} absorption in intestinal epithelium-like Caco-2 monolayers through protein kinase C (PKC) signaling [11, 12]. On the other hand, an activation of ASIC1a led to a suppression of Mg^{2+} absorption across Caco-2 monolayers through

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a Ca^{2+}-signaling dependent pathway [12]. In addition to acid sensors, the purinergic $P2Y_2$ receptor is also involved in mucosal acid sensing and defense of duodenocytes [8]. Activation of G_q-associated $P2Y_2$ stimulated duodenal mucosal HCO_3^- secretion in a Ca^{2+} signaling-dependent mechanism [4]. Besides HCO_3^- secretion, the $P2Y_2$ receptor also regulates Na^+, K^+, and Cl^- transport in various epithelial tissues [13]. However, the role of P2Y on intestinal Mg^{2+} absorption is still unknown.

PPI-induced hypomagnesemia (PPIH) has been reported since 2006 [14–19]. Suppression of intestinal Mg^{2+} absorption is a major underlying mechanism of PPIH in chronic users and animal models [14, 15, 17, 19–21]. We previously reported that a higher apical HCO_3^- secretion contributed to a suppression of intestinal Mg^{2+} absorption in PPIH [21]. Apical acidity in the small intestine [22] required for stabilizing mineral solubility [23] and stimulates intestinal Mg^{2+} absorption [11, 12, 24]. An increase in luminal pH from ~5 to 7.8 led to a decrease in soluble Mg^{2+} from 79.61 to 8.71% [25]; thus, secreted HCO_3^- increases luminal pH and subsequently suppresses Mg^{2+} absorption. Omeprazole, the most common PPI, significantly enhanced HCO_3^- secretion in human duodenum and intestinal epithelium-like Caco-2 monolayers [12, 26]. Antagonists of mucosal HCO_3^- secretion markedly increased duodenal Mg^{2+} absorption in PPIH rats [21]. $P2Y_2$ regulated mucosal HCO_3^- secretion [4], but the involvement of $P2Y_2$ on apical HCO_3^- secretion and Mg^{2+} absorption in PPI-treated intestinal epithelium is unknown.

Therefore, the present study aimed to investigate the role of purinergic P2Y receptors in the regulation of Mg^{2+} absorption in normal and omeprazole-treated intestinal-like Caco-2 monolayers. Caco-2 cells express P2Y receptors, i.e., $P2Y_2$, $P2Y_4$, and $P2Y_6$ [27, 28]. They are also equipped with apical HCO_3^- secretion transporting machineries, e.g., cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+-HCO_3^- cotransporter-1 (NBCe1), which are modulated by parathyroid hormone (PTH) and HCl [12, 29]. In addition, Caco-2 monolayers have been used as a model for studying the regulation of intestinal Mg^{2+} absorption [11, 12, 30, 31].

Methods

Cell culture

Caco-2 cells (ATCC No. HTB-37, passage 25–40th) were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 12.5% 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 in a humidified atmosphere containing 5% CO_2 at 37 °C. Culture medium was changed 3 times a week. For epithelial electrical parameter measurement and Mg^{2+} flux studies, the monolayers were developed by seeding cells (5.0×10^5 cells/cm^2) onto permeable polyester Snapwell™ inserts (12 mm diameter and 0.4 μm pore size filter) (Corning, Corning, NY, USA). After being maintained for 14 days, the Snapwell was inserted into a Ussing chamber (World Precision Instrument, Sarasota, FL, USA). For HCO_3^- secretion studies, the cells were plated (5.0×10^5 cells/cm^2) onto permeable polyester Transwell-clear inserts (Corning) and maintained for 14 days. For western blot analysis, cells were plated (5.0×10^5 cells/well) on 6-well plates (Corning) and maintained for 14 days.

In the omeprazole-treated group, Caco-2 monolayers were grown in a culture medium containing omeprazole (Calbiochem, San Diego, CA, USA), from day 7 to day 14 of culture [31], at concentrations of 200 and 400 ng/ml that resembled those found in human plasma [32].

Bathing solutions

The physiological bathing solution contained (in mM) 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 mannitol.

For the apical to basolateral total 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, whereas the basolateral solution contained (in mM) 1.25 $CaCl_2$, 4.5 KCl, 12 D-glucose, 2.5 L-glutamine, 250 D-mannitol, and 10 HEPES.

For apical HCO_3^- secretion experiments, the composition of the $NaHCO_3$-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; 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.

All solutions were continuously gassed with humidified 5% CO_2 in 95% O_2, maintained at 37 °C, pH 7.4, and had an osmolality of 290–295 mmol kg^{-1} water as measured by a freezing-point depression-based Fiske® micro-osmometer (model 210; Fiske® Associates, Norwood, MA, USA). All chemicals were purchased from Sigma.

Transepithelial electrical resistance

Snapwell™ inserts containing Caco-2 monolayers were rinsed gently, mounted in a Ussing chamber, and bathed on both sides with physiological bathing solution. Transepithelial potential difference (PD) and short-circuit current (*Isc*) were determined by Ag/AgCl electrodes and an epithelial

voltage/current clamp apparatus (model ECV-4000; World Precision Instrument) as previously described [33]. Transepithelial electrical resistance (TEER) was calculated from PD and *I*sc by Ohm's law.

Mg^{2+} transport study

Caco-2 monolayers were rinsed and mounted in a Ussing chamber as described above. After being equilibrated in physiological bathing solution for 15 min, total Mg^{2+} transport studies were performed by substituting the physiological bathing solution with apical and basolateral bathing solutions for Mg^{2+} transport. To investigate the Mg^{2+} channel-independent Mg^{2+} transport, apical sites of Caco-2 monolayers from the same passage and culture plate were pre-incubated for 10 min with Mg^{2+}-channel inhibitor Cobalt(III)hexaammine [Co(III)hex, Table 1], which suppressed Mg^{2+} influx in Caco-2 epithelium and blocked Mg^{2+} channel-dependent Mg^{2+} transport. After that the apical and basolateral solutions were substituted with bathing solution for Mg^{2+} transport. At 30, 60, and 120 min after solution replacements, 50 µl of solution was collected from the basolateral side, as well as from the apical side. The Mg^{2+} concentration and the rate of Mg^{2+} flux were determined by the

Table 1 Agonist, antagonist, or chelator used in the study of Mg^{2+} transport and HCO_3^- secretion across Caco-2 monolayers

Target	Common name	Full name	Concentration used	Manufacturer
Agonist				
Adenylyl cyclase	Forskolin	7*β*-Acetoxy-8,13-epoxy-1*α*,6*β*,9*α*-trihydroxylabd-14-en-11-one	10 µmol/l	Sigma
$P2Y_2$ receptor	MRS2768	Uridine-5′-tetraphosphate δ-phenyl ester tetrasodium salt	10 µmol/l	Tocris
$P2Y_4$ receptor	MRS4062	N^4-Phenylpropoxycytidine-5′-*O*-triphosphate tetra(triethylammonium) salt	100 nmol/l	Tocris
$P2Y_6$ receptor	MRS2693	5-Iodouridine-5′-*O*-diphosphate trisodium salt	100 nmol/l	Tocris
PKC	Carbachol	(2-Hydroxyethyl)trimethylammonium chloride carbamate	500 µmol/l	Sigma
Antagonist/chelator				
Mg^{2+} channel	Co(III)hex	Cobalt(III)hexaammine	1 mmol/l	Sigma
$P2Y_2$ receptor	Suramin	8,8′-{Carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene) carbonylimino]}bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt	150 µmol/l	Sigma
PLC	U-73122	1-{6-[(17*β*-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino] hexyl}-1*H*-pyrrole-2,5-dione	10 µmol/l	Calbiochem
PKC	Gö 6850	2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide	1 µmol/l	Calbiochem
MEK1/2	U-0126	1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene	10 µmol/l	Calbiochem
PI3K	LY-294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	75 µmol/l	Calbiochem
PKA	H89	*N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide	30 µmol/l	Calbiochem
Intracellular Ca^{2+}	BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)	50 µmol/l	Calbiochem
IP_3 receptor	2-APB	2-Aminoethoxydiphenylborane	100 µmol/l	Calbiochem
Voltage-gated Ca^{2+} channel	Nifedipine	1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester	10 µmol/l	Sigma
Membrane-bound CA IX	U-104	4-[[[(4-Fluorophenyl)amino]carbonyl]amino]-benzenesulfonamide	45 nmol/l	Sigma
CFTR	GlyH-101	*N*-2-Naphthalenyl-2-[(3,5-dibromo-2,4-dihydroxyphenyl) methylene]glycine hydrazide	50 µmol/l	Calbiochem
NBCe1	DIDS	4,4′-Diisothiocyanatostilbene-2,2′-disulfonate	500 µmol/l	Sigma
Ca^{2+}-activated K^+ channel	ChTX	Charybdotoxin	100 nmol/l	Tocris
Ca^{2+}-activated Cl^- channel	Benzbromarone	3-(3,5-Dibromo-4-hydroxybenzoyl)-2-ethylbenzofuran	20 µmol/l	Sigma
CA	Methazolamide	*N*-(4-Methyl-2-sulfamoyl-Δ^2-1,3,4-thiadiazolin-5-ylidene) acetamide	1 mmol/l	Sigma

Calbiochem, San Diego, CA; Sigma, St. Louis, MO, USA; Tocris, Tocris Bioscience, Bristol, UK

CA carbonic anhydrase, *CFTR* cystic fibrosis transmembrane conductance regulator, *MEK* mitogen-activated protein kinase, *NBCe1* Na^+-HCO_3^- cotransporter-1, *PKA* protein kinase A, *PKC* protein kinase C, *PLC* phospholipase C, *PI3K* phosphoinositide 3-kinase, *PLC* phospholipase C

method of Thongon and Krishmanra [11]. The rate of Mg^{2+} channel-dependent Mg^{2+} transport was calculated by subtracting the rate of Mg^{2+} channel-independent Mg^{2+} transport from the rate of total Mg^{2+} transport. However, Co(III) hex might somehow interfered Mg^{2+} channel-independent paracellular Mg^{2+} transport, which was not demonstrated in the present study.

In some experiments the monolayers were pre-incubated with agonists or antagonist, as demonstrated in Table 1, for 40 min prior to performing experiments.

Measurements of HCO_3^- secretion

Apical HCO_3^- secretion was studied by the modified method of Thongon et al. [12]. The Caco-2 monolayer was gently rinsed 3 times and incubated for 15 min in the physiological bathing solution. Then, apical and basolateral solutions were substituted with bathing solutions for HCO_3^- secretion. The apical membrane-bound carbonic anhydrase (CA) activity was suppressed by the selective CA IX inhibitor U-104 (Table 1). After 20 min, HCO_3^- secretion was stimulated by adding MRS2768 or forskolin (Table 1) and incubation proceeded for 5 min. After removal of the MRS2768- or forskolin-containing solutions, the monolayers were gently rinsed 3 times and further incubated for 25 min. Aliquots of apical solution at various time points (Fig. 5) were individually sampled. The concentration of HCO_3^- was immediately determined as previously described [12].

In some experiments the monolayers were pre-incubated with agonists or antagonist, as demonstrated in Table 1, for 40 min prior to performing experiments.

Western blot analysis

Western blot analysis was performed as previously described [11]. In brief, protein samples of Caco-2 cells were prepared by using Piece® Ripa Buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein samples (35 µg each) were separated on 12.5% SDS-PAGE gels, and then transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) by electroblotting. Membranes were blocked and probed overnight at 4 °C with 1:1000 rabbit polyclonal antibodies raised against human $P2Y_2$ receptor and CFTR (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were also re-probed with actin monoclonal antibodies (Santa Cruz Biotechnology) diluted at 1:5000. After 2 h incubation at 25 °C with goat anti-rabbit IgG-HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted at 1:10,000, blots were 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.

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 (ANOVA) 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 Inc., San Diego, CA, USA).

Results

Omeprazole modulated paracellular Mg^{2+} transport

Previous Mg^{2+} transport kinetic analysis demonstrated that omeprazole selectively impeded non-saturable passive Mg^{2+} transport but not saturable active Mg^{2+} transport across Caco-2 monolayers [31]. By using competitive Mg^{2+}-channel inhibitor Co(III)hex (Fig. 1), we observed total (white bars), Mg^{2+} channel-independent (gray bars), and Mg^{2+} channel-dependent Mg^{2+} transport (black bars). The results showed that 200 and 400 ng/ml omeprazole significantly suppressed total (Fig. 1d) and Mg^{2+} channel-independent Mg^{2+} transport (Fig. 1e) compared to the control group. Furthermore, we had studied the effect of omeprazole on Mg^{2+} channel-independent Mg^{2+} transport. Previously, it was reported that protein kinase C activator carbachol (CCh) could increase paracellular permeability and decrease TEER in Caco-2 monolayers [34, 35]. In control (Fig. 1a), 200-ng/ml omeprazole-treated group (Fig. 1b), and 400-ng/ml omeprazole-treated group (Fig. 1c), CCh significantly increased total and Mg^{2+} channel-independent Mg^{2+} transport compared to the corresponding vehicle-treated group. The rates of Mg^+ channel-dependent Mg^{2+} transport of all experiments were not changed (Fig. 1a–c, f).

We also studied TEER to confirm the permeability of Caco-2 monolayers. As demonstrated in Fig. 2c, Co(III)hex, which impeded Mg^{2+} channel-dependent Mg^{2+} transport, had no effect on TEER of control and omeprazole-treated monolayers when compared to its corresponding vehicle-treated monolayers. On the other hand CCh, which increased Mg^{2+} channel-independent Mg^{2+} transport, significantly decreased TEER of control and omeprazole-treated (Fig. 2c) Caco-2 monolayers. We further observed the involvement of Ca^{2+}-activated K^+ and Ca^{2+}-activated Cl^- channels on CCh-suppressed TEER by using charybdotoxin (ChTX) and benzbromarone. ChTX and benzbromarone had no effect on CCh-suppressed TEER of control and omeprazole-treated Caco-2 monolayers. However, CCh might somehow modulate some ion channels or transports, which could affect TEER of Caco-2 monolayers. TEER of 400-ng/ml omeprazole-treated monolayers (458.56 ± 14.97 Ω cm^2) was significantly higher than that of vehicle-treated control monolayers

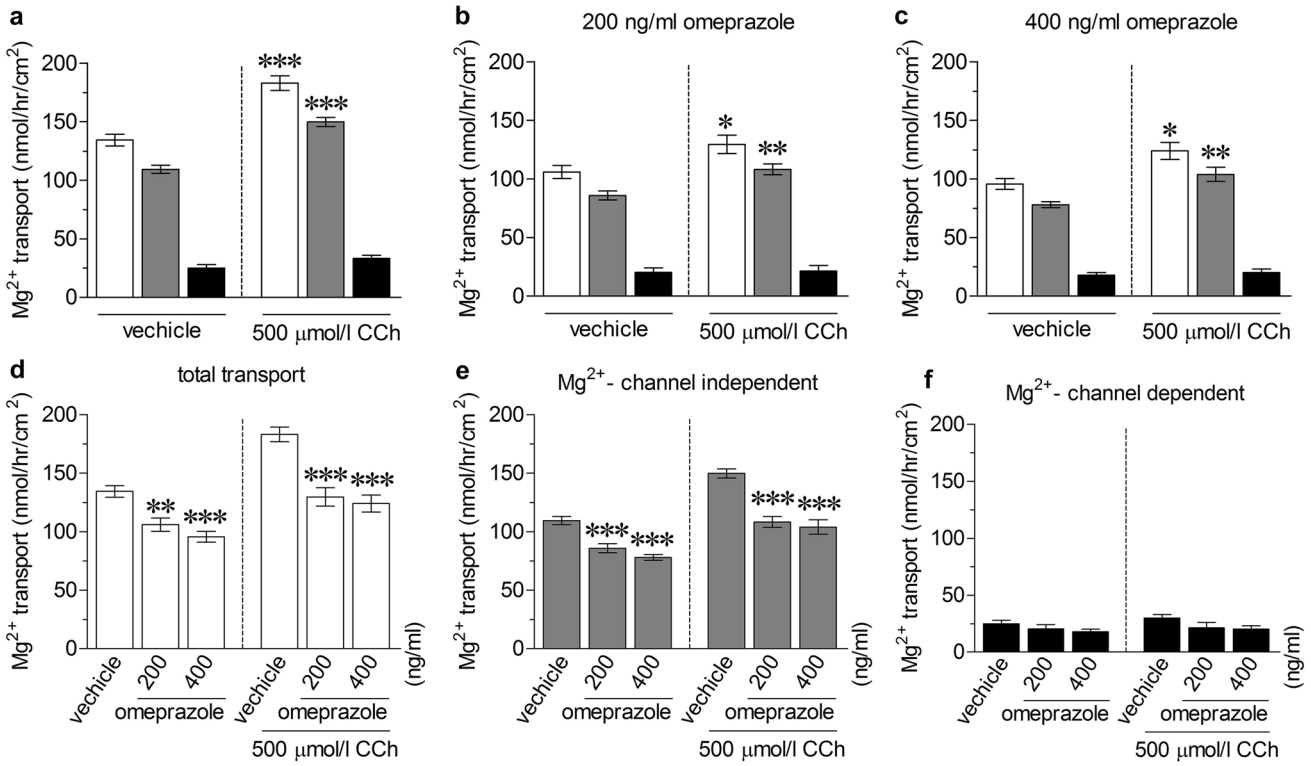

Fig. 1 Effect of omeprazole on Mg^{2+} transport across Caco-2 monolayers. The rate of Mg^{2+} transport across control (**a**), 200-ng/ml omeprazole-treated (**b**), and 400-ng/ml omeprazole-treated Caco-2 monolayers (**c**). White bar; total Mg^{2+} transport, gray bar; Mg^{2+} channel-independent Mg^{2+} transport, *black bar*; Mg^{2+} channel-dependent Mg^{2+} transport. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with its corresponding CCh-untreated group. ($n=6$). The rate of total (**d**), Mg^{2+}-channel independent (**e**), and Mg^{2+}-channel dependent (**f**) Mg^{2+} transport of control and omeprazole-treated Caco-2 monolayers. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with its corresponding control group ($n=6$)

($362.31 \pm 17.69\ \Omega\ cm^2$, $P=0.0003$) (Fig. 2c). This series of experiments suggested that alteration of total Mg^{2+} transport across Caco-2 monolayers was the result of the modulation of Mg^{2+} channel-independent Mg^{2+} transport. These results also agreed with a previous study [31] that omeprazole exclusively suppressed nonsaturable passive Mg^{2+} transport in intestinal epithelium-like Caco-2 monolayers. In addition, TEER could determine the change of Mg^{2+} channel-independent Mg^{2+} transport, but not Mg^{2+} channel-dependent Mg^{2+} transport. Thus, Mg^{2+} channel-dependent Mg^{2+} transport was ignored in the rest of the experiments.

$P2Y_2$ receptor modulated Mg^{2+} transport

To observe the roles of $P2Y_2$, $P2Y_4$, and $P2Y_6$ activities on Mg^{2+} transport across Caco-2 monolayers we incubated the monolayers with selective agonists of $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors (Table 1). In control monolayers (Fig. 3a) the rate of Mg^{2+} transport (in nmol/h/cm^2) of the $P2Y_2$ agonist-treated group (81.14 ± 3.45), but not $P2Y_4$ or $P2Y_6$ agonist-treated groups, was significantly lower than that of the vehicle-treated group (138.89 ± 4.85). As demonstrated in Fig. 3b, the rate of Mg^{2+} transport (in nmol/h/cm^2) of the $P2Y_2$ agonist-treated group (41.94 ± 5.91) was significantly lower than that of vehicle-treated group (106.74 ± 5.12) in the 200-ng/ml omeprazole-treated condition. In 400-ng/ml omeprazole-treated monolayers (Fig. 3c), Mg^{2+} transport (in nmol/h/cm^2) of the $P2Y_2$ agonist-treated group (35.28 ± 2.72) was also significantly suppressed compared to the vehicle-treated group (95.24 ± 4.96). When compared to the corresponding vehicle-treated group, $P2Y_2$ agonist suppressed the rate of Mg^{2+} transport by about 41.58, 60.71, and 62.96% of control, in the 200-ng/ml omeprazole-treated, and 400-ng/ml omeprazole-treated monolayers, respectively. In addition, TEER of MRS2768 treated monolayers was significantly higher than that of the corresponding vehicle-treated control (Fig. 2a) or omeprazole-treated monolayers (Fig. 2b). We further performed a western blotting study to confirm the enhancing effect of omeprazole on $P2Y_2$ receptor activation in suppressing intestinal Mg^{2+} absorption. As demonstrated in Fig. 3d, 200 and 400 ng/ml omeprazole significantly enhanced $P2Y_2$ expression when compared to the control cells.

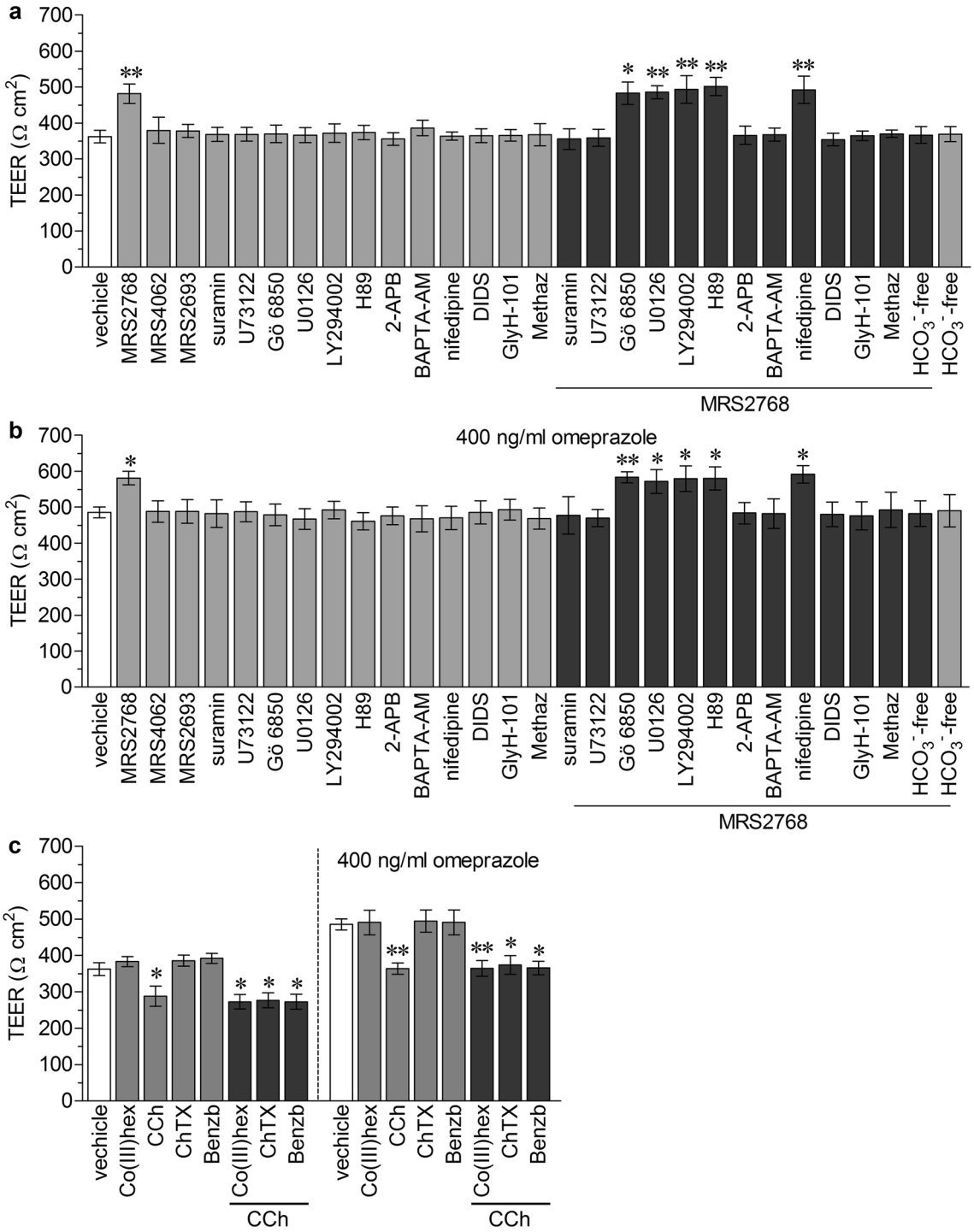

Fig. 2 Transepithelial electrical resistance (TEER). TEER of control and 400-ng/ml omeprazole-treated Caco-2 monolayers in various experiments. $*P<0.05$, $**P<0.01$ compared with its corresponding vehicle-treated group ($n=6$). *Benzb* benzbromarone

Signaling pathway of $P2Y_2$ receptor activation suppressed Mg^{2+} transport

This series of experiments aimed to observe the underlying mechanism by which $P2Y_2$ receptor activation mediated the suppression of intestinal Mg^{2+} absorption. In control (Fig. 4a) and omeprazole-treated Caco-2 monolayers (Fig. 4b), MRS2768 significantly suppressed the rate of total Mg^{2+} transport. $P2Y_2$ receptor antagonist, PLC antagonist, IP_3 receptor antagonist, and intracellular Ca^{2+}

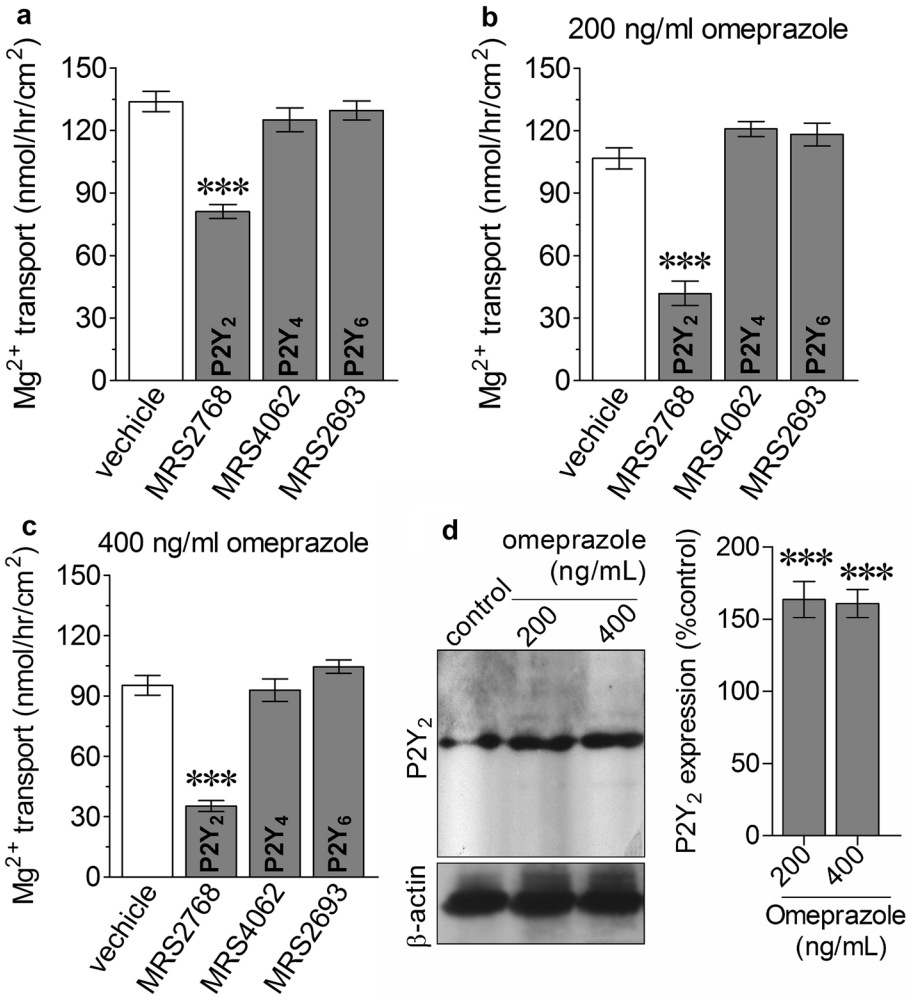

Fig. 3 The effect of P2Y receptor agonists on Mg^{2+} transport across Caco-2 monolayers. The rate of Mg^{2+} transport across control (**a**), 200-ng/ml omeprazole-treated (**b**), and 400-ng/ml omeprazole-treated Caco-2 monolayers (**c**) with agonist of $P2Y_2$ receptor MRS2768, $P2Y_4$ receptor MRS4062, and $P2Y_6$ receptor MRS2693 pre-incubations. Representative immunoblotting and densitometric analysis of $P2Y_2$ expression in control and omeprazole-treated Caco-2 cells (**d**). $^{***}P<0.001$ compared with its vehicle-treated group ($n=6$)

chelator markedly normalized the effect of MRS2768 on Mg^{2+} transport. However, the antagonist of PKC, MEK1/2, PI3K, PKA, or voltage-gated Ca^{2+} channel had no effect on MRS2768-suppressed Mg^{2+} transport in either control or omeprazole-treated Caco-2 monolayers (Fig. 4a–b). In the TEER study, $P2Y_2$ receptor antagonist, PLC antagonist, IP_3 receptor antagonist, and intracellular Ca^{2+} chelator also normalized the effect of MRS2768-increased TEER of control (Fig. 2a) and omeprazole-treated (Fig. 2b) monolayers. These results suggested that $P2Y_2$ receptor activation mediated the suppression of intestinal Mg^{2+} absorption through PLC, IP_3 receptor, and intracellular Ca^{2+} signaling pathway.

Contribution of HCO_3^- secretion on $P2Y_2$ receptor activation suppressed Mg^{2+} transport

Previously, we reported the contribution of mucosal HCO_3^- secretion on omeprazole-suppressed duodenal Mg^{2+} absorption in PPIH rats [21]. Then, we further studied the contribution of HCO_3^- secretion on $P2Y_2$ receptor activation-suppressed Mg^{2+} transport in Caco-2 monolayers. As demonstrated in Fig. 5a and b, $P2Y_2$ agonist significantly suppressed Mg^{2+} transport in control and omeprazole-treated monolayers. The antagonist of NBCe1, CFTR, and CA could relieve the inhibitory effect of $P2Y_2$ activation on Mg^{2+} transport across control and omeprazole-treated monolayers (Fig. 5a–b). By using HCO_3^--free bathing solution in both apical and basolateral sites, the $P2Y_2$ agonist had no effect on Mg^{2+} transport in normal Caco-2 monolayers (Fig. 5a). In omeprazole-treated monolayers (Fig. 5b), the HCO_3^--free condition significantly increased Mg^{2+} transport when compared to its corresponding vehicle-treated group. Under the HCO_3^--free condition, the $P2Y_2$ agonist also had no effect on Mg^{2+} transport in omeprazole-treated Caco-2 monolayers (Fig. 5b). Moreover, the antagonist of CFTR, CA, and NBCe1 also normalized the effect of $P2Y_2$ activation-increased TEER of control (Fig. 2a) and omeprazole-treated (Fig. 2b) Caco-2 monolayers. Therefore, apical HCO_3^- secretion was involved in $P2Y_2$ receptor activation-suppressed Mg^{2+} transport.

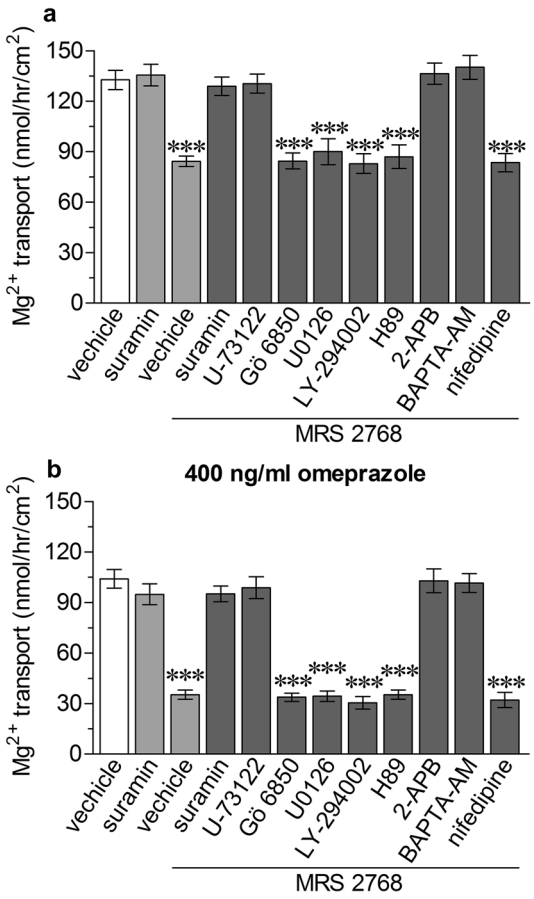

Fig. 4 The signaling pathway of P2Y receptor activation suppressed Mg^{2+} transport across Caco-2 monolayers. The rate of Mg^{2+} transport across control (**a**) and 400-ng/ml omeprazole-treated Caco-2 monolayers (**b**) with agonist or antagonist pre-incubations. ***$P<0.001$ compared with its vehicle-treated group ($n=6$)

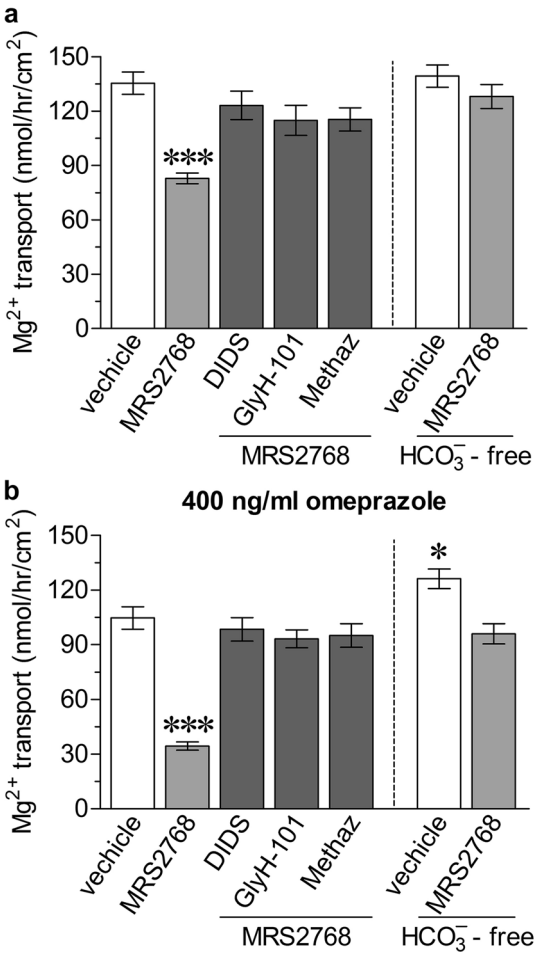

Fig. 5 Contribution of HCO_3^- secretion on P2Y receptor activation suppressed Mg^{2+} transport across Caco-2 monolayers. The rate of Mg^{2+} transport across control (**a**), and 400-ng/ml omeprazole-treated Caco-2 monolayers (**b**) with agonist or antagonist pre-incubations. *$P<0.05$, ***$P<0.001$ compared with its vehicle-treated group ($n=6$)

$P2Y_2$ receptor activation stimulated HCO_3^- secretion

Previously, it was reported that $P2Y_2$ stimulated mucosal HCO_3^- secretion in rat duodenum [4]. The present study observed the effect of $P2Y_2$ receptor activation on apical HCO_3^- secretion in Caco-2 monolayers. Since forskolin stimulated duodenum HCO_3^- secretion in mice [36] and apical HCO_3^- secretion in Caco-2 monolayers [29], we used forskolin as positive control for the stimulation of apical HCO_3^- secretion. Our results showed that $P2Y_2$ agonist MRS2768 and forskolin significantly increased the rate of apical HCO_3^- secretion by control (Fig. 6a, b), 200-ng/ml omeprazole-treated (Fig. 6c, d), and 400-ng/ml omeprazole-treated (Fig. 6e, f) monolayers. Under the HCO_3^--free condition, the rate of basal, forskolin-stimulated, and MRS2768-stimulated HCO_3^- secretions were suppressed in control and omeprazole-treated Caco-2 monolayers (Fig. 6b, d, f). In the pre-stimulating condition (Fig. 6g), the basal HCO_3^- secretions (in μmol/h/cm^2) of 200-ng/ml omeprazole-treated (4.45 ± 0.46) and 400-ng/ml omeprazole-treated (5.19 ± 0.49) monolayers were significantly higher than that of the control monolayers (2.49 ± 0.41). The rate of peak HCO_3^- secretions (in μmol/h/cm^2) in forskolin-stimulated and MRS2768-stimulated conditions (Fig. 6h) of 200-ng/ml omeprazole-treated (12.48 ± 0.46 and 13.01 ± 0.57, respectively) and 400-ng/ml omeprazole-treated (14.12 ± 0.44 and 14.59 ± 0.48, respectively) monolayers were significantly higher than those of the control monolayers (9.16 ± 0.68 and 8.66 ± 0.57, respectively). We further observed the expression of CFTR protein in omeprazole-treated monolayers. As demonstrated in Fig. 7a, omeprazole had no effect on CFTR protein expression in Caco-2 monolayers.

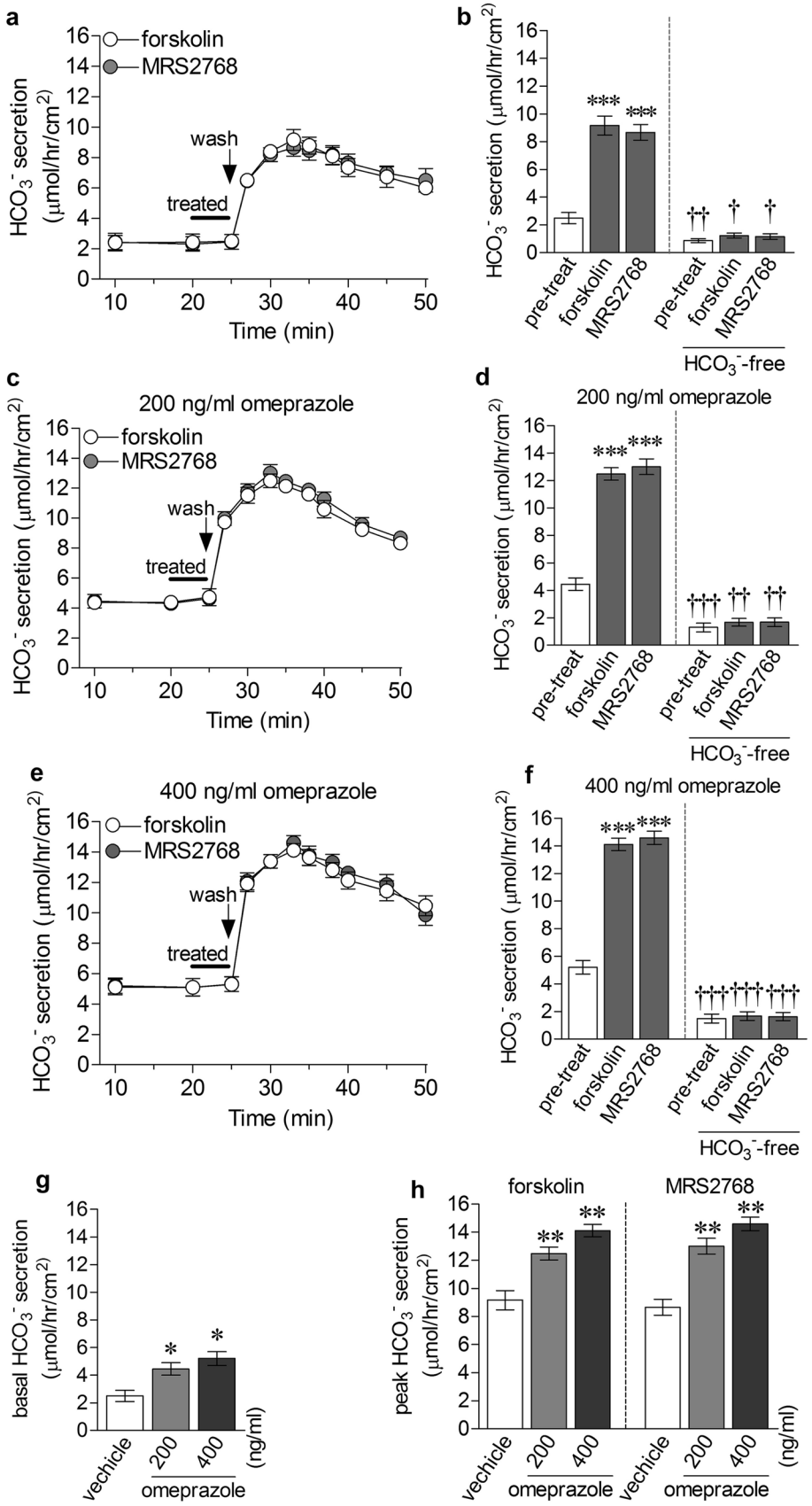

Fig. 6 $P2Y_2$ receptor agonist stimulated HCO_3^- secretion. Time course and rate of apical HCO_3^- secretion by control (**a**, **b**), 200-ng/ml omeprazole-treated (**c**, **d**), and 400-ng/ml omeprazole-treated Caco-2 monolayers (**e**, **f**) that were induced by forskolin or MRS2768. Basal (at 20 min; **g**) and peak forskolin- or MRS2768-stimulated HCO_3^- secretion (at 33 min; **h**) by control or omeprazole-exposed Caco-2 monolayers. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the corresponding control group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared with the pre-treated control group ($n=6$)

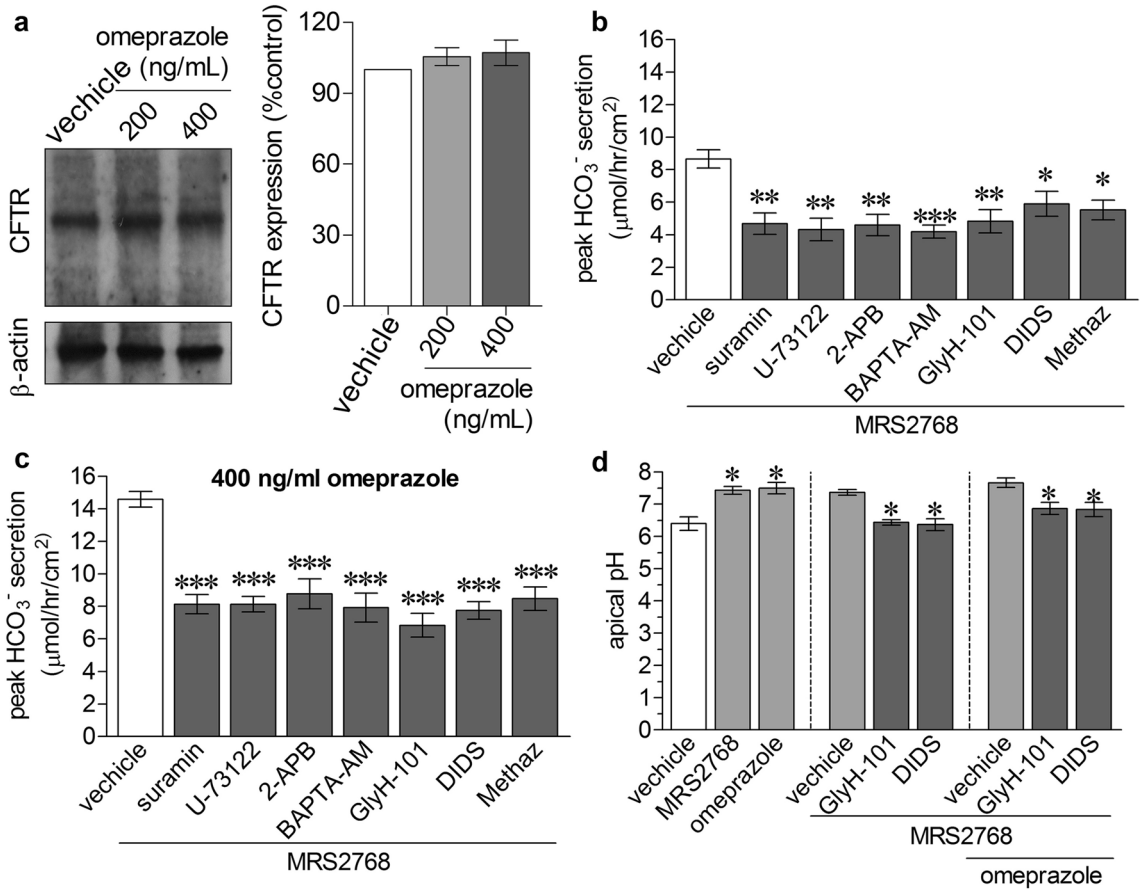

Fig. 7 The signaling pathway of $P2Y_2$ receptor agonist stimulated HCO_3^- secretion. Representative immunoblotting and densitometric analysis of CFTR expression in control and omeprazole-treated Caco-2 cells (**a**). The rate of peak MRS2768-stimulated HCO_3^- secretion by control (**b**), and 400-ng/ml omeprazole-treated Caco-2 monolayers (**c**) that were induced by agonist or antagonist. The pH of apical culture media of Caco-2 monolayers at 24 h after culture media change (**d**). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the corresponding vehicle group ($n=6$)

Signaling pathway of $P2Y_2$ receptor activation stimulated HCO_3^- secretion

Previously, it was found that $P2Y_2$ activation enhanced mucosal HCO_3^- secretion through PLC, IP_3 receptor, and intracellular Ca^{2+} signaling pathway [4]. This series of experiments, therefore, showed the underlying signaling transduction pathway of $P2Y_2$ receptor activation that mediated the stimulation of apical HCO_3^- secretion in Caco-2 monolayers. In control (Fig. 7b) and omeprazole-treated monolayers (Fig. 7c), the rate of peak MRS2768-stimulated HCO_3^- secretion was significantly suppressed in the monolayers treated with the antagonist of $P2Y_2$ receptor, PLC, PI3K receptor, CFTR, NBCe1, and CA, as well as intracellular Ca^{2+} chelator. These results showed that $P2Y_2$ receptor activation mediated the stimulation of HCO_3^- secretion through PLC, IP_3 receptor, and intracellular Ca^{2+} signaling pathway. We further observed the pH of apical culture media of Caco-2 monolayers at 24 h after culture media change. MRS2768 and 400 ng/ml omeprazole significantly increased apical pH compared to vehicle treated group (Fig. 7d). CFTR and NBCe1 antagonists significantly abolished MRS2768 and omeprazole effects on apical pH.

Discussion

Intestinal Mg^{2+} absorption can be processed through saturable transcellular and non-saturable paracellular mechanisms. 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 [1, 37–39] or other transport pathways. Paracellular Mg^{2+} transport is a passive mechanism modulated by the tight junction associated Claudin (Cldn) [11, 40]. However, the regulatory mechanism of intestinal Mg^{2+} absorption is largely unknown.

Our previous study demonstrated that intestinal-associated proton sensors ASIC1a and OGR1 could modulate intestinal-like Mg^{2+} transport across Caco-2 monolayers [12]. OGR1 activation increased Mg^{2+} transport across Caco-2 monolayers while ASIC1a activation decreased it. In the present study we focused on the role of the purinergic $P2Y_1$ receptor family, which was regulating trans-epithelial Na^+, K^+, and Cl^- transport [13], on Mg^{2+} transport across Caco-2 monolayers. The G_q-coupled $P2Y_1$ receptor family is composed of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors. However, Caco-2 monolayers expressed $P2Y_2$, $P2Y_4$, and $P2Y_6$ [27], which are exclusively localized in the apical membrane [28]. Our results suggested that only $P2Y_2$ was involved in the modulation of Mg^{2+} transport across Caco-2 monolayers. Generally, $P2Y_2$ receptors regulate epithelial ion transport through G_q-dependent pathways which activate PLC and stimulate intracellular Ca^{2+} mobilization [4, 13]. In agreement with our results, $P2Y_2$ receptor activation suppressed Mg^{2+} transport through PLC, IP_3 receptor, and the intracellular Ca^{2+} signaling pathway. Li et al. [41] reported the inhibitory role of $P2Y_2$ receptor on Cldn-1 expression. Purinergic P2Y receptor agonist adenosine triphosphate (ATP) rapidly suppressed epithelial paracellular permeability and increased epithelial TEER [42]. In addition, purinergic P2 receptor agonist also suppressed the activity of TRPM6 and TRPM7 [43, 44]. However, the role of $P2Y_2$ on Cldn, TRPM6, and TRPM7 expressions and functions required further studies.

Duodenal mucosal bicarbonate secretion (DMBS) is the critical process of duodenal defense against intermittent duodenal epithelial exposure to a luminal acidic environment (pH < 2). Luminal H^+ is the potent activator of DMBS by stimulating a duodenal associated acid sensor, e.g., ASIC1a [5]. In addition, luminal uridine triphosphate, a $P2Y_2$ agonist, also stimulates DMBS [4]. Previously, apical HCO_3^- secretion had been observed in Caco-2 monolayers [12, 29]. Laohapitakworn et al. [29] reported that PTH rapidly stimulated CFTR-, CA-, and NBCe1-mediated apical HCO_3^- secretion in Caco-2 monolayers. Our group demonstrated that activation of ASIC1a stimulated an apical HCO_3^- secretion CFTR-dependent mechanism [12]. In the present study we reported the activation of $P2Y_2$ receptor stimulating CFTR-, CA-, and NBCe1-mediated apical HCO_3^- secretion in Caco-2 monolayers. Our results agreed with the finding of a previous report [4] that showed $P2Y_2$ receptor activation mediated the stimulation of intestinal HCO_3^- secretion through PLC, IP_3 receptor, and the intracellular Ca^{2+} signaling pathway.

The underlying mechanism of PPI-suppressed intestinal Mg^{2+} uptake is still unclear. A previous mathematically simulated study suggested that only a 1% reduction of intestinal Mg^{2+} absorption could induce 80% Mg^{2+} depletion within 1 year of PPIs used [45]. Previous studies proposed that PPIs mainly affected colonic Mg^{2+} absorption in PPIH mice [2, 20]. Our group reported that PPIs impeded duodenal Mg^{2+} absorption in PPIH rats [21]. We hypothesized that a higher luminal HCO_3^- secretion could lead to a suppression of small intestinal Mg^{2+} absorption in PPIH [12, 21]. Omeprazole markedly enhanced HCO_3^- secretion in human duodenum and intestinal epithelium-like Caco-2 monolayers [12, 26]. Secreted HCO_3^- increased luminal pH and probably decreased Mg^{2+} solubility, since luminal soluble Mg^{2+} decreased from 79.61 to 8.71% when luminal pH increased from ~5 to 7.8 [25]. Therefore, antagonists of CFTR and NBCe1 significantly increased duodenal Mg^{2+} absorption in PPIH rats [21]. These findings agreed with the present study that omeprazole induced basal and peak-stimulated HCO_3^- secretion. Interestingly, in the HCO_3^--free condition the rate of Mg^{2+} transport markedly increased in omeprazole-treated Caco-2 monolayers, suggesting the inhibitory role of secreted HCO_3^- on intestinal Mg^{2+} absorption. However, luminal Mg^{2+} solubility and precipitation in the PPI-treated animal model requires further study.

The present study showed higher $P2Y_2$ expression in omeprazole-treated Caco-2 monolayers. Thus, $P2Y_2$-activated HCO_3^- secretion was also significantly higher in omeprazole-treated monolayers. These results explained the higher degree of suppression of Mg^{2+} absorption in $P2Y_2$-activated 200-ng/ml omeprazole-treated (60.71%) and 400-ng/ml omeprazole-treated monolayers (62.96%) in comparison to control monolayers (41.58%). Moreover, $P2Y_2$ activation enhanced HCO_3^- secretion and suppressed Mg^{2+} absorption were also mediated by PLC, IP_3 receptor, intracellular Ca^{2+} mobilization, CFTR, CA, and NBCe1. In addition to higher HCO_3^- secretion, another possible mechanism of omeprazole-suppressed Mg^{2+} absorption is phosphatidylinositol 4,5-bisphosphate (PIP_2)-mediated TRPM6 function. Since TRPM6 function required an interaction with membrane-associated PIP_2, hydrolysis of PIP_2 through activation of the G_q-protein coupled PLC-dependent pathway fully inactivated TRPM6 channels [46]. The higher G_q-associated $P2Y_2$ expression and function might have induced PIP_2 degradation which then inactivated TRPM6 channels in omeprazole-treated Caco-2 monolayers. However, our results suggested that Mg^{2+} channel-dependent Mg^{2+} absorption could not be involved in omeprazole-suppressed Mg^{2+} transport across Caco-2 monolayers.

Our results in the present study agreed with the previous study [8] that omeprazole suppressed Mg^{2+} channel-independent, but not Mg^{2+} channel-dependent, Mg^{2+} transport across Caco-2 monolayers. There are two possible answers to explain why omeprazole had no effect on Mg^{2+} channel-dependent Mg^{2+} transport across Caco-2 monolayers. Since omeprazole has no effect on TRPM6 expression in Caco-2 cells [21], Mg^{2+} channel-dependent Mg^{2+} transport was maintained in the same fraction. Regarding our recent results, from 100% of total

Mg^{2+} transport, the percentages of Mg^{2+} channel-dependent Mg^{2+} transport were 18.55, 19.02, and 18.58% in control, 200-ng/ml omeprazole-treated, and 400-ng/ml omeprazole-treated monolayers. On the other hand, our recent method may not be sensitive enough to detect a very small change of Mg^{2+} channel-dependent Mg^{2+} transport of omeprazole-treated Caco-2 monolayers.

In conclusion, the present study reported the role of $P2Y_2$ function on the modulation of intestinal Mg^{2+} absorption. $P2Y_2$ agonist enhanced HCO_3^- secretion and suppressed Mg^{2+} transport through the activation of PLC, IP_3 receptor, intracellular Ca^{2+} mobilization, CFTR, CA, and NBCe1. Inhibition of HCO_3^- secretion could restore Mg^{2+} transport in $P2Y_2$ agonist-treated monolayers. The higher $P2Y_2$ expression was found in omeprazole-treated Caco-2 monolayers. Therefore, the higher degree of HCO_3^- secretion and Mg^{2+} transport suppression was demonstrated in $P2Y_2$-activated omeprazole-treated Caco-2 monolayers. Our results propose an inhibitory role of $P2Y_2$ on intestinal Mg^{2+} absorption.

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Author contributions TN designed and performed the experiments, analyzed and interpreted the results, and wrote and edited the manuscript. CS performed the experiments, analyzed the results, and wrote and edited the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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