JCI The Journal of Clinical Investigation

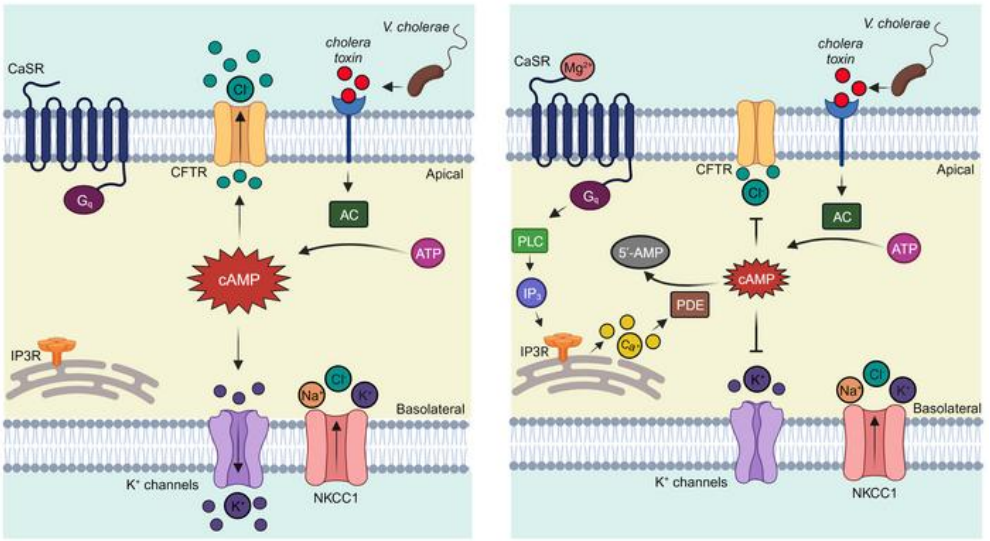
Mg^{2+} supplementation treats secretory diarrhea in mice by activating calcium-sensing receptor in intestinal epithelial cells

Livia de Souza Goncalves, … , Qi Gao, Onur Cil

J Clin Invest. 2023. https://doi.org/10.1172/JCI171249.

Research In-Press Preview Cell biology

Graphical abstract



Mg^{2+} supplementation treats secretory diarrhea in mice by activating calcium-sensing receptor in intestinal epithelial cells

Livia de Souza Goncalves[1]†, Tifany Chu[1]†, Riya Master[1], Parth D. Chhetri[1], Qi Gao[1] and Onur Cil[1*]

*Corresponding author: Onur Cil, M.D., Ph.D.

Department of Pediatrics

University of California, San Francisco

513 Parnassus Avenue, HSE 1244, San Francisco, CA, 94143

Phone: 415-4762423 Fax: 415-4769976

Email: onur.cil@ucsf.edu

Conflict-of-interest statement: The authors have declared that no conflict of interest exists.

[1] Department of Pediatrics, University of California, San Francisco.

†These authors contributed equally to this study.

ABSTRACT

Cholera is a global health problem with no targeted therapies. Ca^{2+}-sensing receptor (CaSR) is regulator of intestinal ion transport and therapeutic target for diarrhea, and Ca^{2+} is considered its main agonist. We found that increasing extracellular Ca^{2+} had minimal effect on forskolin-induced Cl^- secretion in human intestinal epithelial T84 cells. However, extracellular Mg^{2+}, an often-neglected CaSR agonist, suppressed forskolin-induced Cl^- secretion in T84 cells by 65% at physiological levels seen in stool (10 mM). Mg^{2+} effect was via CaSR-Gq signaling that leads to cAMP hydrolysis. Mg^{2+} (10 mM) also suppressed Cl^- secretion induced by cholera toxin, heat-stable *E. coli* enterotoxin and vasoactive intestinal peptide by 50%. In mouse intestinal closed-loops, luminal Mg^{2+} treatment (20 mM) inhibited cholera toxin-induced fluid accumulation by 40%. In mouse intestinal perfusion model of cholera, adding 10 mM Mg^{2+} to the perfusate reversed the net fluid transport from secretion to absorption. These results suggest that Mg^{2+} is the key CaSR activator in mouse and human intestinal epithelia at physiological levels seen in stool. Since stool Mg^{2+} concentrations in cholera patients are essentially zero, oral Mg^{2+} supplementation, alone or in oral rehydration solution (ORS), can be a potential therapy for cholera and other cyclic nucleotide-mediated secretory diarrheas.

Keywords: cholera; Traveler's diarrhea; VIPoma; CFTR; oral rehydration solution

INTRODUCTION

Diarrhea remains an important cause of global mortality, and diarrheal illness accounts for more than 25% of deaths in children under 5 years of age in Sub-Saharan Africa and South Asia (1-3). Secretory diarrhea is a major form caused by diverse etiologies that lead to activation of prosecretory Cl^- channels (such as CFTR) and/or inhibition of proabsorptive ion transporters (such as sodium-hydrogen exchanger 3, NHE3) in the luminal membrane of the intestinal epithelial cells (4). Cholera is a severe form of secretory diarrhea in which the key pathology is increased CFTR-mediated Cl^- secretion and reduced NHE3-mediated Na^+ absorption due to elevated cyclic adenosine monophosphate (cAMP) levels in intestinal epithelial cells (5-10). Current treatment of secretory diarrhea is primarily supportive with fluid and electrolyte replacement by intravenous fluids or oral rehydration solution (ORS). Commonly used glucose-based ORS formulations rely on intact sodium-glucose cotransporter activity in cholera for electrolyte repletion (11, 12). However, ORS has no effect on stool output, which is considered as a key contributor for low ORS use rates globally (13). Current ORS formulations do not directly address the hypersecretion and cholera remains an important cause of mortality despite ORS (14). An "improved" ORS formulation with antisecretory properties, in addition to repleting fluid and electrolytes, can potentially improve clinical outcomes in cholera and other secretory diarrheas.

Extracellular Ca^{2+}-sensing receptor (CaSR) is expressed in many tissues including parathyroid, kidney, bone, brain and gut (15). In the intestine, CaSR is a regulator of fluid and electrolyte transport (16, 17). We recently showed that FDA-approved CaSR activator cinacalcet inhibits CFTR-mediated fluid secretion and promotes NHE3-mediated fluid absorption in human colonic epithelial T84 cells and mouse intestine by promoting cAMP hydrolysis through phosphodiesterases (PDEs). Consistent with this mechanism, cinacalcet was very effective in various mouse models of secretory diarrhea (18, 19). Although CaSR activation is a promising treatment strategy for secretory diarrheas and short-term cinacalcet use for cholera might provide marked clinical benefits, long-term cinacalcet use for chronic diarrheas can cause systemic side effects due to CaSR activation in other organs. CaSR is physiologically activated by organic cations, including Ca^{2+} that the receptor is named after (20). We hypothesized that CaSR activation by enteral supplementation of its endogenous agonists can be used

as a simple, safe and effective treatment for secretory diarrhea. Although Ca^{2+} is considered as the major CaSR agonist, here we found that extracellular Ca^{2+} concentration has minimal effect on CaSR activity in human intestinal epithelial cells and mouse intestine. Interestingly, CaSR activity and cAMP-induced Cl^- secretion in human intestinal epithelial cells and mouse intestine strongly correlated with extracellular concentration of Mg^{2+}, an often-neglected CaSR agonist. Although Mg^{2+} causes osmotic diarrhea at high stool concentrations (>100 mM) (21), here we found that Mg^{2+} largely inhibits cyclic nucleotide-induced Cl^- secretion at physiological levels (10-20 mM) seen in stool. Considering that stool Mg^{2+} concentrations are essentially zero in cholera patients (22), we postulate that oral Mg^{2+} supplementation (either alone or in ORS) can be a potential treatment for cholera and other secretory diarrheas.

RESULTS

Extracellular Ca^{2+} has minimal effect on CFTR-mediated Cl^- secretion in T84 cells

To test the effect of extracellular Ca^{2+} on Cl^- secretion, short-circuit current (I_{sc}) measurements were done in human colonic T84 cells bathed with varying concentrations of Ca^{2+}. Changing extracellular Ca^{2+} concentration had minimal effect on forskolin-induced I_{sc} as suggested by similar maximal forskolin responses in the presence of 0.1 to 20 mM Ca^{2+} (Figure 1A). In Ca^{2+}-free solution, forskolin caused slightly increased I_{sc} changes compared to 10 mM or higher Ca^{2+} concentrations (Figure 1B). Forskolin-induced secretory current in T84 cells was partially reversed by selective CFTR inhibitor ($CFTR_{inh}$-172) treatment (Figure 1A). CFTR activity in T84 cells was not dependent on extracellular Ca^{2+} concentration as suggested by similar $CFTR_{inh}$-172 responses in the presence of 0.1 to 20 mM Ca^{2+} (Figure 1A and C). In the same experiments, CaSR activator drug cinacalcet inhibited forskolin and $CFTR_{inh}$-172-induced I_{sc} changes by 80% (Figure 1B and C). These results suggest that extracellular Ca^{2+} has minimal effect on CaSR activity and CFTR-mediated Cl^- secretion in human intestinal epithelial cells.

CFTR-mediated Cl^- secretion in T84 cells is strictly dependent on extracellular Mg^{2+} concentration

Since Ca^{2+} had minimal effect on CaSR activity and Cl^- secretion in T84 cells, we next investigated the effects of Mg^{2+}, a less studied physiological CaSR agonist. Forskolin-induced I_{sc} in T84 cells inversely correlated with extracellular Mg^{2+} (as $MgCl_2$) concentration (Figure 2A). Mg^{2+} inhibited forskolin-induced maximal I_{sc} by 70% at 10 mM or higher concentrations (Figure 2B), which is the physiological Mg^{2+} concentration in human stool (21). The antisecretory effect of Mg^{2+} was due to CFTR inhibition as suggested by substantially lower I_{sc} responses to $CFTR_{inh}$-172 with increasing Mg^{2+} concentrations (Figure 2A and C). The antisecretory effects of 10 mM or higher Mg^{2+} were comparable to CaSR activator cinacalcet effect as suggested by similar forskolin and $CFTR_{inh}$-172 responses. Altering extracellular Ca^{2+} and Mg^{2+} concentrations simultaneously had inhibitory effects on forskolin (Supplemental Figure 1A and B) and $CFTR_{inh}$-172 responses (Supplemental Figure 1A and C) similar to altering Mg^{2+} alone. Mg^{2+} also had concentration-dependent inhibitory effects on forskolin and $CFTR_{inh}$-172-induced I_{sc} changes when citrate ($MgC_6H_6O_7$, Supplemental

Figure 2A and B) or sulfate ($MgSO_4$, Supplemental Figure 2C and D) salts of Mg^{2+} were used. Increasing solution Mg^{2+} in these experiments slightly rises solution osmolality (<10% for 10 mM $MgCl_2$ compared to 1 mM $MgCl_2$). Although equal osmolality increased by $CaCl_2$ did not have antisecretory effects (Figure. 1), we performed control studies to directly rule out any potential effects of increased solution osmolality on secretory currents in T84 cells. Adding 30 or 60 mM mannitol to the solutions (equivalent to adding 10 or 20 mM $MgCl_2$, respectively) did not affect forskolin or $CFTR_{inh}$-172 responses, whereas 10 mM Mg^{2+} had marked antisecretory effects in side-by-side studies (Supplementary Figure. 3). To rule out any potential effects of Mg^{2+} on barrier permeability, we measured transepithelial electrical resistance (TEER) in the presence of various Mg^{2+} and Ca^{2+} concentrations. Altering extracellular Mg^{2+} or Ca^{2+} concentrations had no effect on TEER under conditions that I_{sc} studies were performed (Supplementary Figure. 4). These results suggest that extracellular Mg^{2+} is the major CaSR agonist and regulator of CFTR-mediated Cl^- secretion in human intestinal epithelial cells.

Extracellular Mg^{2+} exerts its antisecretory effect by indirect inhibition of CFTR through CaSR activation

To investigate whether Mg^{2+} or Ca^{2+} have direct CFTR inhibitory effects, I_{sc} studies were done in CFTR-transfected Fischer Rat Thyroid (FRT-CFTR) cells, which do not express the CaSR (23, 24) and are commonly used to study CFTR modulators (18, 25-27). With basolateral membrane permeabilization and 60 mM basolateral-to-apical Cl^- gradient, forskolin induced a large Cl^- secretory current in FRT-CFTR cells, which was completely reversed by $CFTR_{inh}$-172 treatment (Figure 3A). Increasing extracellular Mg^{2+} (or Ca^{2+}) concentration from 1 to 10 mM had no effect on forskolin or $CFTR_{inh}$-172 responses (Figure 3B), suggesting that Mg^{2+} does not have a direct CFTR inhibitory effect.

The effects of Mg^{2+} and Ca^{2+} on CFTR-mediated Cl^- secretion were also investigated in well-differentiated human bronchial epithelial (HBE) cells that express both CFTR and CaSR, and have robust forskolin-induced Cl^- secretory responses (18). Similar to intestinal epithelial cells, increasing extracellular Mg^{2+} concentration from 1 to 10 mM inhibited forskolin and $CFTR_{inh}$-172 responses in HBE cells by ~50% (Figure 3C and D). However, increasing extracellular Ca^{2+} concentration from 1 to 10 mM had no effects on forskolin and

$CFTR_{inh}$-172 responses in HBE cells, suggesting that Mg^{2+} is also the key CaSR agonist in airway epithelial cells.

Antisecretory effect of Mg^{2+} in T84 cells is through inhibition of apical membrane Cl^- and basolateral membrane K^+ conductance

Cyclic AMP-induced secretory I_{sc} in intestinal epithelial cells involves the coordinated action of apical membrane CFTR Cl^- channel, and basolateral membrane K^+ channels (28). To selectively investigate the effect of Mg^{2+} on apical CFTR conductance, experiments were done in T84 cells with selective basolateral membrane permeabilization and basolateral-to-apical Cl^- gradient (29, 30). Under these conditions addition of 10 mM Mg^{2+} to the bathing solution inhibited forskolin and $CFTR_{inh}$-172-induced I_{sc} changes by 70% (Figure 4A and B). To study the effect of Mg^{2+} on basolateral membrane K^+ channels, experiments were done with selective apical membrane permeabilization and apical-to-basolateral K^+ gradient (29, 31). In this setting, addition of 10 mM Mg^{2+} to the bathing solution largely reduced basolateral membrane K^+ conductance as suggested by 80% reduced I_{sc} changes in response to forskolin and $BaCl_2$ (cAMP-activated K^+ channel inhibitor) (Figure 4C and D). These results suggest that Mg^{2+} exerts its antisecretory effect in T84 cells by inhibition of apical membrane CFTR Cl^- channel and basolateral membrane K^+ channels.

Mg^{2+} reduces cAMP levels in T84 cells through activation of phospholipase C and phosphodiesterases

Activation of phospholipase C (PLC) and consecutive mobilization of intracellular Ca^{2+} by IP3 is the key downstream pathway of CaSR activation, which leads to PDE activation and cAMP hydrolysis in human intestinal epithelial cells (16, 32). To test the role of this mechanism on Mg^{2+} effect, intracellular Ca^{2+} was measured by Fluo-4 fluorescence. Extracellular addition of 10 mM Mg^{2+} resulted in marked intracellular Ca^{2+} elevation, which was abolished by PLC inhibitor U73122 pretreatment (Figure 5A). Mg^{2+} induced intracellular Ca^{2+} release was from intracellular stores, as suggested by prevention of Ca^{2+} increase after endoplasmic reticulum stores are depleted by thapsigargin. Consistent with lack of its antisecretory effects, 10 mM Ca^{2+} had no effect on intracellular Ca^{2+} levels in T84 cells (Figure 5A). IP1 is the stable downstream metabolite of IP3

and its quantification is considered as the standard method to assess CaSR activity (33). Similar to studies above, extracellular Mg^{2+} concentration-dependently increase intracellular IP1 levels in T84 cells whereas Ca^{2+} had minimal effect only at high concentrations (Figure 5B). Since cAMP is the major activator of apical CFTR and basolateral K^+ channels, reduced cAMP levels via CaSR activation might explain the antisecretory effect of Mg^{2+} in T84 cells. Consistent with this mechanism, extracellular addition of 10 mM Mg^{2+} (but not Ca^{2+}) reduced the forskolin-induced cAMP elevation in T84 cells (Figure 5C). Mg^{2+} effect on cAMP levels was completely reversed with the PDE inhibitor IBMX treatment, suggesting PDE activation as the key mechanism of Mg^{2+} effect. Collectively, these results further confirm that Mg^{+2} (but not Ca^{2+}) is the major CaSR agonist in human intestinal epithelial cells and Mg^{2+} exerts its antisecretory effects via the known CaSR signaling pathways including PLC-mediated intracellular Ca^{2+} mobilization and PDE activation.

Mg^{2+} does not affect Cl^- secretion induced by Ca^{2+} agonists

Although elevation of cAMP and hence CFTR activation is the key mechanism for cholera, in certain secretory diarrheas elevation of intracellular Ca^{2+} is the major driver of Cl^- secretion via Ca^{2+}-activated Cl^- channels (CaCC). To test the effects of Mg^{2+} on CaCC activity, we performed I_{sc} studies in T84 cells using cholinergic agonist carbachol. In the presence of 1 or 10 mM Mg^{2+}, carbachol induced comparable secretory currents (Supplementary Figure. 5) suggesting CaSR activation by Mg^{2+} does not affect CaCC activity.

Mg^{2+} inhibits cholera toxin, heat-stable E. coli enterotoxin and vasoactive intestinal peptide induced Cl^- secretion in T84 cells

Cyclic nucleotide (cAMP or cGMP)-mediated CFTR activation and consequent Cl^- secretion is the key pathology in certain secretory diarrheas including cholera, Traveler's diarrhea and VIP-secreting tumors (VIPoma) (5, 6, 34). To test efficacy of Mg^{2+} in these settings, I_{sc} experiments were done in T84 cells treated with cholera toxin, heat-stable E. coli enterotoxin (ST_a toxin) and vasoactive intestinal peptide (VIP) as secretagogues. Increasing extracellular Mg^{2+} concentration from 1 to 10 mM suppressed I_{sc} changes induced by cholera toxin (Figure 6A and B), ST_a toxin (Figure 6C and D) and VIP (Figure 6E and F) by greater than 50%.

Similarly, 10 mM Mg^{2+} treatment resulted in reduced CFTR activity in all experiments as suggested by markedly lower $CFTR_{inh}$-172 responses compared to controls (Figure 6B, D and F, right panels).

Mg^{2+} has antisecretory effects in mouse intestine via CaSR activation

As done for T84 cells, the antisecretory effect of Mg^{2+} was tested in mouse jejunal mucosa. In wild type mice, increasing extracellular Mg^{2+} (but not Ca^{2+}) concentration from 1 to 10 mM reduced forskolin-induced secretory I_{sc} by 40% (Figure 7A). Parallel studies were done in intestinal epithelia-specific CaSR knockout mice (*Vil1-Cre;CaSR-flox*), in which 10 mM Mg^{2+} had no antisecretory effects (Figure 7B). These results suggest that CaSR activation is the key mechanism for the antisecretory effect of Mg^{2+} in mouse intestine. Mg^{2+} also had marked antisecretory effects when applied only to the luminal side of the intestine (Figure. 7C), which suggests its potential efficacy with oral treatment.

Efficacy of Mg^{2+} in mouse models of cholera

The efficacy of Mg^{2+} was tested in intestinal closed-loop model of cholera in mice (Figure 8A, left). In this model, cholera toxin caused marked intestinal fluid accumulation at 3 hours as suggested by increased loop weight/length ratio. Intraluminal 20 mM Mg^{2+} treatment at zero time (together with cholera toxin) inhibited the increase in loop weight/length ratio by 40% (Figure 8A, center and right). We measured the remaining Mg^{2+} concentration in the loops at the end of 3 hour period and found that luminal Mg^{2+} concentration drops to 5.2±1.2 mM, which suggests that luminal Mg^{2+} concentration might have become slightly subtherapeutic in some loops towards the end of this study. A potential approach for using Mg^{2+} for diarrhea treatment is fortifying ORS with Mg^{2+}, which can provide sustained CaSR activation in the intestine for even higher efficacy. To test this idea, we established a mouse intestinal perfusion model of cholera (Figure 8B, left) in which Mg^{2+} concentration of the perfusate is controlled. In this model, cholera toxin administration resulted in net intestinal fluid loss as suggested by negative fluid transport rate. Increasing Mg^{2+} concentration from 1 to 10 mM reversed net secretion into net absorption as suggested by positive fluid transport rates (Figure 8B, right). Mg^{2+} (10 mM) also had antisecretory effects in the perfusion model when WHO ORS solution was used,

particularly at 90 min where cholera toxin effect was fully established (Fig. 8C). These results further support our idea of developing Mg^{2+}-fortified ORS.

DISCUSSION

Here we showed that CFTR-mediated Cl^- secretion in human intestinal epithelial cells and mouse intestine are dependent on extracellular Mg^{2+} concentration, which exerts its effect through CaSR activation (see Fig. 9 for proposed mechanisms). Interestingly, Ca^{2+}, which is considered as the main physiological CaSR agonist, had minimal effect on CaSR activity and Cl^- secretion in intestinal epithelial cells.

The antidiarrheal effect of Mg^{2+} as shown here might sound contradictory since oral Mg^{2+} supplements can cause osmotic diarrhea at high doses (21). Normal range of fecal soluble Mg^{2+} concentration is 10-30 mM in healthy subjects, which increases to 100-150 mM in patients with Mg^{2+}-induced diarrhea (21). Although Mg^{2+} can cause osmotic diarrhea at concentrations >100 mM, our findings suggest that physiological Mg^{2+} concentrations in the intestinal lumen have antidiarrheal effects through CaSR activation. In patients with cholera and VIP-induced diarrhea, there is lack of stool osmotic gap (22, 35), which indirectly suggest the possibility that stool Mg^{2+} concentration might be low in these conditions. Our findings suggest that increasing stool Mg^{2+} to physiological levels (10-20 mM) by oral supplementation might offer a simple, safe and effective therapy for secretory diarrheas. In addition, stool Mg^{2+} concentration can potentially be implemented as a secretory diarrhea biomarker to identify patients with low stool Mg^{2+} who are likely to benefit from Mg^{2+} supplementation. However, we would like to note that the earlier studies mentioned above did not directly measure stool Mg^{2+} concentration in patients, and thus lack of stool osmotic gap in these studies may also be explained by other factors such as potential measurement errors. Future clinical studies formally quantifying stool Mg^{2+} concentration in patients with cholera and other forms of diarrhea may be informative to validate its utility as a biomarker.

Current cholera treatment primarily relies on ORS that was developed after discovery of intact glucose-dependent Na^+ absorption in secretory diarrheas (11, 12). However, ORS does not have any effects on hypersecretion or stool output (4, 13). Based on our results, Mg^{2+} can be used as an adjunct therapy that can reduce fluid secretion and stool output. One potential issue with oral Mg^{2+} treatment is its relatively rapid intestinal absorption as shown in our closed-loop studies, which may require frequent administration in severe

diarrheas such as cholera. Additional dose/frequency determination, pharmacokinetics and pharmacodynamics studies may be informative prior to testing the efficacy of intermittent oral Mg^{2+} treatment. Alternatively, we postulate that addition of 10 mM Mg^{2+} to ORS (*Mg^{2+}-fortified ORS*) can provide sustained CaSR activation in the intestine and reduce stool output, in addition to repleting fluid and electrolytes. Although we present evidence for the efficacy of this approach in an animal model, further preclinical studies are required to optimize the formulation of Mg^{2+}-fortified ORS, which can ultimately be tested side-by-side with traditional ORS in clinical trials. A theoretical concern for using Mg^{2+} in diarrhea treatment is potential hypermagnesemia as a side effect since Mg^{2+} salts have 50-67% oral bioavailability (36). However, serum Mg^{2+} levels are tightly regulated by the kidneys, which can rapidly decrease or increase Mg^{2+} excretion according to the dietary changes (37, 38). Thus the absorbed Mg^{2+} is predicted to be rapidly excreted by the kidneys with minimal or no elevation in serum Mg^{2+}. Another theoretical concern might be that repeated Mg^{2+} treatment may result in depletion of IP3-sensitive Ca^{2+} pool in intestinal epithelia. As discussed above, physiological Mg^{2+} concentration in human stool fluid is 10-30 mM, which potentially suggests constitutive CaSR activation in the intestine under normal conditions. Considering potentially low Mg^{2+} concentrations in cholera and other secretory diarrheas as discussed above, short-term Mg^{2+} treatment may be effective in acute secretory diarrheas by restoring physiological Mg^{2+} concentrations and CaSR activity in the intestine. Future studies testing efficacy of long-term Mg^{2+} treatment and potential tolerance development in chronic diarrheas may be informative to demonstrate its efficacy in the chronic setting.

Earlier studies investigating the roles of CaSR on intestinal fluid transport solely focused on Ca^{2+} and suggested Ca^{2+}-dependence of the forskolin-induced Cl^- secretion in rat colonocytes (32, 39). Based on these results, the effects of Ca^{2+} supplementation in diarrhea were studied in earlier clinical trials. Oral Ca^{2+} supplementation was shown to have mild antidiarrheal effect in Traveler's diarrhea, though mainly by preventing bacterial colonization (40). A large randomized controlled trial in children compared the effects of low calcium (50 mg/day) and regular calcium (440 mg/day) milk on number and duration of diarrhea episodes and found no benefits of higher calcium intake (41). Despite the role of CaSR in diarrhea has been known for

decades (4, 42), there are no large-scale clinical studies showing beneficial effects of Ca^{2+} supplementation. Our findings here suggest that Mg^{2+} (but not Ca^{2+}) is the key CaSR agonist in intestinal epithelia, which can potentially explain lack of antisecretory effects of Ca^{2+} in previous clinical studies.

Certain compounds that elevate intracellular Ca^{+2} (such as cholinergic agonists) induce Cl^- secretion which is thought to be mediated by CaCC. Although CaSR activation by Mg^{2+} also increases intracellular Ca^{2+}, Mg^{2+} did not induce a secretory current in T84 cells, similar to what we have previously found with CaSR activator drug cinacalcet (18). In addition, Mg^{2+} did not affect cholinergic agonist carbachol-induced secretory currents. Although cytosolic Ca^{+2} elevation is a shared mechanism between CaSR and cholinergic agonists, additional unshared signaling pathways, including cross-talk with EGF signaling (43, 44), might be important determinants for the secretory effects of cholinergic agonists, but not CaSR agonists.

Certain secretory diarrheas (such as rotavirus) are characterized primarily by CaCC-mediated Cl^- secretion. Here we found that CaSR activation by Mg^{2+} largely inhibits cyclic nucleotide agonists-induced Cl^- secretion, without any effects on Ca^{2+} agonist-induced Cl^- secretion. Thus, Mg^{2+} may not be effective in secretory diarrheas where CaCC activation is the major driver of intestinal fluid loss. Although our results herein showing marked inhibitory effect of Mg^{2+} on CFTR-mediated Cl^- secretion, CaCC may also play a role in intestinal fluid loss in cyclic nucleotide mediated diarrheas. In addition there can be cross-talk between cAMP and Ca^{2+} pathways, which can lead to activation of both CFTR and CaCC in certain secretory diarrheas (4). Future studies investigating effects of Mg^{2+} on different secretory pathways and cross-talk mechanisms may be informative to further understand its mechanisms of action and potential efficacy in diarrhea patients.

Although we showed here that Mg^{2+} is effective in both cAMP and cGMP-mediated diarrhea models, cyclic nucleotide elevation is not a common pathology in all diarrheas. Thus Mg^{2+} (alone or in ORS) may not be effective as a general anti-diarrheal, but it can potentially be used as a specific and targeted treatment for cyclic nucleotide-mediated diarrheas such as cholera, Traveler's diarrhea, VIPoma and *GUCY2C* mutations.

The majority of earlier studies on CaSR agonists used bovine parathyroid cells or HEK-293 cells transfected with parathyroid CaSR isoform (45). In both settings, Ca^{2+} is 2-3 fold more potent agonist than Mg^{2+} in

suppressing PTH secretion (46-48). Consistent with this, serum ionized Ca^{2+} concentration is the primary determinant of PTH secretion in vivo (49). We show here that in human intestinal and airway epithelial cells natively expressing CaSR, Mg^{2+} is the key agonist for this receptor. These results also suggest that the term "calcium-sensing receptor" might be an oversimplification of the biological roles of this receptor.

In conclusion, we demonstrated that extracellular Mg^{2+} concentration is the major regulator of CaSR activity and cAMP -induced Cl^- secretion in intestinal epithelial cells. Oral Mg^{2+} supplementation, either alone or in ORS, can offer a simple, safe, targeted and effective treatment for cyclic nucleotide-mediated secretory diarrheas such as cholera, Traveler's diarrhea, VIPoma and *GUCY2C* mutations.

METHODS

Cell culture

T84 cells (ATCC CCL-248, human colon carcinoma cells) were cultured as described (18, 29) on inserts (12 mm diameter, 0.4 μm pore size; Corning Life Sciences, Tewksbury, MA) and used for short-circuit current experiments 7 days after plating. Fischer rat thyroid cells stably expressing human wild-type CFTR (FRT-CFTR cells) were cultured as described (50) on inserts and used for short-circuit current experiments 5 days after plating. Well-differentiated human bronchial epithelial (HBE) cells were cultured at an air-liquid interface on inserts as described (51). HBE cells were used for short-circuit current experiments 21 days after plating, when they typically form a tight epithelium (transepithelial electrical resistance - TEER > 1000 Ω cm^2).

Short-circuit current (I_{sc}) measurements

Cells were mounted in Ussing chambers containing bicarbonate-buffered Ringer's solution (pH 7.4, in mM: 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$). $MgCl_2$ and/or $CaCl_2$ concentration of both apical and basolateral solutions were altered (0-20 mM) in separate experiments as indicated in each figure. Secretagogues and ion channel inhibitors were added to both apical and basolateral bathing solutions. In some experiments to measure apical Cl^- conductance, the basolateral membrane was permeabilized with 500 μg/mL amphotericin B for 30 min and 60 mM basolateral-to-apical Cl^- gradient was applied. For these experiments, Ringer's was the basolateral bathing solution (120 mM NaCl) and the apical solution contained 60 mM NaCl and 60 mM sodium gluconate. To measure basolateral membrane K^+ conductance, the apical membrane was permeabilized with 20 μM amphotericin B for 30 min and apical-to-basolateral potassium gradient was applied. The apical solution (pH 7.4) contained in mM: 142.5 K-gluconate, 1 $CaCl_2$, 1 $MgCl_2$, 0.43 KH_2PO_4, 0.35 Na_2HPO_4, 10 HEPES, and 10 D-glucose. In the basolateral solution (pH 7.4) 142.5 mM K-gluconate was replaced by 5.5 mM K-gluconate and 137 mM N-methylglucamine. The solutions were aerated with 95% O_2 /5% CO_2 and maintained at 37°C during experiments. Short-circuit current (Isc) was measured using an EVC4000 multichannel voltage clamp (World Precision Instruments, Sarasota, FL,

USA) via Ag/AgCl electrodes and 3 M KCl agar bridges. In parallel experiments, T84 cells were grown on permeable filters as described above, and bathed with Ringer's solution containing 0-10 mM $CaCl_2$ and/or $MgCl_2$ for 60 min. TEER was measured using a Millicell-ERS Resistance System with dual electrode volt-ohmmeter (Millipore, Bedford, MA) to test the effects of various Ca^{2+} and Mg^{2+} concentrations on epithelial barrier function. Net TEER (ohms/cm^2) was calculated by subtracting the resistance of cell-free media from measured resistance (52).

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except $CFTR_{inh}$-172 (MedChemExpress) and STa toxin (Bachem Americas Inc.).

CaSR activity and cAMP measurements

For intracellular Ca^{2+} measurements, T84 cells were plated in 96-well, black-walled microplates (Corning). Confluent cells were loaded with Ca^{2+} indicator Fluo-4 NW (Invitrogen) per manufacturer's instructions. Fluo-4 fluorescence was measured in each well continuously with a Tecan Infinite M1000 plate reader (Tecan Group) at excitation/emission wavelengths of 495 nm/516 nm after addition of 10 mM $CaCl_2$ or $MgCl_2$. In some experiments cells were pretreated with PLC inhibitor U73122 (10 μM) or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM) for 5 min prior to addition of $MgCl_2$. For IP1 assay, T84 cells were grown in 384-well opaque plates (Perkin Elmer) and treated with 0-20 mM $CaCl_2$ or $MgCl_2$ for 30 min. After that, cells were lysed and IP1 concentration in each well was quantified using IP-One Gq kit according to the manufacturer's instructions (Cisbio). For cAMP assay, T84 cells were grown in clear 24-well plates and pretreated for 20 min with 10 mM $MgCl_2$ (or $CaCl_2$) with or without 500 μM IBMX or vehicle control (0.2% DMSO). After that, cells were treated with 10 μM forskolin (for 5 min) and lysed by repeated freeze/thaw and centrifuged to remove cell debris. The supernatant was assayed for cAMP using the cAMP Parameter immunoassay kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Animals

$Casr^{flox/flox}$ (strain #:030647, C57BL/6 background), Vil1-cre (strain #:004586, C57BL/6 background) and wild-type mice (C57BL/6 or CD1 background) were obtained from Jackson Laboratories (Bar Harbor, ME). Intestinal epithelia-specific Casr knockout mice (Vil1-cre; $Casr^{flox/flox}$) were generated by crossbreeding and genotype was confirmed by PCR. Animals were bred in UCSF Laboratory Animal Resource Center and experiments were done in adherence with NIH Guide for the Care and Use of Laboratory Animals. Both male and female mice were used in all experiments.

Intestinal I_{sc} measurement in mice

Jejunum was excised under anesthesia and soaked in an isoosmolar solution containing 300 mM mannitol and 10 μM indomethacin. Mucosa was stripped from serosa/muscle layers under a dissection microscope and mounted on Ussing chambers containing Ringer's solution on the basolateral side. For apical side, a similar solution was used except 120 mM NaCl was replaced with 60 mM NaCl and 60 mM sodium gluconate, and glucose was replaced with 10 mM mannitol. I_{sc} was measured as described above.

Mouse models of cholera

For closed-loop model, mice (CD1, 8-12 weeks old) were fasted overnight with access to 5% dextrose in water but no solid food. Mice were anesthetized with isoflurane, and body temperature was maintained during surgery at 36–38°C using a heating pad. After a small abdominal incision to expose the small intestine, mid-jejunal loops (2–3 cm in length) were isolated by sutures as described (50, 51). Loops were injected with 100 μl phosphate-buffered saline (PBS, pH 7.4, in mM: 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.8 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$) containing 1 μg cholera toxin or PBS alone. Some loops were injected with PBS±cholera toxin with 20 mM Mg^{2+}. After loop injections, the abdominal incision was closed with sutures and mice were allowed to recover from anesthesia. Intestinal loops were removed at 3 h, and loop length and weight were measured to quantify fluid secretion. Loop fluid was aspirated with syringe and Mg^{2+} concentration was quantified by a colorimetric assay (Sigma-Aldrich).

For intestinal perfusion model, mice (C57BL/6, 10-14 weeks old) were fasted overnight. Under isoflurane anesthesia, proximal duodenum and terminal ileum were cannulated. The small intestine was lavaged gently with saline (at 37°C) to clear the luminal contents. After draining the intestine, the ileal catheter was clamped and cholera toxin (10 μg in 2 mL saline) or saline control was instilled and dispersed through the small intestine. After 2 h, the clamps were opened and whole-gut perfusion was initiated at 0.2 mL/min with Ringer's solution containing 2 mM ferrocyanide ($Fe(CN)_6$, non-absorbable volume marker) and 1 or 10 mM $MgCl_2$ (in separate mice). After equilibration for 60 min, effluent samples were collected from ileal catheter. Ferrocyanide concentration was determined in the infusate ($ferrocyanide_i$) and effluent ($ferrocyanide_e$) via absorbance and net fluid transport (μL/min/cm) was calculated using the following formula as described (53): Perfusion rate - [Perfusion rate x ($ferrocyanide_i$)/($ferrocyanide_e$)]/length of gut (cm) x 1000. In some experiments we used WHO ORS (TRIORAL, Trifecta Pharmaceuticals, in mM: 75 Na^+, 65 Cl^-, 75 glucose, 20 K^+, 10 citrate) with and without 10 mM $MgCl_2$.

Statistical analysis

Experiments with two groups were analyzed using two-tailed Student's t test; for three or more groups, analysis was done with one-way analysis of variance (ANOVA) and post hoc Newman-Keuls multiple comparisons test. In all analyses, p value < 0.05 was considered as statistically significant.

Study approval

The experimental protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Data availability

Values for all data points in graphs are available in the Supporting Data Values file.

Acknowledgments: This study was supported by grants from NIH (DK126070 and DK072517) and Cystic Fibrosis Foundation.

Author contributions:

OC made the original discovery, conceptualized the study and designed the experiments; LDG, TC, RM, PC, QG and OC performed the experiments; LDG, TC and OC analyzed the data; OC obtained funding, supervised the study and wrote the paper; LDG and OC revised the paper. All authors read the paper and agree in the submitted form. The first author position is shared and first authors are listed alphabetically according to their first names.

References

1. Demissie GD, Yeshaw Y, Aleminew W, and Akalu Y. Diarrhea and associated factors among under five children in sub-Saharan Africa: Evidence from demographic and health surveys of 34 sub-Saharan countries. *PLoS One.* 2021;16(9):e0257522.
2. Kotloff KL. The Burden and Etiology of Diarrheal Illness in Developing Countries. *Pediatr Clin North Am.* 2017;64(4):799-814.
3. Charyeva Z, Cannon M, Oguntunde O, Garba AM, Sambisa W, Bassi AP, et al. Reducing the burden of diarrhea among children under five years old: lessons learned from oral rehydration therapy corner program implementation in Northern Nigeria. *J Health Popul Nutr.* 2015;34:4.
4. Thiagarajah JR, Donowitz M, and Verkman AS. Secretory diarrhoea: mechanisms and emerging therapies. *Nat Rev Gastroenterol Hepatol.* 2015;12(8):446-57.
5. Field M, Fromm D, al-Awqati Q, and Greenough WB, 3rd. Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. *J Clin Invest.* 1972;51(4):796-804.
6. Rao MC, Guandalini S, Smith PL, and Field M. Mode of action of heat-stable Escherichia coli enterotoxin. Tissue and subcellular specificities and role of cyclic GMP. *Biochim Biophys Acta.* 1980;632(1):35-46.
7. Subramanya SB, Rajendran VM, Srinivasan P, Nanda Kumar NS, Ramakrishna BS, and Binder HJ. Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum. *Am J Physiol Gastrointest Liver Physiol.* 2007;293(4):G857-63.
8. Hecht G, Hodges K, Gill RK, Kear F, Tyagi S, Malakooti J, et al. Differential regulation of Na+/H+ exchange isoform activities by enteropathogenic E. coli in human intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol.* 2004;287(2):G370-8.
9. Ao M, Sarathy J, Domingue J, Alrefai WA, and Rao MC. Chenodeoxycholic acid stimulates Cl(-) secretion via cAMP signaling and increases cystic fibrosis transmembrane conductance regulator phosphorylation in T84 cells. *Am J Physiol Cell Physiol.* 2013;305(4):C447-56.
10. Yun CH, Oh S, Zizak M, Steplock D, Tsao S, Tse CM, et al. cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an associated regulatory protein. *Proc Natl Acad Sci U S A.* 1997;94(7):3010-5.
11. Nalin DR, and Cash RA. 50 years of oral rehydration therapy: the solution is still simple. *Lancet.* 2018;392(10147):536-8.
12. Sentongo TA. The use of oral rehydration solutions in children and adults. *Curr Gastroenterol Rep.* 2004;6(4):307-13.
13. Rabbani GH. The search for a better oral rehydration solution for cholera. *N Engl J Med.* 2000;342(5):345-7.
14. Collaborators GBDDD. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect Dis.* 2018;18(11):1211-28.
15. Chavez-Abiega S, Mos I, Centeno PP, Elajnaf T, Schlattl W, Ward DT, et al. Sensing Extracellular Calcium - An Insight into the Structure and Function of the Calcium-Sensing Receptor (CaSR). *Adv Exp Med Biol.* 2020;1131:1031-63.
16. Cheng SX, Okuda M, Hall AE, Geibel JP, and Hebert SC. Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion. *Am J Physiol Gastrointest Liver Physiol.* 2002;283(1):G240-50.
17. Cheng SX. Calcium-sensing receptor inhibits secretagogue-induced electrolyte secretion by intestine via the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol.* 2012;303(1):G60-70.
18. Oak AA, Chhetri PD, Rivera AA, Verkman AS, and Cil O. Repurposing calcium-sensing receptor agonist cinacalcet for treatment of CFTR-mediated secretory diarrheas. *JCI Insight.* 2021;6(4).
19. Chu T, Yottasan P, Goncalves LS, Oak AA, Lin R, Tse M, et al. Calcium-sensing receptor activator cinacalcet for treatment of cyclic nucleotide-mediated secretory diarrheas. *Transl Res.* 2023.
20. Jensen AA, and Brauner-Osborne H. Allosteric modulation of the calcium-sensing receptor. *Curr Neuropharmacol.* 2007;5(3):180-6.

21. Fine KD, Santa Ana CA, and Fordtran JS. Diagnosis of magnesium-induced diarrhea. *N Engl J Med.* 1991;324(15):1012-7.
22. Watten RH, Morgan FM, Yachai Na S, Vanikiati B, and Phillips RA. Water and electrolyte studies in cholera. *J Clin Invest.* 1959;38(11):1879-89.
23. Garrett JE, Tamir H, Kifor O, Simin RT, Rogers KV, Mithal A, et al. Calcitonin-secreting cells of the thyroid express an extracellular calcium receptor gene. *Endocrinology.* 1995;136(11):5202-11.
24. Nitsch L, and Wollman SH. Suspension culture of separated follicles consisting of differentiated thyroid epithelial cells. *Proc Natl Acad Sci U S A.* 1980;77(1):472-6.
25. Han ST, Rab A, Pellicore MJ, Davis EF, McCague AF, Evans TA, et al. Residual function of cystic fibrosis mutants predicts response to small molecule CFTR modulators. *JCI Insight.* 2018;3(14).
26. Phuan PW, Haggie PM, Tan JA, Rivera AA, Finkbeiner WE, Nielson DW, et al. CFTR modulator therapy for cystic fibrosis caused by the rare c.3700A>G mutation. *J Cyst Fibros.* 2021;20(3):452-9.
27. Sheppard DN, Carson MR, Ostedgaard LS, Denning GM, and Welsh MJ. Expression of cystic fibrosis transmembrane conductance regulator in a model epithelium. *Am J Physiol.* 1994;266(4 Pt 1):L405-13.
28. Das S, Jayaratne R, and Barrett KE. The Role of Ion Transporters in the Pathophysiology of Infectious Diarrhea. *Cell Mol Gastroenterol Hepatol.* 2018;6(1):33-45.
29. Oak AA, Chu T, Yottasan P, Chhetri PD, Zhu J, Du Bois J, et al. Lubiprostone is non-selective activator of cAMP-gated ion channels and Clc-2 has a minor role in its prosecretory effect in intestinal epithelial cells. *Mol Pharmacol.* 2022;102(2):106-15.
30. Duan T, Cil O, Thiagarajah JR, and Verkman AS. Intestinal epithelial potassium channels and CFTR chloride channels activated in ErbB tyrosine kinase inhibitor diarrhea. *JCI Insight.* 2019;4(4).
31. Rufo PA, Merlin D, Riegler M, Ferguson-Maltzman MH, Dickinson BL, Brugnara C, et al. The antifungal antibiotic, clotrimazole, inhibits chloride secretion by human intestinal T84 cells via blockade of distinct basolateral K+ conductances. Demonstration of efficacy in intact rabbit colon and in an in vivo mouse model of cholera. *J Clin Invest.* 1997;100(12):3111-20.
32. Geibel J, Sritharan K, Geibel R, Geibel P, Persing JS, Seeger A, et al. Calcium-sensing receptor abrogates secretagogue- induced increases in intestinal net fluid secretion by enhancing cyclic nucleotide destruction. *Proc Natl Acad Sci U S A.* 2006;103(25):9390-7.
33. Trinquet E, Bouhelal R, and Dietz M. Monitoring Gq-coupled receptor response through inositol phosphate quantification with the IP-One assay. *Expert Opin Drug Discov.* 2011;6(10):981-94.
34. Fabian E, Kump P, and Krejs GJ. Diarrhea caused by circulating agents. *Gastroenterol Clin North Am.* 2012;41(3):603-10.
35. Kane MG, O'Dorisio TM, and Krejs GJ. Production of secretory diarrhea by intravenous infusion of vasoactive intestinal polypeptide. *N Engl J Med.* 1983;309(24):1482-5.
36. Coudray C, Rambeau M, Feillet-Coudray C, Gueux E, Tressol JC, Mazur A, et al. Study of magnesium bioavailability from ten organic and inorganic Mg salts in Mg-depleted rats using a stable isotope approach. *Magnes Res.* 2005;18(4):215-23.
37. Jahnen-Dechent W, and Ketteler M. Magnesium basics. *Clin Kidney J.* 2012;5(Suppl 1):i3-i14.
38. Nielsen FH. Dietary Magnesium and Chronic Disease. *Adv Chronic Kidney Dis.* 2018;25(3):230-5.
39. Lysyy T, Lalani AS, Olek EA, Diala I, and Geibel JP. The calcium-sensing receptor: A novel target for treatment and prophylaxis of neratinib-induced diarrhea. *Pharmacol Res Perspect.* 2019;7(5):e00521.
40. Bovee-Oudenhoven IM, Lettink-Wissink ML, Van Doesburg W, Witteman BJ, and Van Der Meer R. Diarrhea caused by enterotoxigenic Escherichia coli infection of humans is inhibited by dietary calcium. *Gastroenterology.* 2003;125(2):469-76.
41. Agustina R, Kok FJ, van de Rest O, Fahmida U, Firmansyah A, Lukito W, et al. Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children. *Pediatrics.* 2012;129(5):e1155-64.
42. Cheng SX. Calcium-sensing receptor: A new target for therapy of diarrhea. *World J Gastroenterol.* 2016;22(9):2711-24.
43. Uribe JM, Gelbmann CM, Traynor-Kaplan AE, and Barrett KE. Epidermal growth factor inhibits Ca(2+)-dependent Cl- transport in T84 human colonic epithelial cells. *Am J Physiol.* 1996;271(3 Pt 1):C914-22.

44. Keely SJ, and Barrett KE. ErbB2 and ErbB3 receptors mediate inhibition of calcium-dependent chloride secretion in colonic epithelial cells. *J Biol Chem.* 1999;274(47):33449-54.
45. Nemeth EF. Calcimimetic and calcilytic drugs: just for parathyroid cells? *Cell Calcium.* 2004;35(3):283-9.
46. Habener JF, and Potts JT, Jr. Relative effectiveness of magnesium and calcium on the secretion and biosynthesis of parathyroid hormone in vitro. *Endocrinology.* 1976;98(1):197-202.
47. Brown EM, and Thatcher JG. Adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase and the regulation of parathyroid hormone release by divalent cations and agents elevating cellular cAMP in dispersed bovine parathyroid cells. *Endocrinology.* 1982;110(4):1374-80.
48. Brauner-Osborne H, Jensen AA, Sheppard PO, O'Hara P, and Krogsgaard-Larsen P. The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. *J Biol Chem.* 1999;274(26):18382-6.
49. Chen RA, and Goodman WG. Role of the calcium-sensing receptor in parathyroid gland physiology. *Am J Physiol Renal Physiol.* 2004;286(6):F1005-11.
50. Cil O, Phuan PW, Lee S, Tan J, Haggie PM, Levin MH, et al. CFTR activator increases intestinal fluid secretion and normalizes stool output in a mouse model of constipation. *Cell Mol Gastroenterol Hepatol.* 2016;2(3):317-27.
51. Cil O, Phuan PW, Gillespie AM, Lee S, Tradtrantip L, Yin J, et al. Benzopyrimido-pyrrolo-oxazine-dione CFTR inhibitor (R)-BPO-27 for antisecretory therapy of diarrheas caused by bacterial enterotoxins. *FASEB J.* 2017;31(2):751-60.
52. Cil O, Chu T, Lee S, Haggie PM, and Verkman AS. Small-molecule inhibitor of intestinal anion exchanger SLC26A3 for treatment of hyperoxaluria and nephrolithiasis. *JCI Insight.* 2022;7(13).
53. Subramanya S, Ramakrishna BS, Binder HJ, Farthing MJ, and Young GP. Evaluation of oral rehydration solution by whole-gut perfusion in rats: effect of osmolarity, sodium concentration and resistant starch. *J Pediatr Gastroenterol Nutr.* 2006;43(5):568-75.

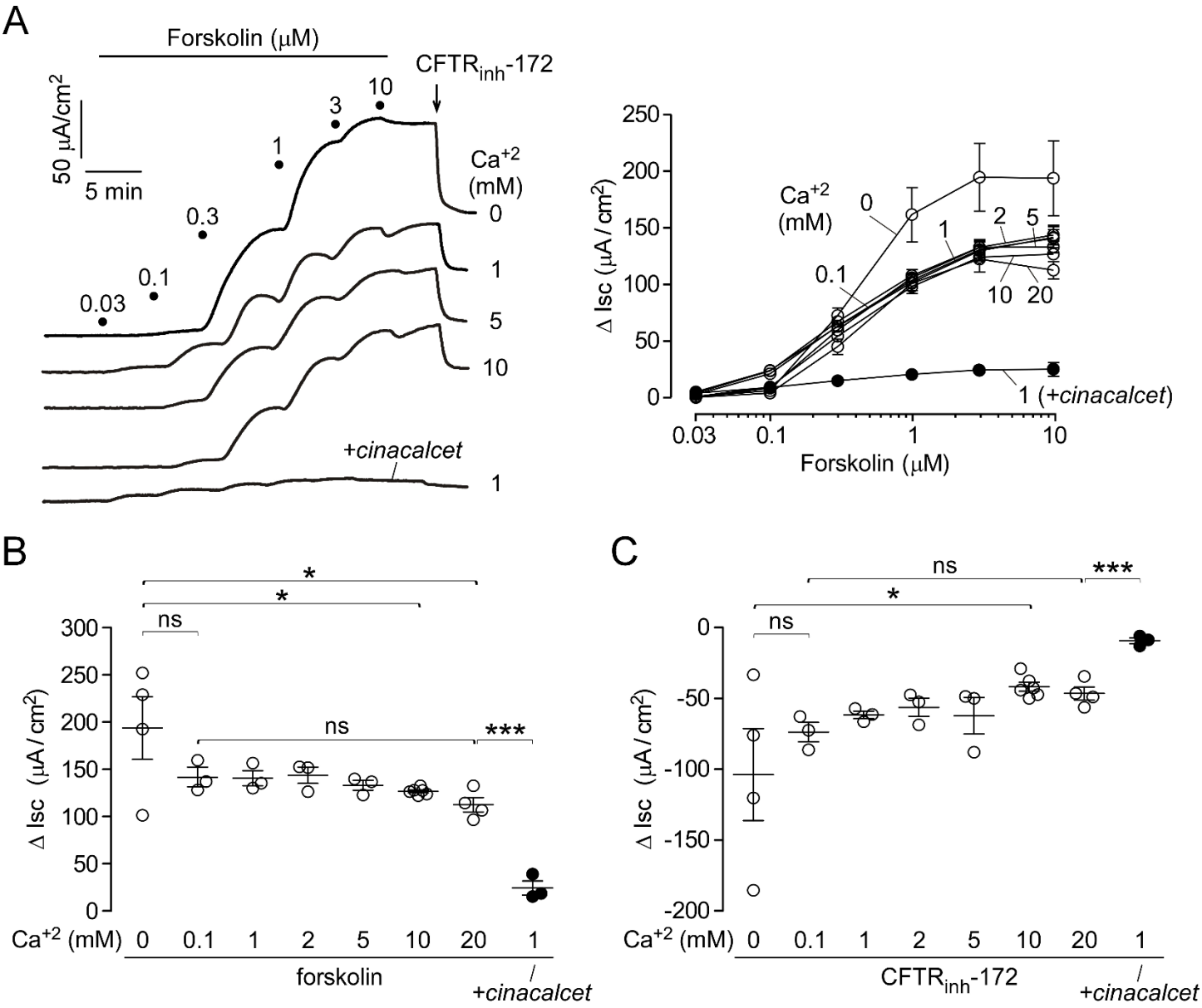


Figure 1

Figure 1. Extracellular Ca^{2+} concentration has minimal effect on forskolin-induced I_{sc} in T84 cells. (A) Short-circuit current (I_{sc}) traces in T84 cells showing forskolin concentration response and $CFTR_{inh}$-172 (10 μM) inhibition following 20 min pretreatment with indicated concentrations of $CaCl_2$ or 30 μM cinacalcet *(left)*. Summary of changes in I_{sc} (ΔI_{sc}) from experiments *(right)*. **(B)** ΔI_{sc} induced by forskolin in the presence of different Ca^{2+} concentrations and cinacalcet. **(C)** ΔI_{sc} induced by $CFTR_{inh}$-172 at in the presence of different Ca^{2+} concentrations and cinacalcet. Mean ± S.E.M., n=3-6 per group, one-way ANOVA with Newman-Keuls multiple comparisons test, *p<0.05, ***p<0.001, ns: not significant.

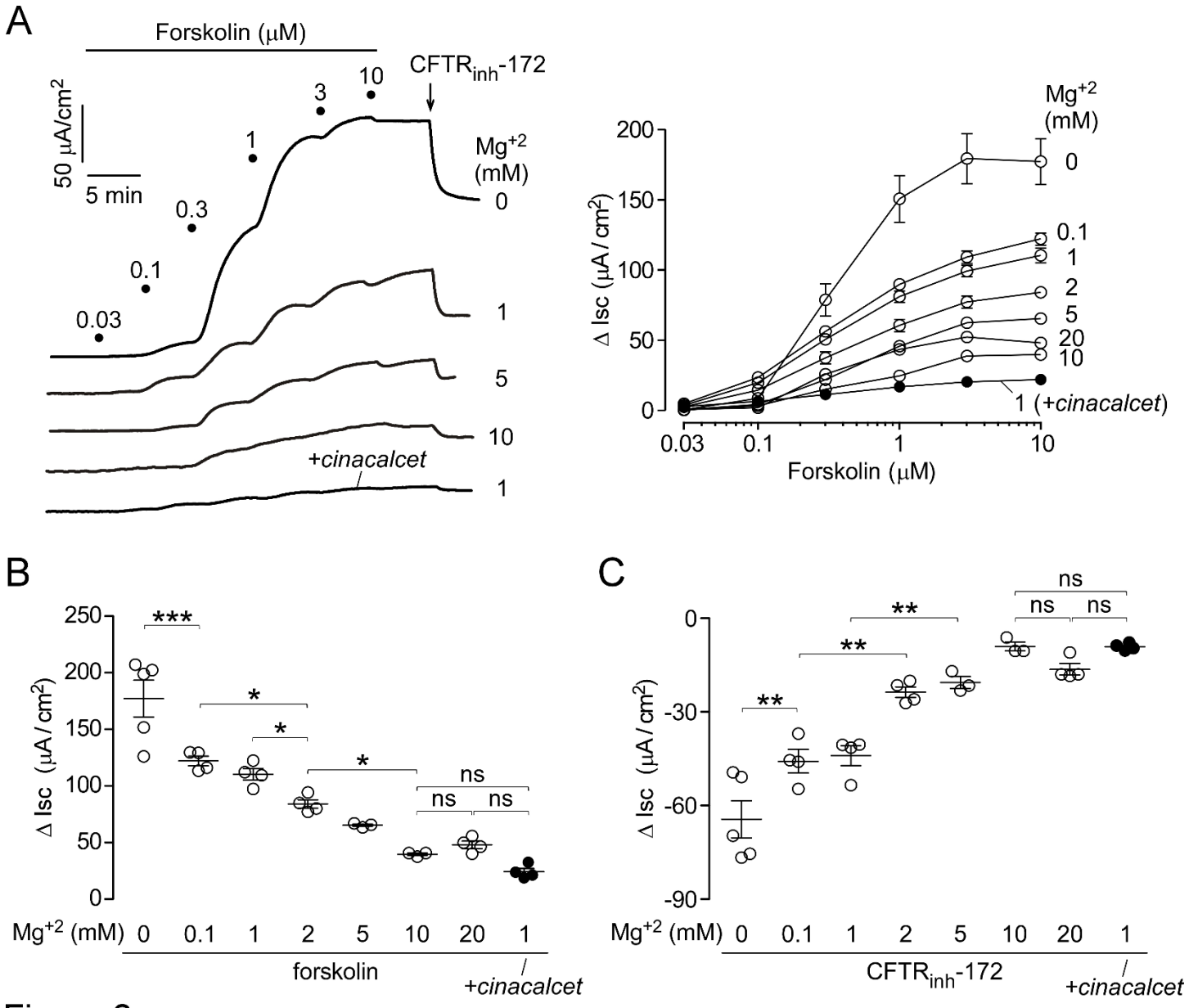


Figure 2

Figure 2. Forskolin-induced I_{sc} in T84 cells is dependent on extracellular Mg^{2+} concentration. (A) Short-circuit current (I_{sc}) traces in T84 cells showing forskolin concentration response and $CFTR_{inh}$-172 (10 μM) inhibition following 20 min pretreatment with indicated concentrations of $MgCl_2$ or 30 μM cinacalcet *(left)*. Summary of changes in I_{sc} (ΔI_{sc}) from experiments *(right)*. **(B)** ΔI_{sc} induced by forskolin in the presence of different Mg^{2+} concentrations and cinacalcet. **(C)** ΔI_{sc} induced by $CFTR_{inh}$-172 at in the presence of different Mg^{2+} concentrations and cinacalcet. Mean ± S.E.M., n=3-5 per group, one-way ANOVA with Newman-Keuls multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ns: not significant.

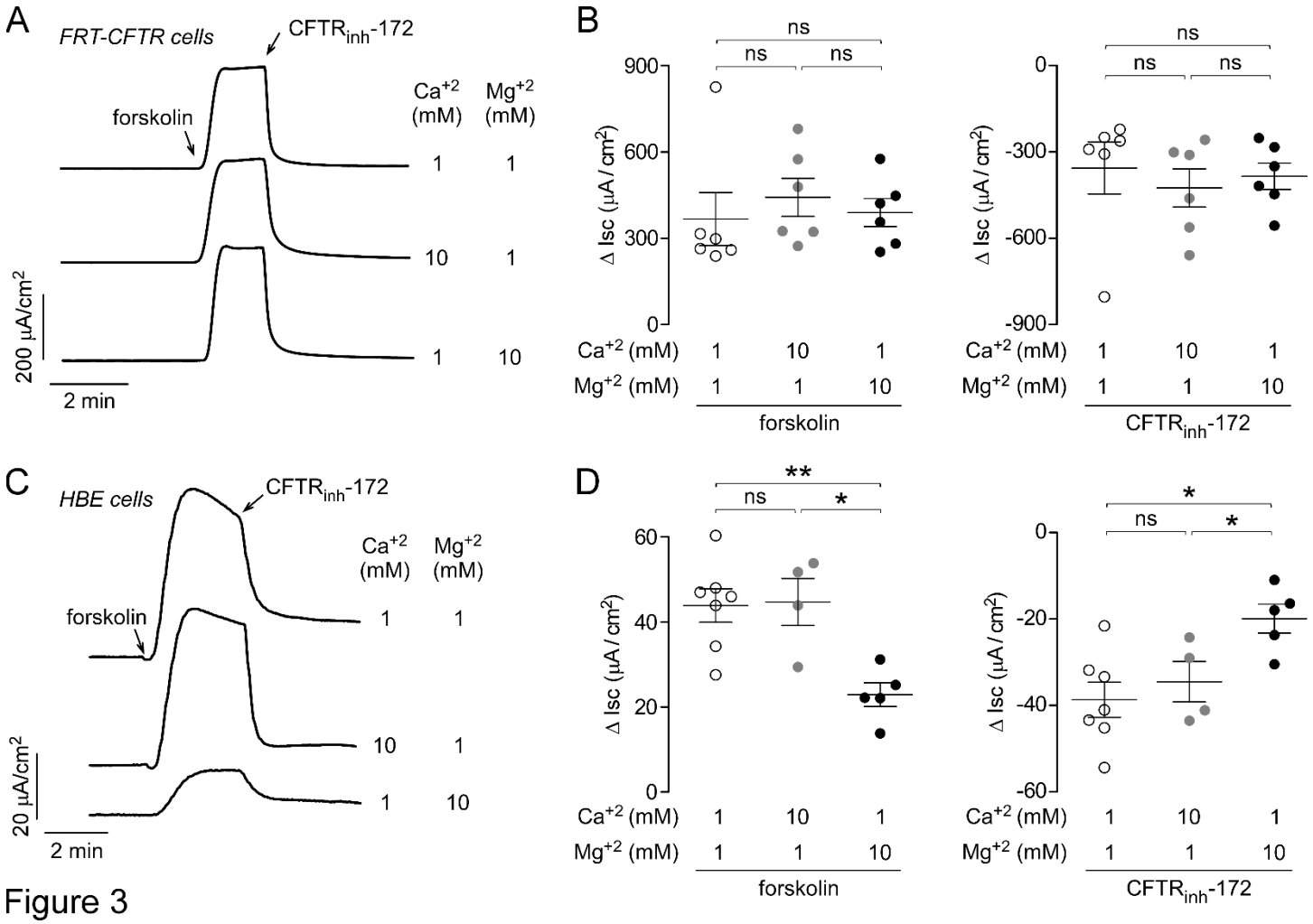


Figure 3

Figure 3. Mg^{2+} inhibition of CFTR-mediated Cl^- secretion is indirect and depends on CaSR activation.

(**A)** Short-circuit current (I_{sc}) traces in CFTR-transfected Fischer rat thyroid (FRT-CFTR) cells, showing responses to maximal forskolin (10 µM) and $CFTR_{inh}$-172 (10 µM) following 20 min pretreatment with indicated concentrations of $CaCl_2$ or $MgCl_2$. **(B)** Summary of changes in I_{sc} (ΔI_{sc}) induced by forskolin *(left)* and $CFTR_{inh}$-172 *(right)* at different Ca^{2+} and Mg^{2+} concentrations. (**C)** I_{sc} traces in human bronchial epithelial (HBE) cells, showing responses to maximal forskolin (10 µM) and $CFTR_{inh}$-172 (10 µM) following 20 min pretreatment with indicated concentrations of Ca^{2+} or Mg^{2+}. **(D)** ΔI_{sc} induced by forskolin *(left)* and $CFTR_{inh}$-172 *(right)* at different Ca^{2+} and Mg^{2+} concentrations. Mean ± S.E.M., n=4-7 per group, one-way ANOVA with Newman-Keuls multiple comparisons test, *p<0.05, ***p<0.001, ns: not significant.

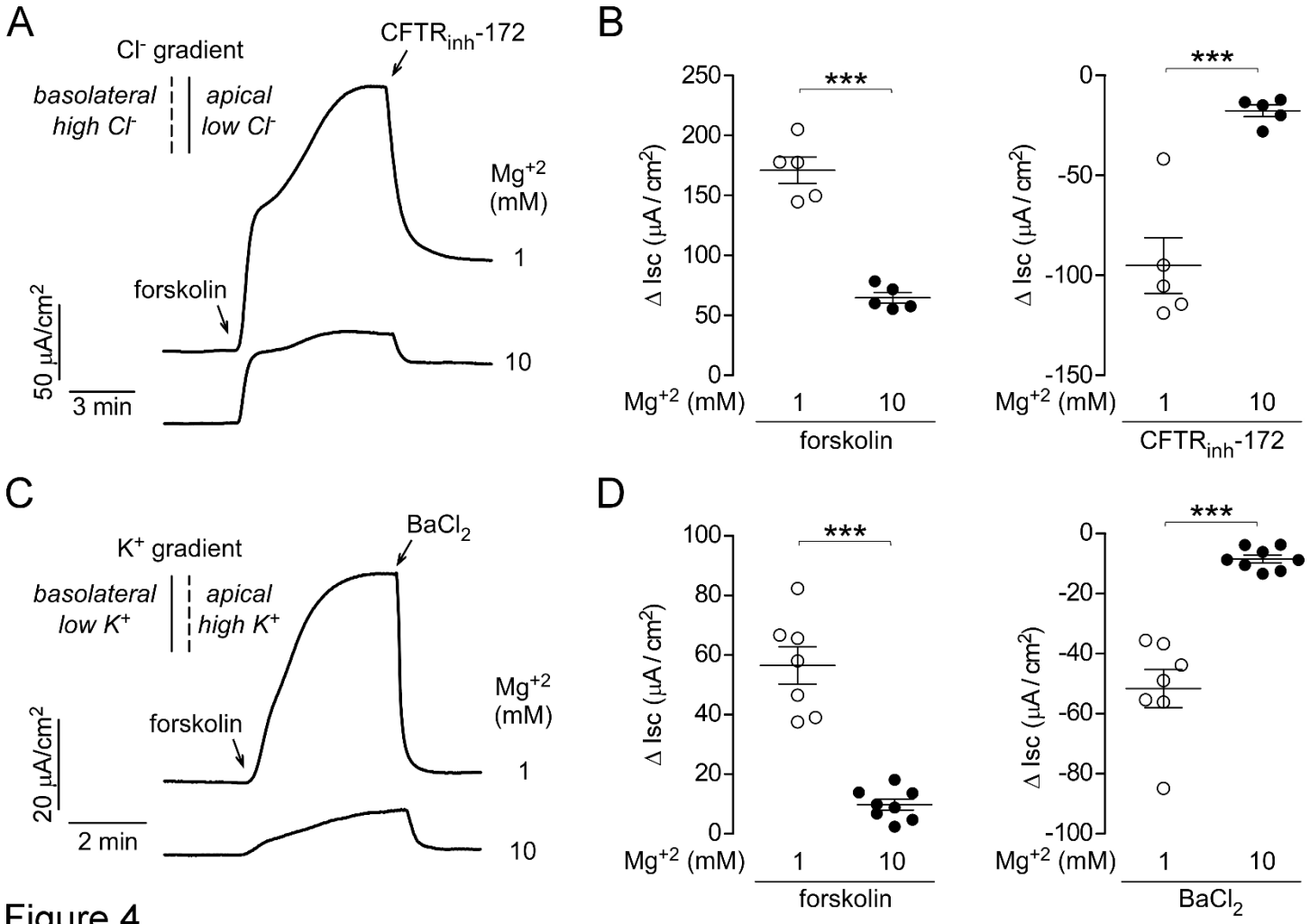


Figure 4

Figure 4. Mg^{2+} inhibits apical membrane CFTR Cl^- channel and basolateral membrane K^+ channels in T84 cells. **(A)** Short-circuit current (I_{sc}) traces in T84 cells with basolateral permeabilization (amphotericin B, 500 µg/mL for 30 min) and 60 mM basolateral-to-apical Cl^- gradient showing responses to 10 µM forskolin and 10 µM $CFTR_{inh}$-172 following 20 min pretreatment with 1 or 10 mM $MgCl_2$. **(B)** Summary of changes in I_{sc} (Δ I_{sc}) induced by forskolin *(left)* and $CFTR_{inh}$-172 *(right)* at 1 or 10 mM Mg^{2+}. **(C)** I_{sc} traces with apical permeabilization (amphotericin B, 20 µg/mL for 30 min) and apical-to-basolateral K^+ gradient showing responses to 10 µM forskolin and 5 mM $BaCl_2$ following 20 min pretreatment with 1 or 10 mM Mg^{2+}. **(D)** Δ I_{SC} induced by forskolin *(left)* and $BaCl_2$ *(right)* at 1 or 10 mM Mg^{2+}. Mean ± S.E.M., n=5-8 per group, unpaired Student's t test, ***p<0.001.

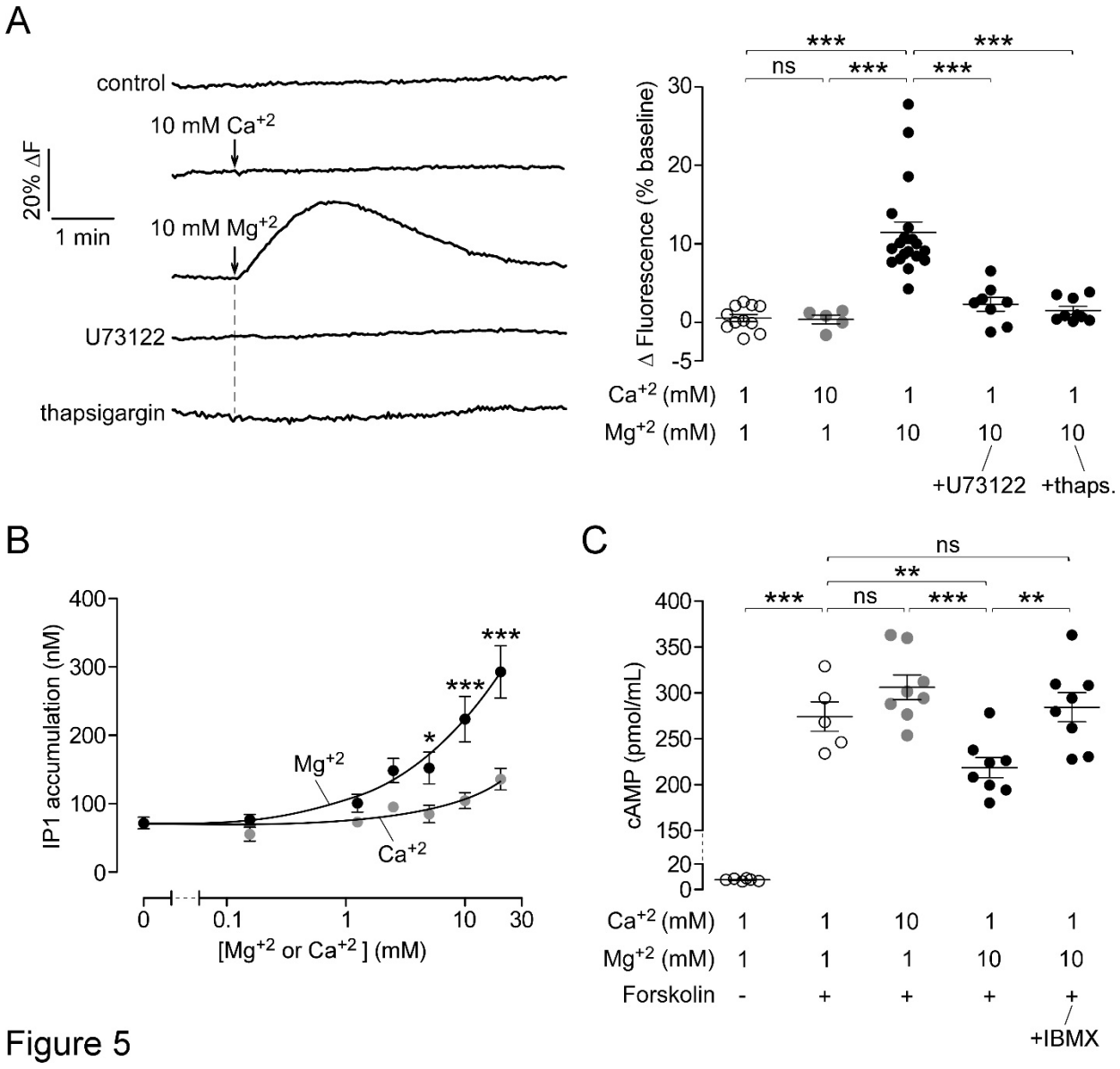


Figure 5

Figure 5. Mg^{2+} increases intracellular Ca^{2+} via Gq and phospholipase C activation, and inhibits forskolin-induced cAMP elevation in T84 cells. (A) Intracellular Ca^{2+} traces measured by Fluo-4 NW fluorescence in T84 cells with vehicle control (0.1% DMSO), $CaCl_2$ (10 mM) or $MgCl_2$ (10 mM) *(left)*. In some experiments, T84 cells were pretreated with PLC inhibitor U73122 (10 μM) or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM) for 10 min before Mg^{2+} addition. Thapsigargin caused a large increase in intracellular Ca^{2+} which is not shown. Summary of data in A presented as maximum changes in Fluo-4 NW fluorescence as percentage of baseline *(right)*. n=5-20 per group. **(B)** IP1 (stable downstream metabolite of IP3) accumulation after 30 min of treatment with 0-20 mM of Ca^{2+} or Mg^{2+}. n=7-11 per concentration, per group. **(C)** cAMP concentration in T84 cell lysates with 10 μM forskolin (±500 μM IBMX, phosphodiesterase inhibitor) treatment in the presence of 20 min pretreatment with indicated concentrations of Ca^{2+} and Mg^{2+}, or vehicle control (0.2% DMSO). n=5-8 per group. Mean ± S.E.M. For panels A and C one-way ANOVA with Newman-Keuls multiple comparisons test was used (**p<0.01, ***p<0.001). For panel B, two-way ANOVA with Bonferroni posttests was used. (*p<0.05 and ***p<0.001 compared to the same concentration of Ca^{2+}, other comparisons are not significant).

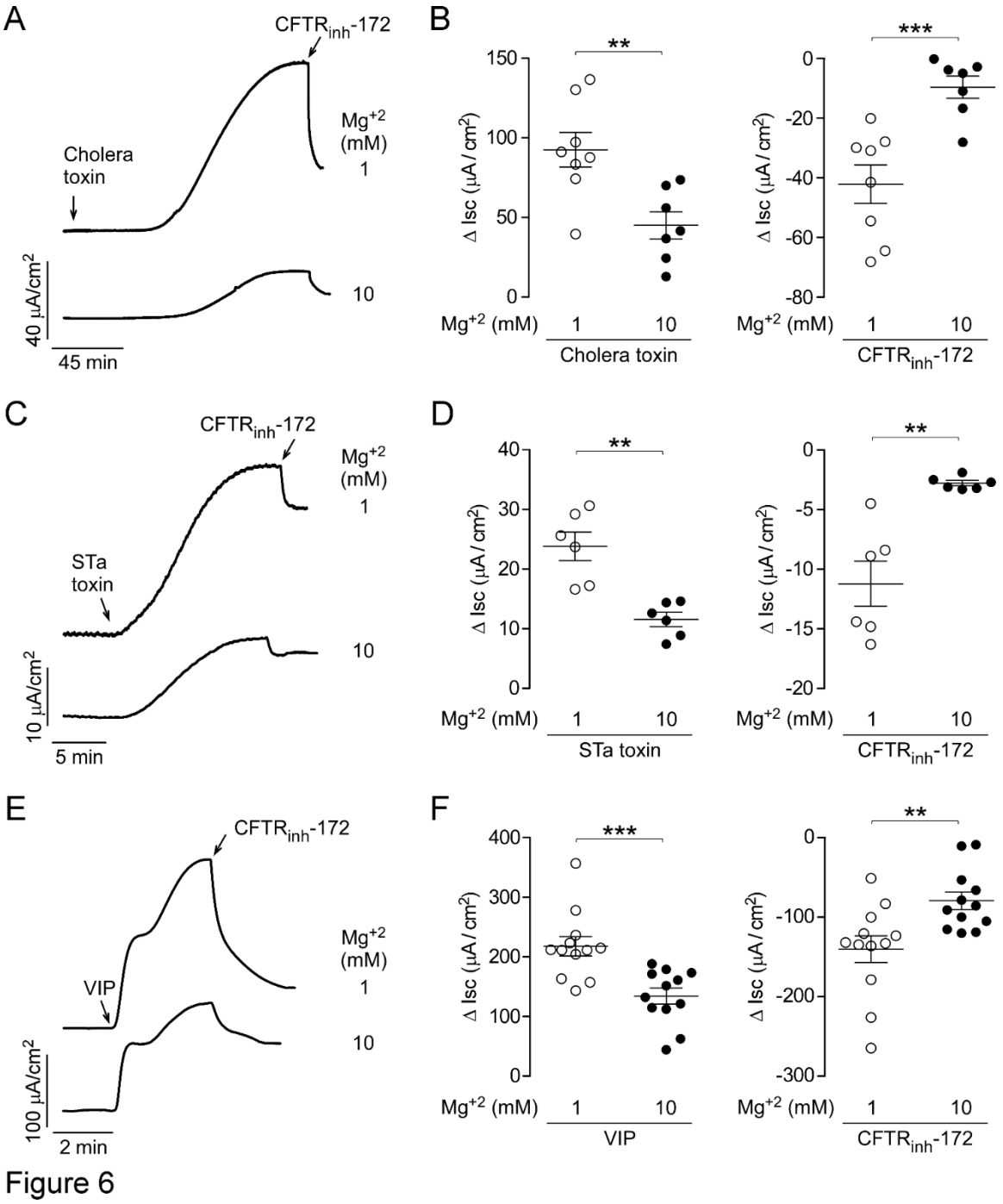


Figure 6

Figure 6. Mg^{2+} inhibits cholera toxin, STa toxin and VIP induced Cl^- secretion in T84 cells. (A) Short-circuit current (I_{sc}) traces showing responses to 1 µg/mL cholera toxin and 10 µM $CFTR_{inh}$-172 with 1 or 10 mM $MgCl_2$ pretreatment for 20 min. **(B)** Summary of data as in A showing changes in I_{sc} (ΔI_{sc}) following cholera toxin *(left)* and $CFTR_{inh}$-172 *(right)*. **(C)** I_{SC} traces showing responses to 0.1µg/mL heat-stable E. coli enterotoxin (STa toxin) and 10 µM $CFTR_{inh}$-172 with 1 or 10 mM Mg^{2+}pretreatment for 20 min. **(D)** Summary of data as in C showing ΔI_{sc} following STa toxin *(left)* and $CFTR_{inh}$-172 *(right)*. **(E)** I_{sc} traces showing responses to 10 nM vasoactive intestinal peptide (VIP) and 10 µM $CFTR_{inh}$-172 with 1 or 10 mM Mg^{2+}pretreatment for 20 min. **(F)** Summary of data as in E showing ΔI_{sc} following VIP *(left)* and $CFTR_{inh}$-172 *(right)*. Mean ± SEM, n=6-12 per group, unpaired Student's t test, **p<0.01, ***p<0.001.

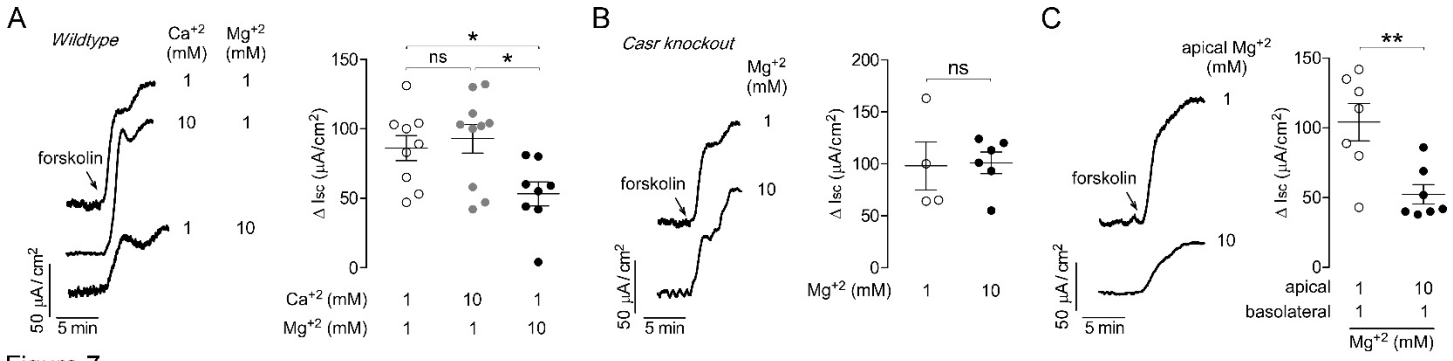


Figure 7

Figure 7. Antisecretory effect of Mg^{2+} in mouse intestine is CaSR dependent. (A) Short-circuit current (I_{sc}) traces *(left)* and summary data *(right)* showing responses to 10 µM forksolin in wildtype C57BL/6 mouse jejunum with indicated concentrations of $CaCl_2$ or $MgCl_2$ pretreatment from both sides for 20 min. **(B)** I_{sc} traces *(left)* and summary data *(right)* showing responses to 10 µM forksolin in jejunum of intestinal epithelia-specific CaSR knockout mice (*Vil1-cre; Casr $^{flox/flox}$*) with 1 or 10 mM Mg^{2+} pretreatment from both sides for 20 min. (**C**) I_{sc} traces *(left)* and summary data *(right)* showing responses to 10 µM forksolin in jejunum of wildtype mice with 1 or 10 mM Mg^{2+} pretreatment from luminal (apical) side for 20 min. For 10 mM Mg^{2+} experiments, solution osmolality was balanced between luminal and basolateral solutions by adding 27 mM mannitol to the basolateral side. Mean ± S.E.M., n=4-10 per group. For panel A, one-way ANOVA with Newman-Keuls multiple comparisons test was used. For panel B and C, unpaired Student's t test was used. *$p<0.05$, **$p<0.01$, ns: not significant.

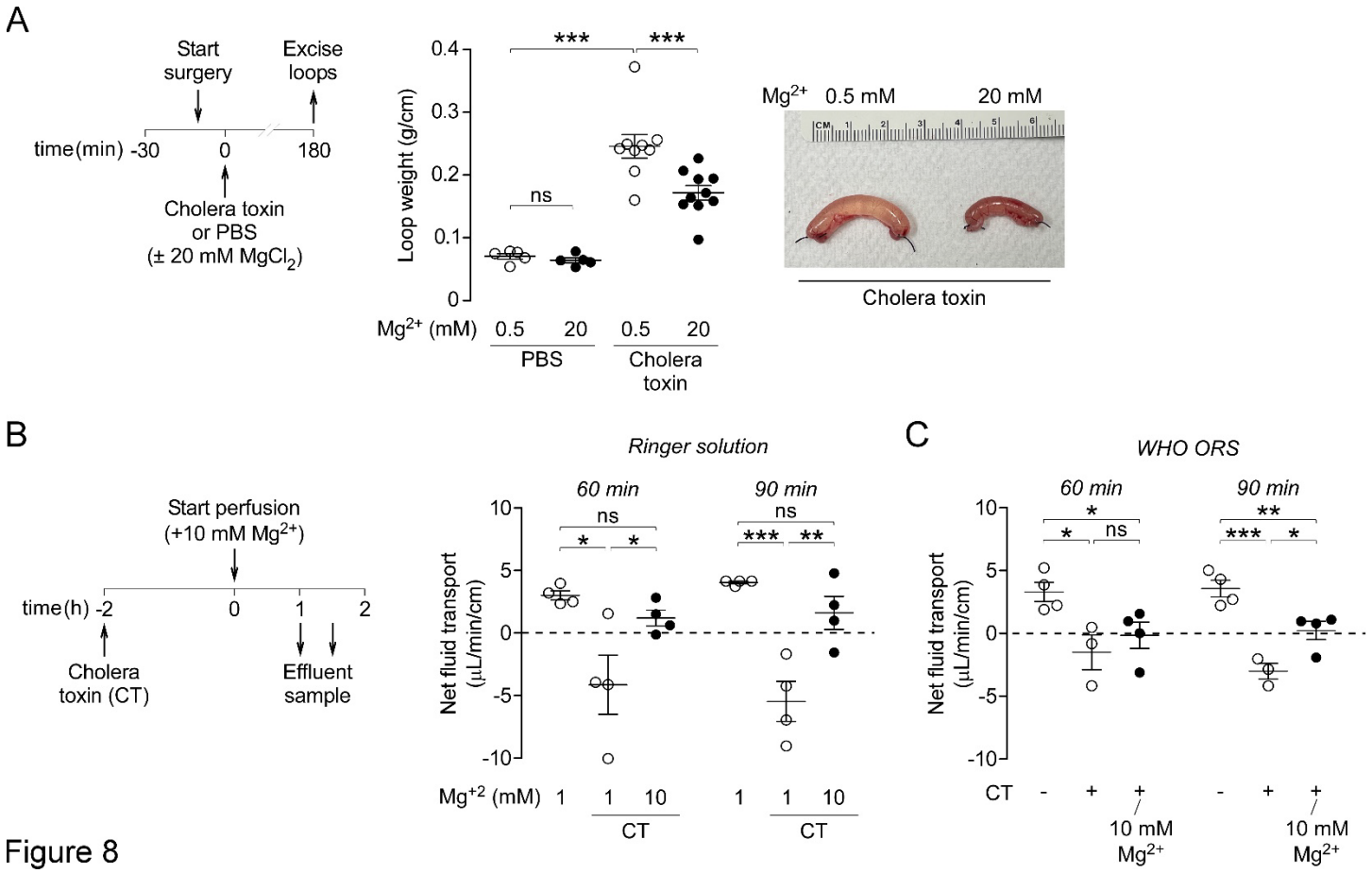


Figure 8

Figure 8. Efficacy of Mg^{2+} in mouse models of cholera. (A) Experimental design (*left*), loop weight/length ratio (*center*) and representative photos of mouse closed-loops (*right*) injected 100 µl phosphate-buffered saline (PBS, ±1 µg cholera toxin) with 0.5 or 20 mM $MgCl_2$. n=5-10 loops per group. **(B)** (*left*) Experimental design of intestinal perfusion model of cholera in mice induced by intestinal instillation of cholera toxin (10 µg/2 mL saline) or saline control at -2 hours. Intestinal perfusion was done with Ringer's solution containing 1 or 10 mM Mg^{2+}. (*right*) Calculated net intestinal fluid transport at indicated time points. (**C**) Perfusion experiments were done as in B, using WHO ORS with and without 10 mM $MgCl_2$. n=3-4 mice per group. Mean ± SEM, one-way ANOVA with Newman-Keuls multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, ns: not significant.

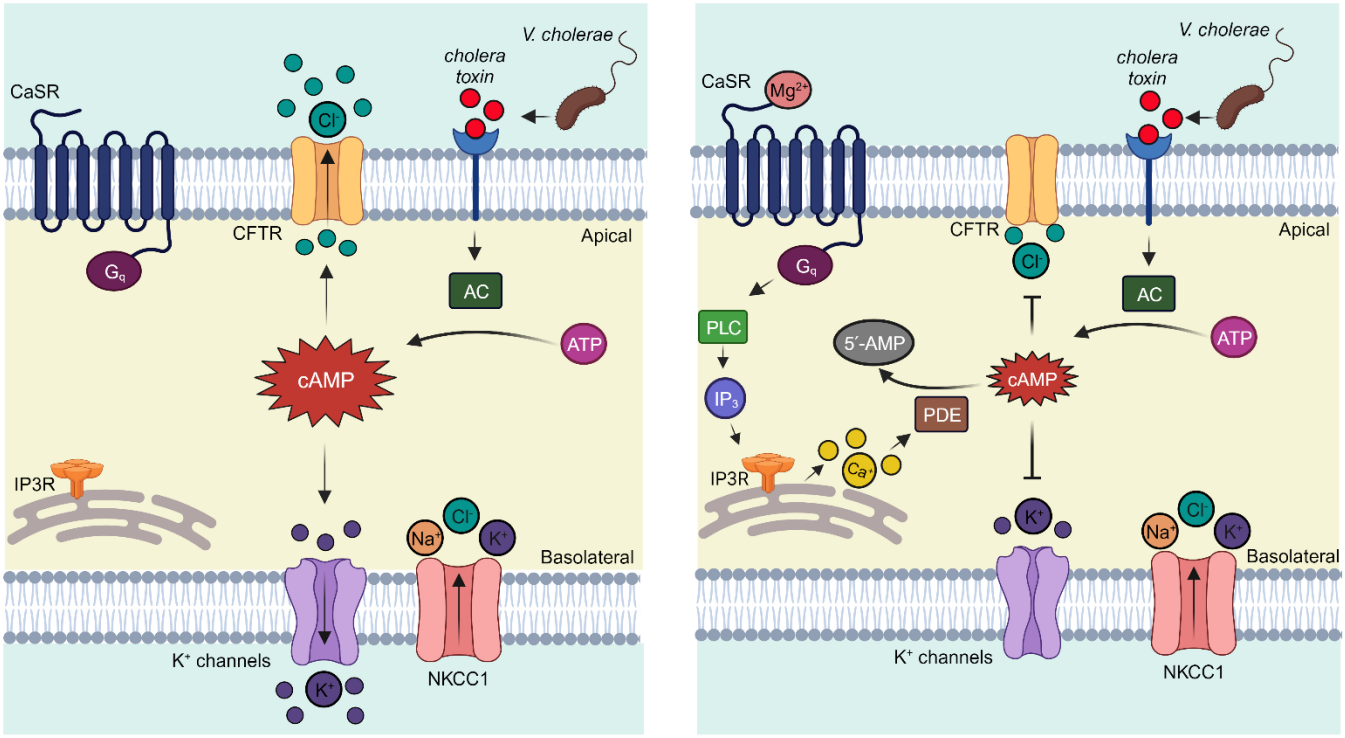


Figure 9. Mechanisms of Mg^{2+} effect in secretory diarrheas. (*left*) Cholera toxin stimulates adenylate cyclase (AC) to elevated cytoplasmic cAMP, which activates apical membrane CFTR Cl^- channel and basolateral membrane K^+ channels to stimulate Cl^- secretion. (*right*) Mg^{2+} treatment activates CaSR which stimulates Gq-PLC-IP3 pathway and promotes cAMP hydrolysis by phsophodiesterases (PDE). This process results in reduced activities of CFTR and K^+ channels, and decreases intestinal Cl^- secretion. Created in Biorender.com.