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REVIEW

Calcium-sensing receptor: A new target for therapy of diarrhea

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Abstract

Management of acute diarrhea remains a global challenge, particularly in resource-limiting countries. Oral rehydration solution (ORS), a passive rehydrating therapy developed approximately 40 years ago, remains the mainstay treatment. Although ORS is effective for hydration, since it does not inhibit enterotoxin-mediated excessive secretion, reduced absorption and compromised barrier function - the primary mechanisms of diarrhea, ORS does not offer a rapid relief of diarrhea symptom. There are a few alternative therapies available, yet the use of these drugs is limited by their expense, lack of availability and/or safety concerns. Novel anti-diarrheal therapeutic approaches, particularly those simple affordable therapies, are needed. This article explores intestinal calcium-sensing receptor (CaSR), a newly uncovered target for therapy of diarrhea. Unlike others, targeting this host antidiarrheal receptor system appears "all-inclusive": it is anti-secretory, pro-absorptive, anti-motility, and anti-inflammatory. Thus, activating CaSR reverses changes of both secretory and inflammatory diarrheas. Considering its unique property of using simple nutrients such as calcium, polyamines, and certain amino acids/oligopeptides as activators, it is possible that through targeting of CaSR with a combination of specific nutrients, novel oral rehydrating solutions that are inexpensive and practical to use in all countries may be developed.

Key words: Secretory diarrhea; Inflammatory diarrhea; Oral rehydration solution; Anti-secretory; Pro-absorptive; Intestinal permeability; Intestinal barrier function; Enteric nervous system; Cholera toxin; *Escherichia coli* heat stable toxin

Core tip: Diarrheal disease remains a leading cause of death in children and the elderly throughout the world. The cause of death is dehydration secondary to severe diarrhea. Intestinal calcium-sensing receptor (CaSR) is a newly uncovered ancient antidiarrheal

receptor system that appears to exert profound effects not only on intestinal secretion, absorption and motility but also on gut permeability and inflammatory responses. Activating this unusual machinery reverses pathophysiological changes of both secretory and inflammatory diarrheas. Considering its unique property of using simple nutrients as activators, it is now possible that through targeting of CaSR and developing novel oral rehydrating solutions that are inexpensive and practical to use in all countries, these diarrhea-associated deaths are reduced or eliminated.

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THERE IS NEED FOR NEW TREATMENTS FOR ACUTE DIARRHEA

Problem

Despite advances and improvements in health care over the past century, diarrheal diseases continue to exert a staggering health burden worldwide, particularly in children and the elderly[1-3]. Globally, there are nearly 1.7 billion cases per year of diarrheal disease in children under 5 years old. Although this number is slightly declining compared to 1.9 billion cases per year 20 years ago[4], it remains a huge challenge. In some developing countries, children may be plagued with 12 or more episodes (a median of 6 episodes) of diarrhea per year by the time they reach 5 years old. In the United States, the costs spent on diarrhea outpatient visits and hospitalizations in between 1993-1996 were $1.2 and 2.2 billion/year, respectively[5]; these costs increased to $3.5 and $4.6 billion/year in between 2001-2006[6]. Adding the costs of ED visits of $1.8 billion/year, the total estimated expense for diarrhea management was nearly $10 billion/year, excluding indirect costs from parents and deaths[6].

Also, diarrheal disease is the second leading cause of child death[1-3]. According to the World Health Organization (WHO), 9%-34% of childhood mortality in developing countries is due to diarrheal diseases[1-3]. Worldwide, approximately 1.5 million people including 620000 children under 5 years old and 320000 adults over 70 years old die each year from diarrhea[3]. Importantly, the majority of these deaths do not result from infection, the most common cause of diarrhea; instead, they are due to associated dehydration, acidosis and other metabolic derangements. These latter are very preventable and treatable. Thus, new methods that effectively reduce the fluid and electrolyte losses from acute diarrhea would offer a major opportunity for improving human health globally.

Success and failure of ORS

Oral Rehydration Solution (ORS) is currently the only oral therapy that is recommended for children with acute diarrhea. A mixture of simple salts and glucose in specific proportions, ORS was developed in 1968 by researchers from U.S. government-funded institutes in Calcutta and Dhaka. This therapy is a result of the basic science discoveries that show that sodium (Na^+) transport and glucose transport are coupled in the intestine so that glucose accelerates fluid absorption. What leads to the proposal and forms the physiological basis for efficacy of ORS in treating diarrhea-associated dehydration is the discovery that the glucose stimulated Na^+ absorption is a cAMP-independent process, which means it remains intact without being inhibited in diarrhea (cholera) while most other absorptive processes are shut down by enterotoxins (*e.g.,* cholera toxin)[7]. Initially, ORS was used as a treatment for cholera. Later, it was found that this solution is efficacious to correcting dehydration by other forms of acute diarrhea, both in adults and in children. Because of the simplicity, inexpensiveness and availability, ORS was rapidly spread worldwide and was used by over 90% of the population who need it. Since it was effective in reducing the morbidity and mortality of acute diarrhea, it was established as the mainstay of therapy for dehydration, especially in developing countries where hundreds and thousands of patients were affected by large-volume diarrhea and IV hydration was not always available. For this reason, the invention of ORS is considered one of the most important medical advancements in the 20th Century. Since its inception, it has saved a minimum of one million lives a year.

Although it is valuable for hydration, ORS neither suppresses intestinal fluid secretion or overly active enteric nerve activity that occurs in secretory diarrhea, nor does it reduce gut permeability or inflammation that are the primary contributors of inflammatory diarrhea. Consequently, ORS does not decrease diarrhea over the short term. It may even paradoxically make diarrhea worse as the patient is rehydrated[8,9]. Because of this perceived failure, caregivers have become reluctant to continue to use ORS but resort to antimicrobial or other agents for treating diarrhea. It is estimated that ORS is currently used by parents and practitioners in only one third of cases that need it[10].

Alternative antidiarrheal therapies

In addition to ORS, there are a few alternative anti-diarrheal therapies in use (Table 1). Based upon their mechanisms of action, these therapies are classified into four types, namely, proabsorptive, antisecretory, antimotility, and anti-inflammatory (Table 1). These four types of therapies target changes in four corresponding host diarrhea-forming mechanisms seen in four types of diarrhea (Figure 1).

Normally, fluid moves across and along the intestine; both processes contribute to diarrhea formation when

Table 1 Current antidiarrheal therapies and their mechanisms of action

Therapies	Mechanisms of action	Comments	Ref.
Proabsorptive			
ORS/RS-ORS	↑glucose/SCFA absorption	Limited efficacy to reduce diarrhea	[98-100]
Antisecretory			
Crofelemer	↓CFTR and CaCC→↓ secretion	Less efficacious as anticipated	[15]
Cholestyramine resin	↓bile salt in lumen→↓secretion	Concerns for vitamin malabsorption	[101]
Bismuth subsalicylate	↓PG synthesis→↓secretion	Safety concerns in children (Reye syndrome)	[102,103]
Antimotility/ENS modulatory			
Loperamide/Diphenoxylate	↑μ opioid receptor →↓motility	Safety concerns in children (ileus)	[13,14]
Hyoscyamine/Dicyclomine	↓Ach action→↓muscle contraction	Safety concerns in children (seizure)	
Alosetron	↓5-HT$_3$R →↓motility and secretion	Safety concerns in children (ischemic colitis)	[104]
Racecadotril	↑encephalin→↓secretion	Not widely available	[105,106]
Clonidine	↑α_2 adrenoceptor→↑absorption	No efficacy and safety study in children	
Anti-inflammatory			
TEN	Unknown mechanism of action	Slow action	[107]
Corticosteroids	Immunosuppression (↓PGs, ↑IL-10)	Concerns for adverse effects	
Anti-TNFα	↓blood and tissue TNFα	Limited by expenses and adverse effects	

ACh: Acetylcholine; CaCC: Ca^{2+} activated chloride channels; CFTR: Cystic fibrosis transmembrane conductance regulator; ORS: Oral rehydration solution; PG: Prostaglandins; RS-ORS: Resistant starch-based oral rehydration solution; SCFA: Short-chain fatty acids; VIP: Vasoactive intestinal polypeptide; TEN: Total enteral nutrition.

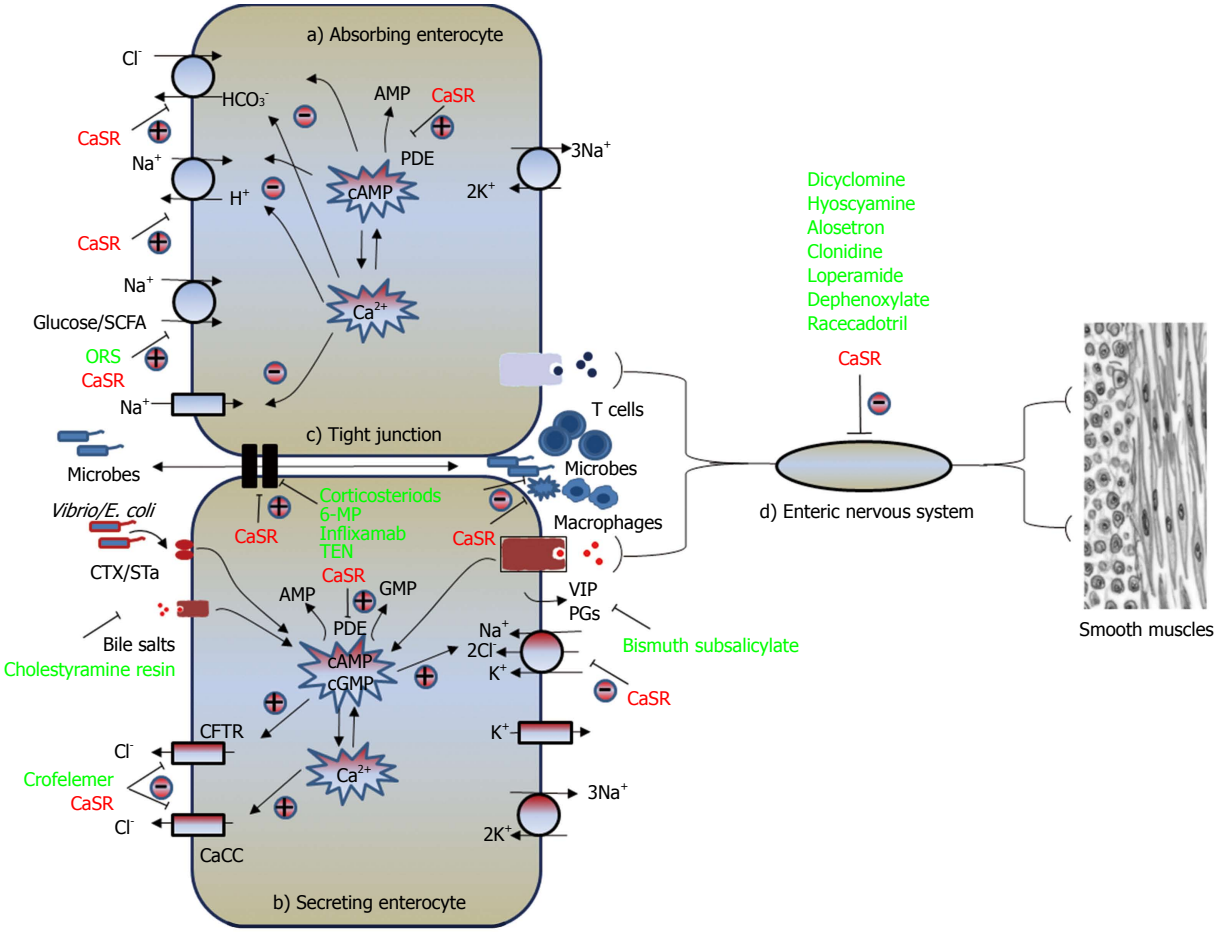

Figure 1 Illustrates the four common pathways leading to formation of diarrhea, including. A: impaired absorption (cause of osmotic diarrhea); B: excessive secretion (leading to secretory diarrhea); C: breakdown of intestinal barrier and enhanced inflammation (implicated in inflammatory diarrhea); D: overly active enteric nervous system (presumed cause of neurogenic diarrhea seen in irritable bowel syndrome). Note that while most current antidiarrheal therapeutics (green-colored) target one individual pathway, CaSR activators (red-colored) have the potential of correcting changes in all the four diarrhea-causing pathways. CaCC: Calcium-activated chloride channel; CaSR: Calcium-sensing receptor; CFTR: Cystic fibrosis transmembrane conductance regulator; CTX: Cholera toxin; ORS: Oral rehydration solution; PDE: Phosphodiesterase; PG: Prostaglandin; SCFA: Short-chain fatty acid; STa: Heat stable toxin; VIP: Vasoactive intestinal peptide.

they are disturbed. While these are inter-connected and not separated processes, the fluid movement across the intestine (absorption or secretion) is driven by active epithelium transport of electrolytes, mainly Na^+, Cl^- and HCO_3^-, and solutes, mainly glucose in the small intestine and short-chain fatty acids (SCFA) in the large intestine (Figure 1), and the fluid moving along the intestine (anterograde or retrograde) is governed by gut motility. Enteric nervous system (ENS), the brain of the gut, controls both processes, with absorption/secretion being primarily controlled by the submucosal Meissner's plexus whereas motility by the myenteric Auerbach's plexus. Diarrhea results when excessive secretion, impaired absorption and/or overly active motility/ENS activity occur. All these changes have been described in secretory diarrheas, exemplified in *Vibro cholera*, enterotoxigenic *Escherichia coli*, and *rotavirus* infections.

A fourth mechanism that leads to formation of diarrhea is compromised intestinal barrier function, a mechanism commonly seen in inflammatory diarrheas (*e.g., Shigella, Salmonella, Campylobacter,* enteroinvasive and enterohemorrhagic *Escherichia coli* infections as well as inflammatory bowel disease). Situated between adjacent intestinal epithelial cells of the mucosa is the apical junctional complex, *i.e.,* the tight junction (Figure 1) and the adherens junction. These intercellular structures along with the layer of epithelium composing the intestinal mucosa act as a barrier separating the luminal contents from the submucosal compartment, which is home of gut immune system. Breaching of this barrier function may result in excessive exposure of submucosal immune system to luminal microbes and foreign antigens, leading to intestinal inflammation. Thus, in addition to the aforementioned mechanisms, breakdown of intestinal barrier is a primary mechanism that causes inflammatory diarrheas. Detailed description of the diarrhea-forming mechanisms can be found in a recent review by Thiagarajah *et al*[11].

While alternative anti-diarrheal therapies, described in Table 1, are helpful for symptomatic treatment of diarrhea, the use of these drugs is limited by their expense, lack of availability, toxicities, and other safety concerns, particularly in pediatric age patients (Table 1). For example, the use of antimotility agents in children should be considered with caution[12-14]. These agents are generally not recommended for any child at any age when acute infectious diarrhea or colitis is suspected as there are reports, albeit rare, of toxic megacolon associated with the use of these agents. Antimotility agents are suspected, though not proven, to increase the risk of hemolytic-uretic syndrome in children with *Escherichia coli O157:H7* infection. Many of the proabsorptive/antisecretory drugs lack pediatric studies determining efficacy and safety (*e.g.,* clonidine) or optimal dosing for children (*e.g.,* racecadotril). Also, some of the newly developed antisecretory drugs (*e.g.,* crefelemer) are not as efficacious as originally anticipated[15].

Use of antibiotics: While anti-microbial therapy is useful in some cases (*e.g.,* dysentery), selection for appropriate antibiotics requires lab detection of the organisms, which is often unavailable. Misuse or abuse of antibiotics can lead to development of resistance, and antibiotics are contraindicated in certain enteric infections (*e.g.,* salmonella). Due to their delayed onset of action, antibiotics do not prevent immediate dehydration; in fact, it is generally not the enteric infections but the dehydration and electrolyte imbalance that cause most of the diarrhea-associated morbidity and deaths.

In addition to diarrhea-related dehydration, repeated diarrheal episodes in children often cause malnutrition, which in turn leads to more severe and more frequent diarrhea. This diarrhea-malnutrition-diarrhea cycle causes almost half of the deaths associated with diarrheal diseases in children under five. Except for TEN (total enteral nutrition), none of the current diarrhea therapies, including the proabsorptive ORS, the antisecretory, the antiinflammatory and the antimotility therapies, have the notable capacity to break this vicious cycle. Therefore, novel anti-diarrheal therapies, particularly those simple nutrient-based "child-friendly" therapeutic approaches are needed.

UNDERSTANDING OF THE PHYSIOLOGICAL ROLES FOR CASR IN GASTROINTESTINAL BIOLOGY MAY LEAD TO THE DEVELOPMENT OF NOVEL COST-EFFECTIVE THERAPIES FOR TREATING DIARRHEA

Extracellular calcium-sensing receptor (CaSR)[16] is a well-conserved ancient G protein-coupled cell surface receptor (GPCR) of class C, originally cloned from bovine parathyroid[16] and subsequently found to be expressed in diverse tissues in mammalian[17], birds[18], amphibians[19,20], and marine species[21,22]. As the name implies, CaSR is a key regulator of tissue responses for calcium homeostasis[16]. Later, it was found that CaSR also plays a crucial role for fluid balance[23,24] and osmotic regulation[21]. The primary physiological ligand for CaSR is extracellular ionized calcium (Ca^{2+}_o), providing a mechanism for Ca^{2+}_o to function as a first messenger. Importantly, CaSR also functions as a general sensor of the extracellular milieu due to allosteric modification of Ca^{2+}_o affinity and efficacy by polyamines, L-amino acids, oligo-peptides, pH and ionic strength[25].

In marine species, CaSR is expressed in the intestine and tissues that are critical for water preservation[21]. There, it acts as a calcium/osmo/salinity-sensor[21], helping fish preserve water from loss to their hyperosmotic surroundings and protect against calcium overload from high calcium sea water[26].

CaSR is also highly expressed in the mammalian

gut, including the transporting epithelial cells[27-30], the fluid/motility-modulating enteric nerves[27,31], and the cells that regulate gut inflammation[32,33]. Over the past several years, studies have shown that the water preserving, or anti-dehydrating function, of CaSR is conserved along with its calcium homeostatic function in terrestrial animals[28,29,31,34-36]. These data strongly support the notion that targeting of CaSR may be a new approach for the development of novel anti-diarrheal therapies.

Activation of intestinal CaSR reverses changes in epithelial transport in diarrhea

Increased anion secretion and decreased salt absorption are two major abnormalities found in electrolyte handling by the intestine during diarrhea, particularly in secretory diarrhea[37,38]. CaSR is expressed in both absorbing surface cells and secreting crypts of the intestine, suggesting critical roles in regulating intestinal absorption and secretion. In enteric epithelial cells, CaSR has been identified on both the apical and basolateral membranes of human[29,39] and rat colonocytes[27,29]. Receptors in both membrane domains of these polarized epithelia are functionally active and can be activated by Ca^{2+}_o[28,29], amino acids and peptides[40,41], polyamines[28,29] and the specific pharmacological CaSR agonist (also called calcimimetic) R568[34].

CaSR agonists inhibit anion secretion

In rat colonic crypts, CaSR activation from either mucosal or serosal side by extracellular calcium, spermine or R568 inhibits net fluid secretion[28,29,34] and cyclic nucleotide accumulation[34] induced by synthetic/natural secretagogues. These secretagogues include forskolin[42] and guanylin[43], which generate cAMP and cGMP, respectively. CaSR activation also blocks the effects of bacterial enterotoxins[34] such as cholera toxin[44], a potent activator of membrane bound adenylyl cyclase leading to elevated intracellular levels of cAMP, and STa[45], which enhances cytosolic cGMP accumulation through the guanylyl cyclase C-type guanylin receptor. Similarly, activation of CaSR by extracellular Ca^{2+} or R568 inhibits net fluid secretion induced by cholera toxin and guanylin in colon mucosa of wild type mice; such effects are abolished in CaSR null mice[34]. Pharmacological inhibitor studies show that these CaSR anti-secretory effects depends on receptor-mediated increases in intracellular Ca^{2+} and require the presence of phosphodiesterase (PDE)[34] suggesting that CaSR activation may reverse secretagogue-stimulated fluid secretion through a signaling pathway that activates phospholipase C (PLC) and degrades cyclic nucleotides by PDE.

CaSR agonists inhibit apical anion channel activity: Fluid secretion is driven primarily by transepithelial anion secretion[37,46,47] (Figure 1). Anion secretion into the lumen of colonic crypts depends on movement of anions across the luminal plasma membrane through anion channels such as cystic fibrosis transmembrane conductance regulator chloride channels (CFTR), and mice deficient in CFTR lack a secretory response to cholera toxin[48]. Secretagogue-induced increases in cellular accumulation of cAMP and cGMP enhance PKA and PKG phosphorylation processes, respectively, which drives translocation of activated CFTR channels to the luminal plasma membrane[37]. Because CaSR agonists reduce cyclic nucleotide accumulation, activation of CaSR would reverse increased apical anion channel activity induced by secretagogues. Indeed, by measuring short circuit current responses to pharmacological inhibitors of anion channels in the apical membrane of colonic mucosa mounted in Ussing chambers, it has been shown that the cyclic nucleotide-dependent NPPB/glibenclamide-sensitive apical anion channel activity is inhibited by activation of CaSR[49], although it remains unknown whether it is directly inhibited by CaSR or indirectly via the reversal of changes in cyclic nucleotide by the activation of the receptor.

CaSR agonists inhibit basolateral anion entry pathway mediated by NKCC1: Equally critical for transepithelial anion (Cl^-) transport during secretagogue-stimulated fluid secretion is increased Cl^- entry into cells from the basolateral fluid via the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (NKCC1; ref[37]). Mice lacking NKCC1 exhibit impaired secretory responses to cAMP and STa[50]. Using perfused colonic crypt model and by measuring Cl^--sensitive MQAE fluorescence, it has been shown that basolateral addition of bumetanide abolishes forskolin stimulated basolateral Cl^- entry into colonic crypt cells consistent with bumetanide inhibition of cAMP activated NKCC1[34]. Addition of R568 to the basolateral fluid also significantly reduces the rate of forskolin-stimulated Cl^- entry[34]. A similar inhibition of Cl^- entry via NKCC1 is seen in the presence of cholera toxin with increasing extracellular calcium[34], demonstrating that activation of the CaSR inhibits NKCC1 activity.

CaSR agonists inhibit secretagogue-induced HCO_3^- secretion: In addition to Cl^- secretion, HCO_3^- secretion is markedly increased in cholera and other secretagogue-induced diarrheal diseases[51,52]. This enhanced intestinal HCO_3^- secretion can result in not only fluid loss and dehydration but also HCO_3^- deficit and metabolic acidosis[51,52], another most common cause (additional to dehydration and systemic volume depletion) of the morbidity and mortality associated with these clinical conditions. To assess if CaSR activation inhibits secretagogue-induced HCO_3^- secretion as it does for the secretagogue-induced Cl^- secretion, CaSR effect is examined in a tissue model (colonic mucosa) of secretagogue-induced

secretory diarrhea. In this study, forskolin is used as a secretagogue to stimulate HCO_3^- secretion, and HCO_3^- secretory response is monitored by measuring HCO_3^- secretory rate (J_{HCO3}) and by recording I_{sc}. Forskolin stimulates both J_{HCO3} and I_{sc} in colon mucosa of rats, wild type mice, and CaSR null mice; subsequent addition of R568 to either luminal or basolateral fluid decreases forskolin-induced HCO_3^- secretion in colon mucosa of rats and wild type mice but not in colon mucosa of CaSR null mice[49]. The results indicate that targeting of CaSR may be useful in arresting not only intestinal Cl^- but also HCO_3^- losses associated with diarrheal diseases.

CaSR agonists enhance absorption

Secretory diarrhea results not only from enhanced fluid secretion, but also from reduced fluid absorption[37,38] (Figure 1). CaSR is expressed in absorbing villus/surface cells, suggesting critical roles in regulating intestinal absorption. To address this, colonic mucosa epithelia from rat and mice are isolated and are used as models[34]. Since both absorption and secretion can occur in the same epithelial cells, to minimize interference from secretion, tissues are first treated with basolateral bumetanide to block secretion before absorption is studied. In the absence of secretagogues, addition of bumetanide to the basolateral fluid of perfused crypts slightly increases the absorptive $^{net}J_V$ due to inhibition of a small remaining fluid secretion. This basal fluid secretion is likely due to the low levels of cell cyclic nucleotides that remain even in the absence of secretagogues. Thus, in the presence of bumetanide, $^{net}J_V$ measurements represent the absorptive component of fluid transport. This absorptive fluid movement is substantially reduced by addition of cAMP or forskolin, which would importantly contribute to secretagogue-induced diarrhea. Either increasing extracellular calcium to 2 mM and/or addition of R568 to the basolateral bath significantly abrogates the cAMP-mediated reduction in fluid absorption, demonstrating that activation of CaSR is able to reverse the reduced absorption caused in secretagogue-induced diarrhea.

CaSR agonists stimulate apical NHE activity: A major component of fluid absorption in the colon (and small intestine) is mediated by parallel Na^+/H^+ (sodium-hydrogen exchanger, NHE)[37,38] and Cl^-/HCO_3^- exchange[56,57] located at the apical plasma membranes. Cyclic nucleotides reduce this Na^+-dependent fluid absorption in ileum and colon by inhibiting NHE activity and Cl^-/HCO_3^- exchange (Figure 1; also ref[11]), and this event contributes importantly to severity of fluid and electrolyte losses in secretory diarrheas[11,37,38]. To examine whether CaSR agonists reverse the cyclic nucleotide diminished NHE activity, the effects of Ca^{2+} or R568 on Na^+-dependent proton extrusion from colonocytes in the presence of forskolin are examined[34]. NHE activity is found significantly increased by raising basolateral bath Ca^{2+} from 0.1 to 2 mmol/L, and addition of R568 to the 2 mmol/L Ca^{2+}-containing bath has resulted in a further increase in NHE activity[34].

CaSR agonists stimulate apical: Cl^-/HCO_3^- exchange. To address whether CaSR regulates Cl^-/HCO_3^- exchange, colonic mucosa are isolated, mounted into Ussing chamber and perfused, and Cl^- absorption mediated by Cl^-/HCO_3^- exchange is then recorded by measuring lumen Cl^--dependent HCO_3^- secretion using pH stat technique[49]. The latter technique measures the amount of acid delivered per unit time per surface area to neutralize the secreted HCO_3^- in order to maintain a constant lumen pH. A luminal Cl^--dependent DIDS-sensitive HCO_3^- secretory mechanism (Cl^-/HCO_3^- exchange) is observed in colon mucosa of rats and mice. Activation of CaSR by R568 stimulates Cl^-/HCO_3^- exchange activity in colons of rats and wild type mice; such an effect is abolished in CaSR null mice[49], suggesting that activation of CaSR also stimulates Cl^-/HCO_3^- exchange.

CaSR agonists stimulate apical SCFA/HCO3-exchange: Short-chain fatty acids (SCFA) are the major anion or solute in stool. SCFA is produced in colon by bacteria fermentation of unabsorbed carbohydrates. SCFA absorption stimulates Na^+, Cl^- and water absorption and this occurs via a process involving apical membrane Na^+/H^+, Cl^-/HCO_3^- and $SCFA/HCO_3^-$ exchanges[53,54]. Thus, SCFA production and absorption represents another major mechanism in the colon to conserve fluid and electrolytes, and is target for some modified forms of ORS (*e.g.*, resistant starch-based ORS). To examine if activating CaSR affects absorption of this physiologically and clinically important solute in the colon, SCFA absorption mediated by $SCFA/HCO_3^-$ exchange has recently been studied by measuring lumen isobutyrate-dependent HCO_3^- secretion using the same Ussing chamber-pH stat technique mentioned above, and its responses to absence or presence of R568 compared[49]. Similar to the R568 effects on Na^+/H^+ and Cl^-/HCO_3^- exchanges, isobyturate-dependent HCO_3^- secretion is found significantly stimulated by R568 in colons of rats and wild type mice but not CaSR null mice[49].

Although the effects on these transporters can be explained by reversal changes in second messengers, as increases in cAMP/cGMP-dependent PKA/PKG activity are associated with phosphorylation, and thereby stimulation, of these transporters in transport epithelia (Figure 1), the stimulation of CaSR also appears to directly affect their function. Besides the aforementioned transporters, apical Na^+ and K^+ channels as well as basolateral K^+ channels and Na^+, K^+-ATPase (Figure 1) also play critical roles in epithelial absorption and secretion. For example, in

Cl^- secreting epithelia, the basolateral K^+ channels facilitate basolateral Cl^- entry (*via* cycling back the K^+ for NKCC1) as well as apical Cl^- exit (by maintaining a favorable transepithelial electrical gradient) whereas the Na^+, K^+-ATPase pumps the Na^+ entered by NKCC1 out of the cell. Whether CaSR also affects these transporters activity and function remains to be determined.

ACTIVATION OF INTESTINAL CASR REDUCES OVERLY ACTIVE ENTERIC NERVE ACTIVITY AND MOTILITY

In humans and rodents, at least 50% of the fluid secreted in cholera, rotavirus, and other forms of infectious diarrhea are caused and mediated by activation of the enteric nervous system (ENS)[38,55-58]. For example, cholera toxin-induced fluid secretion/diarrhea is blocked by tetrodotoxin (TTX)[38,55], an inhibitor of neurotransmission[38,55]. Rotavirus-induced diarrhea is blocked by lidocaine[57, 59], an inhibitor of voltage-gated Na^+ channels in the ENS. This ENS-evoked secretion is also found to contribute to diarrhea formation in patients with irritable bowel syndrome[60], inflammatory bowel disease[61], and intestinal allergies. On this basis, a dual-pathway model for fluid secretion in intestine is proposed: (1) a non-neuronal fluid secretory response due to the binding of enterotoxins directly to enterocytes, leading to generation of cyclic nucleotides, which is TTX/lidocaine-insensitive; and (2) a neuronal secretory response that is mediated by stimulation of the ENS, which is TTX/lidocaine-sensitive. The antidiarrheal CaSR is expressed in both non-neuronal and neuronal tissues, and recent studies have suggested that CaSR agonists also appear to produce their antidiarrheal effects in two ways: (1) direct inhibition of epithelium-mediated diarrheal responses, which is TTX/lidocaine-insensitive (see previous section); and (2) indirectly via inhibiting the ENS, which is TTX/lidocaine-sensitive (see below).

CaSR agonists inhibit ENS-mediated secretion

By measuring TTX-sensitive short-circuit current (I_{sc}) responses of intact ENS-containing colon segments in Ussing chambers, it has been shown that ENS-mediated secretion is abolished by R568[31]. First set of experiments are performed to test diarrhea "treatment" effect of CaSR agonist. In these experiments, forskolin or cholera toxin is added to stimulate secretion before R568 is added. TTX-sensitive I_{sc} is employed as a measure of ENS-mediated anion secretion. Consistent with active regulation of secretion by the ENS, a significant proportion of I_{sc} in the proximal and distal colon is inhibited by serosal TTX, both at basal and under cAMP (forskolin or cholera toxin)-stimulated conditions. TTX-sensitive I_{sc} is substantially increased by addition of forskolin or cholera toxin; subsequent addition of R568 to the basolateral bath abolishes the secretagogue-stimulated TTX-sensitive I_{sc}.

Second set of experiments are performed to test diarrhea "prevention" effect of CaSR agonist. In these experiments, R568 is added prior to the secretagogue stimulation. R568 pretreatment reduces both basal and stimulated secretion. Thus, CaSR agonist may be useful not only for diarrhea treatment but also for diarrhea prevention.

CaSR agonists inhibit motility

CaSR is present in the ENS[27,31], not only in the submucosal Meissner's plexus that mainly controls fluid secretion by the epithelium, but also in the myenteric Auerbach's plexus that is thought to primarily control the gut motility. Thus, CaSR may have important roles in gastrointestinal motility and constipation. Indeed, calcium, the primary ligand of CaSR, is well-known for its constipation-causing effects in humans. People taking high calcium diets are often constipated as are patients with hypercalcemia. Chronic opiate use inhibits the motility of the gastrointestinal tract; opiate withdrawal reverses the inhibited motility, causing diarrhea. In mice, co-administration of calcium and magnesium effectively blocks the signs of morphine withdrawal[62]. Polyamines, another class of agonists for CaSR, are also shown to have a profound impact on the motility[63,64] and are effective in slow down of the gastrointestinal transit in several rodent models of diarrhea-dominant irritable bowel syndrome[65-67]. Using LoxP and Nestin-Cre conditional gene targeting technology, mice lacking the neuronal CaSR in the ENS are generated. We are now characterizing these mice. By comparing fecal pellet output rates, it seems that mice deficient in the neuronal CaSR have enhanced colonic propulsive activity, as evidenced by significantly faster bowel movements than their wild-type littermates (unpublished observation). Also useful of these mice is to determine how much of the effects of calcium and polyamines are mediated through activation of CaSR.

ACTIVATION OF INTESTINAL CASR SUPPRESSES GUT INFLAMMATION

Depending on the presence or absence of inflammation, diarrhea can be pathologically grouped into inflammatory (as observed in enterocolitis caused by *Salmonella* and *Shigella*, as well as inflammatory bowel diseases) and non-inflammatory (*e.g.,* osmotic as seen in lactose intolerance; secretory as seen in cholera, travelers' diarrhea, and rotavirus). The CaSR is also expressed in inflammatory cells, as well as other cells that regulate inflammatory diarrhea[32,33], suggesting a potential protective role in this setting. Indeed, by characterizing the gut-specific CaSR knockout mice, it has been shown that this highly conserved, nutrient-sensing mechanism also plays a critical role in maintaining

intestinal barrier function integrity and reducing gut permeability[35] - central to the pathogenesis of inflammatory diarrhea.

CaSR regulates claudin-2 expression and intestinal barrier function integrity

Mice lacking intestinal CaSR have a decreased colonic expression of tight junction molecules (*e.g.,* claudin-2) and diminished intestinal barrier function, with the transepithelial electrical resistance reduced and the permeability to FITC-dextran increased, the results that are consistent with CaSR regulation of tight junction assembly in cultured MDCK cells[68]. Moreover, microflora composition in CaSR null mice is altered; abundance of beneficial flora (*e.g.,* Lactobacilli and Clostridia) is reduced and of harmful phylum (*e.g.,* Deferribacteres) increased.

CaSR regulates Reg3b and Reg3g expression and bacterial translocation and dissemination

Importantly, mice lacking CaSR have significantly decreased epithelial expression of Reg3b and Reg3g, which encode secreted C-type lectins that bind and protect against translocation and dissemination of Gram-negative[69] and Gram-positive bacteria[70,71], respectively. As a consequence, more bacteria are found to translocate and disseminate into peripheral organs of CaSR null mice compared to CaSR wild type mice, and immune responses (*e.g.,* CD11b+ dendritic cell, Th1 and Th17 responses) are activated and are skewed to pro-inflammatory, both locally and systemically[35].

CaSR regulates Wnt5a-Ror2-TNFR1 expression

The colon is an organ in a constant state of inflammation. The latter is largely controlled by the integrity of intestinal barrier function. In addition to its direct action on intestinal barrier shown in 3.1, CaSR can produce its effect indirectly *via* epithelial receptors for inflammatory mediators[72], such as TNFR1, a known modulator of barrier function. MacLeod has compared wild type and "global" CaSR knockout mice and found that TNFR1 signaling is inhibited by CaSR[72]. This occurs by two distinct mechanisms: CaSR increases secretion of wnt5a from subepithelial myofibroblasts, which interacts with Ror2, an orphan tyrosine kinase and receptor for Wnt5a in epithelial cells and leads to a decrease in TNFR1 expression. CaSR also inhibits secretion of TNFα from macrophages, thereby interrupting TNFR1 signaling.

CaSR regulates intestinal inflammation

Because of the aforementioned anti-inflammatory properties of intestinal CaSR, mice lacking intestinal CaSR are found to have more severe spontaneous and induced colitis compared to their littermate counterparts[35,72].

These studies demonstrate that CaSR is a key molecule expressed in gut epithelial and other cells that contributes to the preservation of intestinal epithelial cell integrity, and maintenance of immune homeostasis in the gut, the disruption of which results in intestinal inflammation. Consistent with this, dietary supplementation with calcium, spermine and tryptophan, activators of CaSR, delay the onset, reduce the severity, and accelerate recovery of animals with DSS colitis[73], whereas inhibition of the receptor by depletion of dietary calcium enhances gut inflammation in animal models of induced colitis[74], even if a recent study suggests a different role for the CaSR in murine bone marrow-derived macrophages/monocytes[75].

EFFECTS OF CaSR -/- MICE

While based on observations made from isolated perfused crypts and intestinal tissues in Ussing chambers CaSR certainly seems to have an array of effects on many intestinal aspects leading to diarrhea, it should be noted that so far there has been no report of any associating mutations or SNPs in CaSR for diarrheal conditions. This may suggest redundancy with other pathways. Given these limitations and the fact that studying these effects in isolation can be artificial, it is critical that future studies should be directed in the organismal and systemic levels using whole animals and mice lacking the CaSR in order to better define the effects of under and over activation of this gene.

Currently, there are three types of CaSR null mouse models that are available: single global, double global, and intestine-specific. Since deletion of the CaSR gene results in early death from the toxic effects of unregulated release of parathyroid hormone (PTH) from parathyroid chief cells as well as from the pathological effects of the consequent hypercalcemia[76], double knockouts with simultaneous ablation of additional PTH gene (as in CaSR-/- PTH-/- double knockout mice[77]) or gene that regulates PTH (*e.g.,* Gcm2 as in CaSR-/- Gcm2-/- double knockout mice[78]) are generated that "rescue" the lethal CaSR-deficient phenotype. Also available are intestinal-specific CaSR knockouts in the floxed mice[79].

Preliminary studies in global[72] and intestinal-specific CaSR knockout mice[35] have shown development of spontaneous intestinal inflammation and exaggerated immune responses in these CaSR-/- animals (see above sections for details). So far, no study has ever provided evidence of developing diarrhea in these animals. Given the redundancy of mechanisms/pathways implicated in regulation of diarrhea formation as well as the global nature and multiple confounding factors involved in double CaSR knockout mice, the use of these global CaSR knockout animals may be difficult to discern the intestinal fluid changes that are attributed to intestinal CaSR. For example, besides being a Ca^{2+}-regulating hormone,

Table 2 Clinical evidence for dietary calcium-sensing receptor activators as antidiarrheals in animals and humans[1]

CaSR agonists	Antidiarrheal efficacy	Ref.
Calcium	↑intestinal resistance, ↓bacterial colonization and translocation to *Salmonella* infection in rats	[81-84]
	↓intestinal permeability in rats	[108]
	↓diarrhea severity in *Salmonella* enterocolitis in rats	[82]
	↓diarrhea onset, ↓severity, ↑ recovery in DSS colitis in rodents	[73,74]
	↓gut permeability & diarrhea in immune-mediated colitis in HLA-B27 transgenic rats	[85]
	↓induced intestinal inflammation in mice	[35,72]
	↓stool volume and duration of diarrheas by viruses or parasites in humans (children)	[36]
	↓stool weight and duration of diarrhea by ETEC in humans (adults)	[87]
	↓diarrhea frequency in patients with calcitonin-secreting medullary thyroid cancer	[88]
Calcium and magnesium	↓intestinal motility and diarrhea symptoms of morphine withdrawal in mice	[62]
Polyamines	↓intestinal motility in mice	[63,64]
	↓gastrointestinal transit and diarrhea of irritable bowel syndrome in mice	[65-67]
	↓DSS colitis in rodents	[73]
Tryptophan	↓intestinal inflammation in mice	[109]
	↓DSS colitis in rodents	[73]

[1]The naturally occurring calcium-sensing receptor (CaSR)activators described are all friendly minerals or nutrients and generally safe. Except for chemically synthesized polyamines, no adverse events other than mild GI discomforts (*e.g.*, constipation[36,88], flatulence[88] and bloating[88]) were reported. DSS: Dextran sodium sulfate; ETEC: Enterotoxigenic *Escherichia coli*.

cAMP-stimulating PTH also activates CFTR-mediated anion secretion in intestinal epithelial cells[80]. Thus, both CaSR-/- PTH-/- and CaSR-/-Gcm2 -/- double mice deficient in both cAMP-stimulating PTH and cAMP-inactivating CaSR may not be developing diarrhea. Additionally, in order to sustain their systemic serum calcium these animals are normally maintained in high calcium diet, which may well generate constipation *via* CaSR independent mechanisms. Accordingly, use of conditional CaSR knockout mice for characterization of intestinal fluid movement would be more appropriate. In this regard, hyperplastic elongated secreting crypts have been noted in intestinal epithelium-specific CaSR-deficient mice[79]. In a preliminary study, we show that mice deficient in neuronal CaSR display enhanced colonic propulsive activity, as evidenced by significantly faster bowel movements than their wild-type littermates (unpublished observation). While the result from the former study is in keeping with the anti-secretory effect of epithelial cell CaSR, the data from the latter are consistent with the active control of colonic motility by neuronal CaSR. We are now performing studies to further characterize these animals both under basal *vs* challenged conditions with *vs* without presence of CaSR activators and inhibitors.

In summary, intestinal CaSR is an antidiarrheal GPCR receptor in the gut that, when activated, appears to exert profound effects not only on intestinal secretion, absorption and motility but also on gut permeability and inflammatory responses. As such, activating intestinal CaSR may reverse changes in both secretory and inflammatory diarrheas in animals and humans.

DATA FROM ANIMALS AND CLINICAL TRIALS ON HUMANS

Indeed, in rodents, increased dietary calcium intake is found to reduce diarrheas that are caused by infectious pathogens (*e.g.*, *Salmonella* enterocolitis[81-84]) or induced chemically (*e.g.*, DSS colitis[73]) or immune-mediated (*e.g.*, colitis in HLA-B27 transgenic rats[85]). In contrast, lowering dietary calcium intake in mice was found to increase the severity of diarrhea, at least in DSS colitis[74] (Table 2).

Three human studies also support the concept that CaSR agonists have the anti-diarrheal potential. The 1st study has tested the primary agonist calcium in children with viral or parasitic diarrhea[36]. These children are immune-compromised, and present with persistent enteric infections, hypocalcemia, and protracted diarrhea. When hypocalcemia is corrected, diarrhea stops[36]. In this study, calcium equivalent to 1x RDA (recommended daily allowance) is used. Within 12-24 h following administration, diarrhea stops or significantly reduces. It is safe within the period of 10 d treatment without causing hypercalcemia[36]. In keeping with the unique CaSR agonist-induced receptor feed-forward mechanism[86] (also see below for further explanation), calcium therapy can be repeated in a same patient multiple times without reduction in efficacy or development of resistance. Although the number of the patients tested in this study is small, the result proves the principle.

The 2nd study is a randomized controlled trial in young adult volunteers who ingest attenuated live enterotoxigenic *Escherichia coli* (ETEC)[87]. In this study, 32 subjects are randomized to receive placebo or calcium (about 1x RDA, in the form of cow's milk) for 10 d before they are infected by ETEC. Diarrhea develops in both groups. However, diarrhea recovers significantly faster in calcium treated vs untreated groups: it recovers within one day in calcium treatment group *vs* more than two days in the placebo control group. The authors have also generated evidence that the bulk of the calcium ingested remain in the lumen

of the gut unabsorbed, producing local effect, and are subsequently excreted in feces. Urinary calcium measurements do not show any significant difference between the two groups, demonstrating the safety of this treatment. The only adverse effect associated with this treatment is mild reversible constipation. Neither hypercalcemia nor kidney stone formation are evidenced.

Secretory diarrhea is common in hormone-secreting neuroendocrine tumors. Recently, a group from M.D. Anderson Cancer Center in Houston, TX has tested the efficacy of oral calcium (in the form of aluminosilicate salt) in reducing secretory diarrhea associated with calcitonin-secreting medullary thyroid cancer[88]. Of the 7 patients evaluated, 5 have considered calcium antidiarrheal a success. The mean number of bowel movements/day is reduced from baseline by 7%-99%. Adverse effects are mild and include flatulence, bloating, heartburn, and constipation.

Despite the excitement and promise of the proof-of-concept studies, so far, no human studies have tested antidiarrheal efficacy and safety of CaSR pharmaconutritional agonists other than calcium. Clearly, more randomized controlled trials are warranted on this new simple and promising antidiarrheal therapy.

TRANSLATION ADVANTAGES OF CASR-BASED ANTIDIARRHEAL THERAPEUTICS

Compared to other antidiarrheal therapies in use and in development, CaSR-based antidiarrheals have many advantages. First, as discussed in aforementioned sections, CaSR is an inclusive antidiarrheal mechanism that is both anti-secretory and pro-absorptive while being anti-motility and anti-inflammatory. This is in contrast to other antidiarrheal agents that target only one individual transporter or diarrhea-causing pathway (Figure 1). Accordingly, activating this mechanism may reverse pathophysiological changes of both secretory and inflammatory diarrheas.

Second, CaSR is a unique GPCR that uses simple nutrients as agonists. It uses calcium as a primary or orthosteric activator and polyamines, L-amino acids, and oligo-peptides as secondary or allosteric activators. Thus, unlike most other GPCRs, CaSR must function in and respond to the continuous presence of nutrients/activators. This is possible because CaSR adopts unusual mechanisms regulating its receptor expression, trafficking, and degradation[86]. For example, instead of inducing internalization and causing desensitization in other GPCRs, continuous elevation of the primary activator/ligand or addition of allosteric activators is found to increase the number of plasma membrane-localized CaSRs and sensitizes receptor function[86]. This unique property of not inducing receptor desensitization would make CaSR agonists potentially useful as potent antidiarrheals. Indeed, addition of as low as 1 pmol/L of R568 to the lumen or bath perfusate of perfused colonic crypts is found to inhibit the forskolin-stimulated fluid secretion, with the EC_{50} values being only 5 (if added luminally) to 20 (if added from blood side) pmol/L[34]. Also, raising $[Ca^{2+}]_o$ to a slightly supra-physiological concentration of 2 mmol/L, either luminally or basolaterally, completely reverses the forskolin or cholera toxin-induced cyclic nucleotide accumulation[34] and secretion[29,34]. Similar potency and efficacy are observed for polyamines[28]. For example, in the presence of physiological or near physiological concentrations of 0.5-1 mmol/L Ca^{2+}_o, as low as 1 nmol/L of spermine, added luminally or basolaterally, is able to reverse the secretagogue-induced secretion[28], with the 50% of maximal reversal effect (EC_{50}) of the polyamine being achieved in only 0.5-2 μmol/L[28]. The latter are the polyamine concentrations most often seen in breast milk[89-91] but not in infant formulas [in which the polyamine concentration is at least 1 order of magnitude lower than in breast milk and 2-3 orders of magnitude lower than the polyamine concentration in the lumen of the intestine shortly after ingestion of a typical adult human meal (see reviews[92-94]). Thus, it is conceivable that supplementation of ORS or infant formulas with polyamines and/or other CaSR agonists may be beneficial in treating children with diarrhea and is currently under investigation. A significant challenge for drugs targeting the enterocyte extracellular surface is convective washout in which secreted fluid in intestinal crypts washes away antidiarrheal drugs, preventing the drugs from diffusing into the target in the surface of enterocyte to effect. To overcome this barrier, an orally administered, surface-targeted antidiarrheal agent requires high drug affinity or low EC_{50} to its target in order to obtain sufficiently high luminal drug concentration (usually > 100-fold EC_{50})[95]. In this regard, the calcimimetic R568 and the polyamine have extremely low EC_{50} values, and washout would not be a concern.

Third, in contrast to many other GPCRs that exist in either an "on" or "off" conformation, there is evidence that CaSR adopts multiple active conformations stabilized by different agonists to generate a set of distinct intracellular signals and biological effects[96]. Consequently, super-agonism (*i.e.,* more than 100% efficacy) and biased-agonism (*i.e.,* selective activation or inactivation of one function over others) may occur when a combination of different agonists are used to influence the receptor function. These are important because the unusual properties enable exploration of different CaSR agonist combinations to design an ideal anti-diarrheal therapy - a therapy that produces maximal therapeutic and minimal unwanted outcomes[97].

Finally, naturally occurring nutrients represent an attractive source of antidiarrheal therapeutics, because they are safe, widely available and generally inexpensive, and have the potential for rapid translation

into clinical practice. The CaSR agonists are naturally occurring nutrients. Because they are all child-friendly, they may be particularly useful in pediatric population. Malnutrition is one of the sequelae of diarrhea and contributes significantly to the morbidity and mortality. Except for TEN (total enteral nutrition), none of the current diarrhea therapies has the notable capacity of treating this important diarrheal complication. Considering the unique property of CaSR using simple nutrients as activators, it is now possible that through targeting of CaSR with child-friendly nutrients, used alone or in combination with the calcimimetics, diarrhea and malnutrition may both be treated.

CONCLUSION

Based upon recent studies, it appears that the intestinal calcium-sensing receptor (CaSR) has an inclusive antidiarrheal function. Activating CaSR is anti-secretory, pro-absorptive, anti-motility, and anti-inflammatory. As such, diarrhea therapies that are based on CaSR may have potentials in reversing pathophysiological changes of both secretory and inflammatory diarrheas and reducing morbidity and mortality associated with these diarrheal diseases. Considering its unique property of using simple nutrients as activating ligands, it is now possible that through targeting of CaSR with nutrients, alone or in combination with calcimimetics, novel oral rehydrating solutions that target central diarrhea-forming pathways that are inexpensive and practical to use in all countries can be developed. Clearly, randomized clinical trials using these pharmaconutritional agonists are warranted.

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