Calcimimetic acts on enteric neuronal CaSR to reverse cholera toxin-induced intestinal electrolyte secretion

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Acute secretory diarrheal illnesses, such as cholera, affect millions of children and adults each year and morbidity and mortality from the large fluid and electrolyte losses remain high1,2. The extracellular calcium-sensing receptor (CaSR)3 is an ancient G protein-coupled cell surface receptor that is expressed in diverse tissues in mammalian4 and marine5 species and is a key regulator of tissue responses required for calcium homeostasis3, salt and water balance5, and osmotic regulation5. The primary physiological ligand for the CaSR is extracellular ionized calcium ([Ca2+]o), providing a mechanism for [Ca2+]o to function as a first messenger. The CaSR also functions as a more general sensor of the extracellular milieu due to allosteric modification of [Ca2+]o affinity by polyamines, L-amino acids, pH and ionic strength8.

Enteric mucosal cells along the entire small and large intestines express the CaSR. These include enterocytes9–12, neurons in the enteric nervous system (ENS)9,13 and enterochromaffin (EC) cells14 and other enteroendocrine (EE) cells (refer to a recent review by Tang et al.15 for refs). Studies that characterize the physiologic function of the CaSR in the gastrointestinal (GI) tract have just begun15,16. CaSR has been identified on both the apical and basolateral membranes of human11,17 and rat colonocytes9,11. Receptors localized to both membrane domains of this polarized epithelium are functionally active and can be activated by [Ca2+]o, calcimimetics such as R568, and other polycations such as spermine with similar potency and EC50 values10,11. In isolated microperfused colonic crypts, CaSR activation from either the mucosal or serosal side inhibited net fluid secretion10,11,18 and cyclic nucleotide accumulation18 induced by synthetic/natural secretagogues such as forskolin and guanylin, which generate cyclic AMP and cyclic GMP, respectively. CaSR activation also blocks the effects of bacterial enterotoxins18 such as cholera toxin (CTX), a potent activator of membrane-bound adenyl cyclase leading to elevated intracellular levels of the cyclic AMP. It also blocks the effects of heat stable Escherichia coli enterotoxin (STa)18, which enhances cytosolic cyclic GMP accumulation through the guanylyl cyclase C-type guanylin receptor. CaSR has also been localized to the neurons in the ENS9,13, the brain of the gut18, and thus could regulate neural secretory responses. However, studies that characterize the role of CaSR in the enteric neurons have been limited.

In rodents and humans, the ENS, mainly the submucosal plexus, releases neurotransmitters [e.g., vasoactive intestinal peptide (VIP) and acetylcholine (ACh)] that regulate fluid secretion (see reviews20-22). There is compelling evidence from in vivo experiments that the ENS modulates intestinal fluid secretion induced by

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bacterial enterotoxins (e.g., CTX, STa)\textsuperscript{21–23}, as well as by viral enterotoxins [e.g., rotavirus nonstructural protein 4 (NSP4)]\textsuperscript{24,25}. For example, cholera toxin-induced fluid secretion is blocked by tetrodotoxin (TTX) or lidocaine, inhibitors of neural activity\textsuperscript{26}, or hexamethonium, an inhibitor of cholinergic nicotinic receptor antagonist\textsuperscript{27}. Based upon this, a dual pathway model for fluid secretion and diarrhea formation has been proposed: (1) a non-neuronal fluid secretory response due to binding of enterotoxins directly to enterocytes, leading to generation of cyclic nucleotides, which is TTX/lidocaine-insensitive; (2) a neuronal secretory response that is mediated by stimulation of the neurons in the ENS, which is TTX/lidocaine-sensitive. The calcimimetic R568 is a specific CaSR agonist. Using isolated rat colon segments containing intact ENS mounted in Ussing chambers, we have previously shown that R568, when applied serosally, inhibited basal and forskolin-induced secretory currents in the absence, but not presence, of TTX\textsuperscript{13}. This suggests that this anti-secretory agent acts on the intestine through neurons within the ENS. Although the finding from this latter study on calcimimetic is consistent with the dual-pathway regulation model, it is also possible that R568’s anti-secretory effect results from its binding to the receptor in the basolateral membrane of mucosal epithelial cells. Furthermore, R568 might inhibit TTX/lidocaine-dependent secretory current indirectly, by acting on the CaSR on mucosal epithelial cells that then interact with TTX/lidocaine-sensitive enteric neurons. Finally, there is a small likelihood that R568’s anti-secretory effect is due to a non-specific off-target effect of this agent\textsuperscript{28}.

To test the hypothesis that R568 acts on neuronal CaSR of the ENS to inhibit electrolyte secretion by the intestine, the present study employed a tissue-specific knockout mouse approach in conjunction with Ussing chamber methods for further studying the effect of this calcimimetic on basal and secretagogue-evoked intestinal secretion. In addition to forskolin, we also employed cholera toxin. We selected cholera toxin because it is a secretagogue known to activate enteric neuronal reflexes\textsuperscript{21}. The two tissue-specific CaSR mutant mouse lines used were: (1) Mucosal epithelial cell CaSR-specific conditional knockout mice (\textit{villin\textsuperscript{Cre/Casr\textsuperscript{flox/flox}} mice})\textsuperscript{29}, and (2) Neuronal cell CaSR-specific conditional knockout mice (\textit{nestin\textsuperscript{Cre/Casr\textsuperscript{flox/flox}} mice})\textsuperscript{30}. Our results show that CaSR activity on enteric neurons is required for R568-mediated inhibition of cholera toxin-induced electrolyte secretion by the intestine. This is the first demonstration that the CaSR expressed by enteric neurons is functional. Thus, apart from their direct mucosal epithelial effects shown in our previous studies using ENS-absent colonic epithelial crypts\textsuperscript{10,11,18}, our present data show that the pro- and anti-secretory effects of cholera toxin and R568 on electrolyte secretion by the intestine could also be mediated, indirectly, through opposing actions of the two agents on the activity of the ENS.

**Results**

**Calcimimetic inhibits basal $I_{sc}$ in mouse colon via CaSR on enteric neurons.** Under basal conditions, mucosal distension during normal digestion or mechanical stimulation during experimental preparations can activate neuronal reflex pathways in the ENS, evoking Cl$^-$ secretion\textsuperscript{20,31}. This reflex-evoked Cl$^-$ secretion can be measured in Ussing chamber experiments as increases in TTX/lidocaine-sensitive short-circuit current ($I_{sc}$)\textsuperscript{13}. To test the hypothesis that CaSR is expressed on enteric neurons to restrict neurally mediated secretory responses, we generated a neuronal tissue-specific CaSR conditional knockout mouse line (i.e., \textit{nestin\textsuperscript{Cre/Casr\textsuperscript{flox/flox}} mice or nestin mice}) using Cre recombinase. For comparison, we also generated an epithelial tissue-specific CaSR conditional knockout (i.e., \textit{villin\textsuperscript{Cre/Casr\textsuperscript{flox/flox}} mice or villin mice}). Colons from these knockouts were isolated, mounted onto Ussing chambers, and Cl$^-$ secretory $I_{sc}$ responses to R568 were measured and compared with their wild-type littermates. The results are shown in Fig. 1. Consistent with the presence of neurogenic Cl$^-$ secretion, a large TTX/lidocaine-sensitive $I_{sc}$ was detected in these mouse colons. In wild-type mice, R568 (10\,$\mu$M, added to
the serosal bath) inhibited basal secretory \( I_{sc} \) and abolished the inhibitory effect of TTX/lidocaine (2 µM/1.6 mM, added to the serosal bath) on \( I_{sc} \). In neuronal CaSR knockout mice or nestin mice, however, R568 did not significantly inhibit \( I_{sc} \), although it caused significant inhibition on \( I_{sc} \) in epithelial CaSR knockout mice or villin mice. In all three groups, inhibitory effects of TTX/lidocaine on basal \( I_{sc} \) were unaffected (mean % inhibition of \( I_{sc} \) by TTX/lidocaine in wild-type, nestin and villin mice: 55 ± 12% (6), 63 ± 7% (8) and 43 ± 8% (6), \( P > 0.05 \)). These results suggest that the calcimimetic acts on CaSR of enteric neurons, not mucosal cells, to inhibit TTX/lidocaine-sensitive electrolyte secretion by the intestine.

Calcimimetic inhibits cholera toxin-evoked \( I_{sc} \) in mouse colon via CaSR on enteric neurons. To strengthen the hypothesis that R568 acts on CaSR of enteric neurons to limit secretion, we also performed experiments using cholera toxin to enhance the neurally mediated secretory state. In a pilot study, we found that mouse colons did not survive the long (90 min) incubation required for cholera toxin to have an effect in Ussing chambers. Accordingly, we adopted a two-step protocol previously described by Gabriel et al.\(^3\), i.e., pretreating mouse intestines with cholera toxin, \textit{in vivo}, in live animals to induce hypersecretion (see Methods), followed by examining \( I_{sc} \) changes of pre-treated intestines, \textit{ex vivo}, in Ussing chambers. Summarized are steady-state \( I_{sc} \) changes of these pre-treated intestines in response to the sequential serosal additions of R568 (10 µM) and lidocaine (Lido, 1.6 mM)/TTX (2 µM). The R568 anti-secretory effect is present in colons of wild-type mice and mice expressing neuronal CaSR (\textit{villin} \textit{Cre/Casr}\textit{flox/flox} or villin mice) but is absent in mice lacking neuronal CaSR (\textit{nestin} \textit{Cre/Casr}\textit{flox/flox} or nestin mice). NS, no significance.

### Table 1. \( I_{sc} \) responses to cholera toxin in wild-type and CaSR mutant mouse colons. Data shown are means ± SEM (n).

|                | Wild-type | \textit{nestin} \textit{Cre/Casr}\textit{flox/flox} | \textit{villin} \textit{Cre/Casr}\textit{flox/flox} |
|----------------|-----------|-----------------------------------------------------|-----------------------------------------------------|
| Control        | 48 ± 10 (6) | 41 ± 10 (8)\(^{ns}\) | 65 ± 6 (6)\(^{**}\) |
| Cholera toxin  | 116 ± 12 (6)\(^{**}\) | 60 ± 10 (8)\(^{ns}\) | 113 ± 17 (6)\(^{**}\) |

Table 1. \( I_{sc} \) responses to cholera toxin in wild-type and CaSR mutant mouse colons. Data shown are means ± SEM (n). \(^{**}\)\( P < 0.01, \)\(^{*}\)\( P < 0.05 \) vs. Control. \(^{zz}\)\( P < 0.01, \)\(^{z}\)\( P < 0.05 \) vs. Wild-type. \(^{**}\)\( P < 0.01, \)\(^{*}\)\( P < 0.05 \) vs. \textit{villin} \textit{Cre/Casr}\textit{flox/flox}.

Calcimimetic inhibits forskolin-evoked \( I_{sc} \) in mouse colon via CaSR on enteric neurons. In colonic crypts\(^4\) as well as in kidney\(^5\) and parathyroid cells\(^6\), CaSR exerts its regulatory actions via reductions of the cyclic AMP. Thus, to provide insights into the messenger systems involved in the actions of the CaSR in enteric neurons, we also studied effects of R568 on forskolin-induced \( I_{sc} \) (Fig. 3). We chose forskolin also because in enteric neurons this agent has long been shown to simulate the neuroexcitatory effects of 5-hydroxytryptamine (5-HT), substance P, VIP and many other neurotransmitters released in neuronal reflexes\(^7\). Indeed, forskolin...
(500 nM, added to the serosal bath) significantly increased \( I_{sc} \) in colons of wild-type mice and both knockouts. Subsequent addition of R568 significantly diminished forskolin-induced increases in \( I_{sc} \) in wild-type mice and villin mice, but not in nestin mice. In all three groups, inhibitory effects of TTX/lidocaine on forskolin-evoked \( I_{sc} \) were unaffected [mean \% inhibition of \( I_{sc} \) by TTX/lidocaine in wild-type, nestin and villin mice: 52 ± 6\% (12), 36 ± 11\% (12) and 67 ± 8\% (6), \( P > 0.05 \)]. These results suggest that activation of adenylate cyclase by forskolin and subsequent elevation of intra-neuronal cyclic AMP mimic the effects of cholera toxin-induced neuronal cell excitation on Cl\(^-\) secretion, thus supporting the possibility that reductions of cyclic AMP might be involved in the actions of this neural CaSR.

Neural CaSR is required for enteric neurons to communicate with the mucosal epithelium. Enteric neurons communicate with the mucosal epithelium in not one- but two-way directions. In addition for the mucosa (e.g., EC cells) secreting neuroactive agents (e.g., 5-HT) to control the activity of enteric neurons, enteric neurons also release neurotransmitters (e.g., VIP, somatostatin) to influence the function of the mucosal cells (e.g., EC cells)\(^{36,37}\). Thus, after characterizing its role in regulating the functionality of enteric neurons, we asked if CaSR on enteric neurons also regulates the functions of the mucosal epithelium. For this, we compared \( I_{sc} \) changes in response to cholera toxin (which was administered to the mucosal side) and forskolin (which was administered to the serosal side). The rationale is that cholera toxin requires mucosa to activate a secretory neural reflex whereas forskolin does not. Accordingly, if CaSR on enteric neurons regulated the functionality of the mucosa, then cholera toxin should increase \( I_{sc} \) differently in nestin mice compared with wild-type and villin mice, whereas forskolin should have a similar effect on \( I_{sc} \) in all three groups. Indeed, although both pro-secretory agents increased \( I_{sc} \) in all three animal groups, cholera toxin increased \( I_{sc} \) to a significantly lesser extent in nestin mice (Fig. 2 and Table 1), whereas forskolin had a similar effect on \( I_{sc} \) in all three groups (Fig. 3 and Table 2). Thus, in nestin mice, the mean \% increase in \( I_{sc} \) induced by cholera toxin was only 20 ± 12\% (8), which is significantly smaller than 141 ± 59\% (6) in wild-type (\( P < 0.05 \)) and 83 ± 28\% (6) in villin mice (\( P < 0.05 \)). In contrast, the mean \% increases in \( I_{sc} \) induced by forskolin were 126 ± 25\% (12), 120 ± 23\% (12), and 86 ± 29\% (6), respectively (\( P > 0.05 \)). These results suggest that, in addition to regulating neuronal function, the CaSR expressed by neuronal cells also modulates the function of the mucosa, highlighting the importance of this receptor in GI physiology and pathophysiology.
Discussion

Our data confirm that CaSR in enteric neurons is functional and can modulate neurogenic secretory responses. Using a rat colon model, we have previously shown that the serosally applied calcimimetic R568 inhibits forskolin-induced secretory Isc in the absence, but not presence, of the neurotoxin TTX 15, suggesting that this anti-secretory agent might act on the intestine via enteric neurons within the ENS. In the present study, we extend these observations by showing that in mouse colons this same small molecule compound also inhibits basal and secretagogue (forskolin and cholera toxin)-induced Isc in a neuronal CaSR-dependent manner, i.e., R568 inhibited basal or evoked secretory Isc in colons of wild type mice and mutant mice that contained CaSR in neurons (i.e., villinCre/Casrflx/flx mice), but not in colons of nestinCre/Casrflx/flx mice in which CaSR in neuronal cells was eliminated, providing further support for potential roles that this neuronal CaSR may play in the control of ENS activity and GI function.

The primary function of the GI tract is to digest food and absorb nutrients. To aid in digestion, the GI tract secretes fluid. It is estimated that following the intake of a meal, intestinal secretion can be increased eightfold 24. There is compelling evidence that the ENS has a major role in enhancing this secretory state 25. This enhanced secretion helps lubricate the surface of the lumen of the intestine, ensuring the appropriate mixing and flow of digest along its length. Once digestion is completed and nutrients are extracted, these secretions along with released nutrients must be reabsorbed while also ensuring that further post-digestive secretions do not occur. These processes are highly regulated and coordinated; failure to do so may result in diseases such as mal-digestion, mal-absorption, constipation, or diarrhea. How these functions are regulated and coordinated remains a focus of study. We have presented evidence from this and other studies that CaSR may play a major role in the control of these processes 26 (see a recent review by Tang et al 27). CaSR is a highly conserved G protein-coupled cell surface receptor (GPCR) that is coupled to multiple G proteins including Gq/11, Gi/o, and G12/13, and its downstream factors, and, when activated, turns on various signaling pathways regulating cellular behaviors. These include an increase of intracellular Ca2+, alteration of the intracellular cyclic AMP, phosphorylation of ERK1/2, and activation of small GTPase RhoA 28. However, CaSR is an unusual GPCR in that it uses extracellular nutrients (e.g., calcium, polyamines, certain amino acids and peptides) as its ligands. Accordingly, unlike most otherGPCRs, CaSR must function in the chronic presence of agonists. This is possible because CaSR adopts unusual mechanisms to regulate receptor expression, trafficking, and degradation 29. For example, instead of inducing internalization and causing desensitization in other GPCRs, continuous elevation of agonist increases the number of CaSR in the plasma membrane via the so-called agonist-driven insertion signaling and sensitizes receptor function 30. This is important because this unique property enables CaSR the ability to continuously monitor the ion and nutritional compositions of the extracellular milieu in the gut and modulate the intestinal secretion/absorption in accordance with the status of digestion. Accordingly, local nutrient signals released from digestion, such as calcium, amino acids, and polyamines, can act on CaSR on neurons to function as a negative modulator of these physiological secretory actions and provides a mechanism for modulating net fluid movement during normal digestion. Based on this, we predict that over-activation of CaSR in this process may contribute towards under-activation of CaSR may contribute to diarrhea. Indeed, people with high-calcium diets or taking excessive calcium are often constipated 31, as are patients with hypercalcemia 32. By contrast, those who are starved tend to hypersecretes in their small and large intestines 33–34.

It is worth noting, however, that although this present study shows the primary effect of R568 is via neuronal CaSR, part of the inhibitory effect of this compound may be due to an action on epithelial CaSR. The latter is evidenced by the following: First, R568 produced a significantly less pronounced inhibitory effect on basal Isc in villinCre/Casrflx/flx mutants than wild type mice 35 (compare right versus left panels of Fig. 1; mean inhibition: 24±5% (6) versus 40±5% (6); P<0.05), even though such a difference on cholera toxin [compare right versus left panels of Fig. 2; mean inhibition: 24±7% (6) versus 29±4% (6); P>0.05] or forskolin stimulated Isc [compare right versus left panels of Fig. 3; mean inhibition: 40±5% (6) versus 40±5% (12); P>0.05] was not statistically significant. Second, R568 reduced forskolin-evoked Isc and this clearly diminished the inhibitory effect of subsequent TTX/lidocaine addition in wild-type mice [Fig. 3 left panel; mean inhibition by TTX/lidocaine: 22±5% (12)]. However, the ability of R568 to reduce the inhibitory effect of TTXLidocaine was significantly less pronounced in villinCre/Casrflx/flx knockout colons (Fig. 3 right panel; mean inhibition by TTX/lidocaine: 46±10% (6); P<0.05). This suggests that epithelial CaSR may be involved, albeit to a lesser extent, in the inhibitory effect of R568.

Also noted was the finding that R568 failed to inhibit basal and secretagogue-induced Isc in colons of nestinCre/Casrflx/flx mice (Figs 1–3, middle panels). The reason is unknown. One possibility is the thickness of the tissue preparations used, which may prevent this hydrophobic calcimimetic drug from diffusing into the epithelium layer to access to the receptor 36–37. An alternative explanation would be that the receptor function in the enterocyte might also be lost or its level may be down-regulated in this neuron-specific mutant mouse line. In support of this possibility, Crane et al. recently showed that the use of nestinCre mice also drove expression in the colonic epithelium 38. Accordingly, the expression of Casr gene in the colonic epithelium may be reduced in nestinCre/Casrflx/flx knockouts. Finally, epithelial stem cell production of the many different types of epithelial cells is under neural control so that any mechanism that alters neural activity can be expected to modify the epithium and whether this affects EC or

mal-absorption, constipation, or diarrhea. How these functions are regulated and coordinated remains a focus of study. We have presented evidence from this and other studies that CaSR may play a major role in the control of these processes 26 (see a recent review by Tang et al 27). CaSR is a highly conserved G protein-coupled cell surface receptor (GPCR) that is coupled to multiple G proteins including Gq/11, Gi/o, and G12/13, and its downstream factors, and, when activated, turns on various signaling pathways regulating cellular behaviors. These include an increase of intracellular Ca2+, alteration of the intracellular cyclic AMP, phosphorylation of ERK1/2, and activation of small GTPase RhoA 28. However, CaSR is an unusual GPCR in that it uses extracellular nutrients (e.g., calcium, polyamines, certain amino acids and peptides) as its ligands. Accordingly, unlike most other GPCRs, CaSR must function in the chronic presence of agonists. This is possible because CaSR adopts unusual mechanisms to regulate receptor expression, trafficking, and degradation 29. For example, instead of inducing internalization and causing desensitization in other GPCRs, continuous elevation of agonist increases the number of CaSR in the plasma membrane via the so-called agonist-driven insertion signaling and sensitizes receptor function 30. This is important because this unique property enables CaSR the ability to continuously monitor the ion and nutritional compositions of the extracellular milieu in the gut and modulate the intestinal secretion/absorption in accordance with the status of digestion. Accordingly, local nutrient signals released from digestion, such as calcium, amino acids, and polyamines, can act on CaSR on neurons to function as a negative modulator of these physiological secretory actions and provides a mechanism for modulating net fluid movement during normal digestion. Based on this, we predict that over-activation of CaSR in this process may contribute towards under-activation of CaSR may contribute to diarrhea. Indeed, people with high-calcium diets or taking excessive calcium are often constipated 31, as are patients with hypercalcemia 32. By contrast, those who are starved tend to hypersecretes in their small and large intestines 33–34.

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Finally, epithelial stem cell production of the many different types of epithelial cells is under neural control so that any mechanism that alters neural activity can be expected to modify the epithium and whether this affects EC or EE cell numbers or properties is not known. The CaSR knockouts used here are whole of life effects and hence it might be expected that there are significant differences in neural regulation of epithelial differentiation and hence insensitivity to calcimetics. This may also explain their reduced sensitivity to cholera toxin (see below).

What is the most surprising was the finding on the effect of cholera toxin in nestinCre/Casrflx/flx mice. In these animals, cholera toxin increased Isc to a significantly lesser extent than it did in wild-type and villinCre/Casrflx/flx mice (Fig. 2 and Table 1). This suggests that this neuronal CaSR may also influence the function of the mucosa, particularly the EC cells. Generating an effective secretory response to cholera toxin requires not only activation of secretomotor reflexes but also release of 5-HT from EC cells 39. Thus, two possibilities may exist in nestinCre/Casrflx/flx intestine: a defect occurred in the ENS or in the mucosa. The fact that only cholera toxin (which acts in the
mucosa and ENS) but not forskolin (which acts in the ENS) caused the blunted $I_\text{sc}$ response seems to suggest the latter, rather than the former. Indeed, via synaptic connections, the mucosa (e.g., EC cells) communicates with enteric neurons in a not mono- but bi-directional manner. In addition for EC cells secreting neuroactive agents (e.g., 5-HT) to control the activity of enteric neurons, enteric neurons also release neurotransmitters to influence the function and fate of EC cells. Numerous receptors for neurotransmitters have been shown to be present on the basolateral aspect of EC cells. When stimulated, these receptors modify the release of 5-HT. While some receptors (e.g., GABA and somatostatin) inhibit, others (e.g., VIP/PACAP) enhance, 5-HT release. Thus, neurogenic influences on EC cells may be altered in $\text{Cre}^{\text{nestin}}/\text{Casr}^{\text{flx/flx}}$ intestines such that the balance is shifted to more inhibitory in these animals. In addition, EC cell proliferation and differentiation are under neural control. For example, enteric neurons may release PACAP/VIP to stimulate EC cell proliferation. Thus, without influences from neuronal CaSR, EC cells may become hypoplastic and/or hypotrophic. Future studies need to characterize what exact changes occur to EC cells in $\text{Cre}^{\text{nestin}}/\text{Casr}^{\text{flx/flx}}$ intestines and understand how CaSR elimination from enteric neurons leads to such changes.

The mechanisms by which CaSR inhibits the ENS activity are currently under investigation. In a recent study, Sun et al. examined the R568 effect on the myenteric neuron c-fos expression, a biochemical measurement of neuronal excitability, and found that activation of CaSR by R568 significantly reduced neuronal cell c-fos expression in colons of wild-type mice and $\text{Cre}^{\text{nestin}}/\text{Casr}^{\text{flx/flx}}$ knockouts, but not $\text{Cre}^{\text{nestin}}/\text{Casr}^{\text{flx/flx}}$ knockouts. Thus, one possibility would be to operate by reducing neuronal excitability. Consistent with this, we show in the present study that activation of CaSR on enteric neurons inhibited the neurogenic secretion caused by the neuroexcitatory agent forskolin (Fig. 3). Forskolin is known to excite enteric neurons via activation of neuronal membrane adenyl cyclase and subsequent elevation of the intra-neuronal cyclic AMP, which inhibits Ca2+ influx and closes Ca2+-dependent K+ channels, causing membrane depolarization. Thus, CaSR might inhibit neuronal excitability by reversing this cellular process through receptor-mediated inhibition of adenyl cyclase and/or reduction cyclic AMP, as described in enterocytes and many other cell types. Recent studies on CaSR mechanisms on neurons of the CNS also seem to support this possibility.

Alternatively, CaSR, particularly those expressed on nerve terminals of the ENS, may exert its inhibitory effect on neurons through inhibition of voltage-gated sodium channels (Na+) and thereby synaptic transmission and neurotransmitter release, as in the CNS. Decreases in Ca2+-a primary ligand of CaSR, have long been recognized to increase the likelihood of action potential initiation. The CaSR location in nerve terminals of enteric neurons, the TTX/lidocaine-sensitive nature of the CaSR-mediated suppression of neurogenic secretion (present study), and the abolition of the veratridine-evoked $I_\text{sc}$ by R568 (Tang, unpublished observations) also seem to suggest this possibility. In the CNS, cyclic AMP regulates excitability/neurotransmission in two different pathways: action potential-dependent, which involves PKA-dependent phosphorylation and activation of Na+, and action potential-independent, which involves PKA-independent Epac-dependent modifications of other mechanisms such as increasing Ca2+ influx. Currently, it is unknown which pathway (s) is utilized by CaSR in the ENS.

Cyclic AMP is triggered by increases in intracellular Ca2+, which not only leads to the release of neurotransmitters but also increases synaptic H+ (co-released with neurotransmitters from vesicles) and Ca2+ (resulting from Ca2+/H+ exchange mediated via presynaptic plasma membrane Ca2+/ATPase). While the primary function of neurotransmitters is to excite postsynaptic effector cells, we do not know the roles and fates of these ions. The finding that enteric neuronal activity was inhibited by calcimimetics revealed in the present and the previous studies leads us to speculate that these synaptic Ca2+ and H+ ions may represent an internal ‘brake’ to limit synaptic excitation. Considering the known effects on CaSR of Ca2+ (which activates receptor) and H+ (which inhibits receptor via allosteric modification of Ca2+ affinity), we further hypothesize that CaSR is the receptor that detects and transduces changes in these synaptic ions into changes in cellular excitability. Furthermore, the resultant changes in cellular activity can be of either the presynaptic neuron or the postsynaptic neuron/epithelial cell or both.

The finding of the present study may have important pathophysiological significances. Bacterial enterotoxins, such as cholera toxin and STa, enhance intestinal fluid secretion and cause severe diarrhea. They do so through both direct enterocyte generation of cyclic nucleotides and indirect stimulation of the ENS to release neurotransmitter secretagogues such as VIP. Remarkably, our studies show that nearly all the secretory responses induced by cholera toxin are reversed upon activation of CaSR, acting either on the enterocyte or on the ENS (present study). Based on these findings, we predict that whether a patient develops diarrhea or not is determined not only by the toxin that provokes it but also by the activity of CaSR that prevents it. We also predict that, when this CaSR-mediated anti-secretory protection is decreased or lost, such as in children with a negative calcium balance or nutrient deprivation, this and other diarrheal diseases would be anticipated to be more common. Additionally, the diarrheal symptoms would be more severe, and the disease duration would be longer, as shown. This CaSR protection theory may also explain why increasing calcium intake is effective in reducing the severity and duration of diarrhea resulting from enterotoxigenic Escherichia coli and other enterotoxin-producing infections.

Effectively reducing the life-threatening intestinal fluid loss in enterotoxin-induced diarrhea remains a major challenge. The novel pathway for modulating intestinal Cl− secretion through the colonic CaSR may lead to new pharmaco–nutritional therapies for prevention or treatment of certain clinical diarrheal diseases (i.e., cholera and other cyclic nucleotide-associated diarrheal diseases). Although the present study was conducted in the colon, the finding should apply to the small intestine, as CaSR is similarly expressed in the epithelium and neurons of the small and large intestines. Similarly, the primary virulence factor and diarrhea inducer of the Vibrio cholerae cholera toxin affects both the small and large intestine (present study), including the human colon, even if the non-invasive pathogen colonizes primarily the small intestine. Given that ENS-mediated secretory response is also critically implicated in many other forms of diarrhea, including viral [e.g., rotavirus], neurogenic [e.g., irritable bowel disease], and immunogenic/inflammatory diarrhea [e.g., inflammatory bowel disease], the CaSR-mediated inhibition of the ENS-dependent secretory response observed in the present study might be of clinical importance in treating those forms of diarrhea as well.
In light of the present study and previously published data\textsuperscript{10,11,13,18,29}, we propose a new paradigm for CaSR inhibition of cholera toxin-induced hypersecretion (Fig. 4). According to this paradigm, cholera toxin produces its effect on electrolyte secretion by the intestine using two pathways and CaSR agonists block both pathways. (1) CTX induces a non-neuronal fluid secretory response due to binding of toxin directly to enterocytes, leading to the generation of cyclic AMP and activation of CFTR and NKCC1. This direct secretory response is blocked by activation of CaSR on enterocytes, either apically or basolaterally, through enhancing cyclic nucleotide destruction. (2) CTX induces a neurally mediated secretory response by stimulation of secretomotor reflex pathways in the ENS. This neurally mediated indirect secretory response is blocked by activation of CaSR on neurons (cell bodies, indicated in filled red circles, and nerve terminals, indicated in black lines) possibly by modulations of cyclic nucleotide metabolism and ion channel properties, leading to inhibition of neuronal cell excitability and/or synaptic excitation (see text for more discussions). Besides enterocytes and neurons, the cholera toxin-induced hypersecretion requires the release of 5-hydroxytryptamine (5-HT, indicated by blue dots) from enterochromaffin (EC, indicated in red) cells of the mucosa, which also express CaSR. It remains to be addressed whether cholera toxin-evoked release of 5-HT from EC cells is blocked by CaSR on EC cells.

**Materials and Methods**

**Animals.** Experiments were performed using non-fasting male/female C57BL/6 mice (wild-type and Casr mutants). Mice lacking CaSR expression in intestinal epithelial cells (villin\textsuperscript{Cre/Casrflox/flox} mice) and mice lacking CaSR expression in intestinal neurons (nestin\textsuperscript{Cre/Casrflox/flox} mice) and their wild-type littermates were bred and maintained in-house at the University of Florida Communicore Animal Facility. Mutant villin\textsuperscript{Cre/Casrflox/flox} mice and nestin\textsuperscript{Cre/Casrflox/flox} mice were generated as previously described\textsuperscript{30,71}. Briefly, CaSR flox/flox mice were bred with transgenic mice expressing Cre Recombine under the control of the villin 1 or nestin promoter and genotyped prior to all experiments after an approximate 10–12 generations. Mice were used at 5–10 weeks of age in accordance with the Animal Welfare Act and the Public Health Policy on Human Care. Animals were fed and maintained on regular chow (Harlan) with free access to water before sacrifice. In some experiments, mice were
induced for hypersecretion by cholera toxin (10 µg in 100 µl 7% NaHCO₃ administered via intra-gastric gavage) 6 hrs prior to sacrifice. Cholera toxin–induced hypersecretion/diarrhea was confirmed by the formation of loose stool and/or accumulation of clear fluid in the intestines as previously described[21,22,24]. Animals were sacrificed with standard CO₂ inhalation and killed by cervical dislocation before colons were removed. The use of animals, as well as the protocols for cholera toxin treatment and colon tissue isolation, was approved by the Institutional Animal Care and Use Committee (IACUC 201307567) at University of Florida.

**Ussing chamber I<sub>sc</sub> measurements from colonic segments.** Segments of colons were quickly isolated. Segments were cut open along the mesenteric border into a flat sheet and flushed with ice-cold basal HEPES–Ringer solution containing 1.25 mM Ca<sup>2+</sup> and Mg<sup>2+</sup>. Intact full-thickness segments containing all the layers of middle colons (between proximal and distal colons) were used. Proximal and distal colons were distinguished grossly by the presence of oblique mucosal folds in the former and longitudinal folds in the latter. In a pilot study, we tested proximal and distal colons separately and found no significant difference in their I<sub>sc</sub> responses to R568. The intestinal segments were then mounted between two halves of a modified Ussing chamber (Physiologic Instruments, San Diego, CA) and short–circuited continuously by a voltage clamp (VCC MC6, Physiologic Instruments, San Diego, CA) with correction for solution resistance. The exposure area was 0.3 cm<sup>2</sup>. The mucosal and serosal surfaces of the tissue were bathed in reservoirs with 3–5 ml HEPES–Ringer solution containing 110 mM NaCl, 5 mM KCl, 1.25 mM CaCl<sub>2</sub>, 1.25 mM MgCl<sub>2</sub>, 10 mM glucose, and 22 mM Hepes, pH 7.4, maintained at 37 °C and continuously bubbled with 100% O<sub>2</sub>. On average, an interval of 10 min elapsed between euthanizing the animal and mounting the tissue into the chamber. Tissues were allowed a minimum of 15-minute stabilization period before test reagents or vehicles were added to the mucosal and/or serosal sides of the intestine. Trans–epithelial potentials generated by the tissues were continuously clamped to < 0 mV, except for brief interruption to record opening–circuit potential (V<sub>oc</sub>, mV). Tissue conductance (G<sub>T</sub>, μS/cm<sup>2</sup>) was calculated as the ratio of the measured short–circuit current (I<sub>sc</sub>, μA/cm<sup>2</sup>) to V<sub>oc</sub> from Ohm’s law. Data were acquired via DATAQ™ instruments and were stored on a PC and processed using the program Acquire & Analyze™. I<sub>sc</sub> is defined as the current flow through the tissue when the tissue is short–circuited (i.e., when the voltage across the tissue is zero). Magnitudes (μA/cm<sup>2</sup>) of change in I<sub>sc</sub> were determined before and after additions of test reagents or vehicle, with a positive sign representing net cation absorption and/or net anion secretion, and % changes in I<sub>sc</sub> were calculated. In the present study, a two-step protocol was employed to induce hypersecretion by cholera toxin. To estimate the % changes in I<sub>sc</sub> induced by cholera toxin, the basal I<sub>sc</sub> from colons that were not pretreated with cholera toxin were pooled and used as controls.

In a pilot study, it was found that I<sub>sc</sub> in mouse colons was less sensitive to TTX. Thus, in studies involving mouse colons, TTX was used in combination with lidocaine, another inhibitor of neural activity[24]. For measurements of TTX/lidocaine-sensitive neurally mediated I<sub>sc</sub>, 2μM TTX and 1.6 mM lidocaine were applied to the serosal solutions and the differences in stable I<sub>sc</sub> before and after TTX/lidocaine treatment were compared and calculated and were defined as TTX/lidocaine-sensitive I<sub>sc</sub>. According to a pilot experiment in which a dose–response effect on I<sub>sc</sub> by TTX/lidocaine was sought, these doses of TTX and lidocaine were the lowest concentrations that maximally blocked the activity of the ENS without directly affecting the function of the epithelium[13,14,25]. Previous studies using Cl<sup>−</sup> free Ringer solutions and pharmacologic inhibitors established that under present experimental conditions both the basal and secretagogue-stimulated I<sub>sc</sub> primarily reflected Cl<sup>−</sup> secretion[2].

**Chemicals.** Forskolin, veratridine, cholera toxin and lidocaine were obtained from Sigma, and stock solutions were prepared in dimethyl sulfoxide (DMSO) (forskolin, veratridine) or in water (cholera toxin). TTX was purchased from Enzo Life Science (Plymouth Meeting, PA), and 2 mM stock solutions were prepared in 10 mM acetic acid. R568 was purchased from Tocris Bioscience (Ellisville, MI), and 100 mM stock solutions were prepared in DMSO.

**Statistical Analysis.** Values are given as means ± SEM of n experiments. Statistical comparisons between two means were performed by Student’s t-test, whereas comparisons among multiple means were by one-way ANOVA with Tukey’s post hoc tests. Both tests were performed either using Microsoft Excel 2016 for Windows or using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA). P < 0.05 was considered significant.

**Data availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

S.X.C. conceptualized the study, L.T., L.J., and S.X.C. designed the study, L.T., L.J., E.P., M.E.M., and S.X.C. performed the experiments, L.T., L.J., and S.X.C. analyzed the data, and L.T. and S.X.C. drafted and revised the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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