Dual regulation of heat-stable enterotoxin-mediated cGMP accumulation in T84 cells by receptor desensitization and increased phosphodiesterase activity

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Abstract **We report the regulation of cGMP accumulation induced by the heat-stable enterotoxin, STh, in the T84 human colonic cell line. STh binding to its receptor, guanylyl cyclase C (GCC), leads to elevated intracellular levels of cGMP. Prolonged exposure of T84 cells to STh induced refractoriness to further cGMP accumulation, without significant receptor internalization, but with reduced STh-induced cGMP synthesis by the receptor. Significantly, increased degradation of cGMP by a cGMP-specific phosphodiesterase was observed in desensitized cells. This is the first report on the desensitization of GCC, as well as the role of the Type V phosphodiesterase in inducing cellular refractoriness.**

Key words: Guanylyl cyclase C; Heat-stable enterotoxins; Cellular refractoriness; cGMP-binding, cGMP-specific phosphodiesterase

1. Introduction

The heat-stable enterotoxins (ST) are a family of low molecular weight, methanol soluble, cysteine rich peptides, produced by *E. coli* and other pathogenic bacteria, that cause diarrhea in farm animals and in man [1]. Binding of the toxin to a receptor present in intestinal cells leads to elevation of intracellular cGMP and a consequent efflux of chloride and fluid from the cells, resulting in diarrhea [2]. The receptor for *E. coli* STh, GCC, has been cloned from rat and human intestinal cells [3,4], as well as from human colonic cell lines [5]. Based on sequence homology studies predicted from the nucleotide sequence of the cloned ST receptors, the ST receptor (GCC) is a member of the family of membrane bound guanylyl cyclases [6].

A characteristic feature of any ligand receptor-mediated signal transduction process is the phenomenon of desensitization whereby cellular response to a stimulus is attenuated, or demonstrates refractoriness, following prior exposure of the cells to the ligand in question. ST-mediated diarrheas are transitory in nature, but the mechanism of this refractoriness, presumably induced in the intestinal cells which have been exposed to ST peptide, is unknown. Whether such refractoriness involves receptor desensitization, or other intracellular mechanisms has not been investigated till date.

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Abbreviations: cGB-PDE, cGMP-binding, cGMP-specific phosphodiesterase; GCC, guanylyl cyclase C; PDE, phosphodiesterase; IBMX, isobutyl methyl xanthine; STh, stable toxin of the human variety; STY72F, mutant ST peptide where the C-terminal tyrosine is mutated to phenylalanine

Cellular refractoriness to a given stimulus could be mediated by an altered rate of synthesis or degradation of the second messenger, for example cAMP or cGMP. Synthetic rates are presumably regulated by the receptor, which in the case of guanylyl cyclase coupled receptors, serves as the enzyme per se. The rate of degradation of cAMP has been shown to play an important role in inducing the refractoriness of Sertoli cells to follicle stimulating hormone [7], by the hyperactivation of a cAMP-specific phosphodiesterase (PDE). No such mechanism of regulation has been reported, to our knowledge, for cGMP-mediated mechanisms, and hence the ST response in T84 cells could serve as a useful model system to study such cellular refractoriness.

The T84 cell line has been used by us to study the STh receptor, and we have shown that a single class of receptor with high affinity (K_d 0.1 nM) is expressed in these cells [8,9]. In this study we report that T84 cells show refractoriness to fresh stimulation by ST, following prolonged incubation with the peptide. This refractoriness is at the level of the degradation of the second messenger, cGMP, as a result of increased activity of a cGMP-specific PDE, as well as receptor inactivation at the level of guanylyl cyclase activity.

2. Materials and methods

All fine chemicals were from Sigma Chemical Co., USA, and tissue culture media from Life Technologies, USA.

2.1. Culture and maintenance of T84 cells

T84 cells were obtained from ATCC (CCL 247), and maintained in Dulbecco's Modified Eagle's medium:F12 containing 5% new born calf serum, penicillin and streptomycin as described in detail earlier [8]. Cells were plated in 24-well culture dishes at a concentration of 10^4 cells/well, and were used at approximately 75–90% confluency, after 4–7 days in culture.

2.2. Purification and radiolabeling of ST peptides

STh peptide was purified from the culture supernatant of *E. coli* cells which overproduced the toxin, as described earlier [10]. Purified peptides were quantitated by aminoacid analysis [11]. An analog of STh, STY72F, was used as the radioligand for receptor-binding analysis, and was prepared as reported earlier in detail [9]. The specific activity of the radiolabeled STY72F was 2000 Ci/mmol, and the radiolabeled peptide was used within a month of preparation.

2.3. Desensitization of T84 cells

Cells were grown to confluence in 24-well plates and incubated with the STh (3×10^{-7} M) for 18 h in serum-free DMEM:F12 at 37°C in a 5% CO_2 humidified incubator. Wells were washed thrice with warm, serum-free medium and incubated with the same medium in the absence of any PDE inhibitor, or in the presence of either 1 mM IBMX, or the specific PDE inhibitors zaprinast, phenothiazine, milrinone or Ro 20-1724 (50 μM), for 30 min at 37°C. Cells were then restimulated for 15 min at 37°C with STh (3×10^{-7} M) and the reaction was terminated by aspiration of media and addition of 0.1 M citric

acid to each well. Cells were lysed, and cGMP in the lysates measured without succinylation of the samples by radioimmunoassays using ^{125}I-labeled cGMP prepared as described previously [12]. Stimulation for 15 min with this concentration of STh produce low or undetectable levels of cGMP in the extracellular medium of the cells [8].

2.4. Preparation of membranes from T84 cells and guanylyl cyclase assays

Membranes were prepared from cell lysates (100 000 g pellet) of confluent cultures of T84 cells, following prior exposure of monolayers to STh (3×10^{-7} M) or medium [9], and used for the measurement of labeled STY72F-binding activity as well as guanylyl cyclase activity. To monitor the receptor in membranes prepared from control and desensitized cells, membrane protein (50 μg) was incubated with radiolabeled STY72F peptide (10^{-10} M) in the presence or absence of unlabeled STh (10^{-7} M) as described earlier, [9], and then filtered through GF/C filters. For in vitro guanylyl cyclase assays, membrane protein (50 μg) was incubated in the presence or absence of STh (10^{-6} M) in 60 mM Tris-HCl buffer, pH 7.6, along with 4 mM $MgCl_2$, 2 mM GTP, 500 μM IBMX and a GTP regenerating system consisting of 20 μg creatine phosphokinase and 7.5 mM creatine phosphate. In some cases, membranes were treated with 0.3% Lubrol-PX for 10 min at 25°C before addition of substrate. $MnCl_2$ (4 mM), which is known to activate the receptor non-specifically [13] was also used in some cases instead of $MgCl_2$. Incubations were continued for 10 min at 37°C and the reaction terminated by the addition of 400 ml of 50 mM sodium acetate buffer, pH 4.5. Samples were heated in a boiling water bath for 10 min, and the supernatant taken for assay of cGMP.

2.5. In vitro PDE assays

Cells were cultured in 24-well dishes and exposed to STh (3×10^{-7} M) for 18 h. Cell monolayers were washed in serum-free medium, and harvested in 50 mM Tris-HCl, pH 7.5, containing 1 μg/ml leupeptin, 1 mM benzamidine, 2 mM EDTA, 10 nM okadaic acid, 10 mM sodium vanadate and 5 mM 2-mercaptoethanol. The cells were homogenized and homogenates were used for assay of PDE activity, in the absence or presence of zaprinast (20 μM). The assay was performed essentially as described earlier, with some modifications [14,15]. Assay mixtures contained 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, 300 μg/ml bovine serum albumin and 150 000–200 000 cpm ^{3}H-cGMP (0.2 μM) (New England Nuclear, USA) which had been purified earlier by ion exchange chromatography [16]. Assays were conducted for 15 min at 30°C, following which the samples were boiled for 5 min. *Crotalus vulgaris* snake venom toxin was added (20 μg/tube), and incubation continued for a further 30 min. ^{3}H-guanosine generated was monitored by ion exchange chromatography through DEAE-Sephadex A-25, which had been equilibrated with 20 mM ammonium formate [16].

3. Results

Application of STh to T84 cells leads to a rapid elevation of intracellular cGMP in cells, which declines 3 h following addition of STh [8]. We exposed T84 cells to STh for 18 h and then restimulated the cells with fresh peptide. The intracellular levels of cGMP at the end of 18 h were low, and addition of fresh STh did not lead to a significant increase in intracellular cGMP levels (Fig. 1A), indicating cellular desensitization. High levels (>500 pmol) of cGMP were detected in the extracellular medium, indicating that the cells had responded to STh over 18 h, and much of the cGMP produced during that time had been secreted by the cells. The low intracellular levels of cGMP after 18 h of incubation with STh could be due to degradation of STh over 18 h. We therefore compared cGMP production induced by STh present in the spent medium of cells which had been exposed to STh for 18 h, with that following application of fresh STh. As can be seen in Fig. 1B, no significant difference in the levels of cGMP production was observed, indicating that low levels of cGMP within cells following 18 h incubation with STh could not be attributed to degradation of peptide during the period of the incubation.

To detect receptor internalization and degradation as a means of contributing to the desensitization of T84 cells, we monitored radiolabeled STY72F binding to membranes prepared from control and desensitized cells. Results showed no significant reduction in the binding in desensitized membranes when compared to the control membranes (Fig. 2A; $P>0.1$), and therefore a reduction in total receptor content could not account for the >90% reduction in cGMP accumulation in desensitized cells. This suggested that intracellular changes, either at the level of synthesis or degradation of cGMP, had occurred in T84 cells resulting in the refractoriness to further STh stimulation.

We first investigated GCC desensitization in terms of its ability to synthesize cGMP and performed in vitro guanylyl cyclase assays with membranes prepared from control and desensitized cells. Detergents are known to be non-specific activators of membrane associated guanylyl cyclases, even in the absence of ligand and this has also been observed in the case of GCC [13]. We preincubated membrane preparations

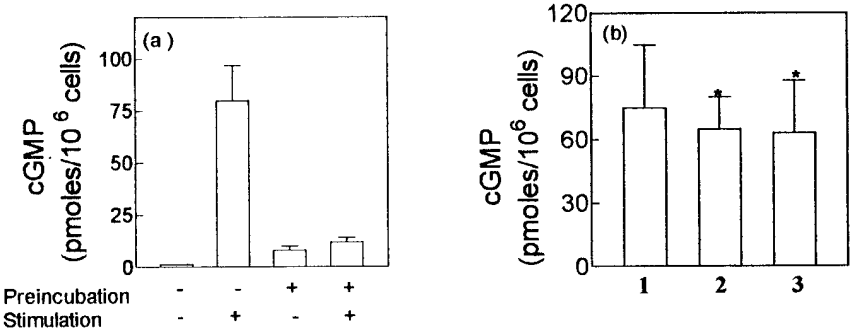

Fig. 1. Homologous desensitization of the T84 cells. A: Confluent monolayers of T84 cells were incubated with STh (3×10^{-7} M) for 18 h. Cells were washed and cGMP levels monitored with or without restimulation with STh (3×10^{-7} M). Control cells were incubated in medium without serum and basal cGMP levels measured directly, or on stimulation with STh. Values represent the mean ± S.D. of duplicate determinations with each experiment repeated thrice. B: To check the degradation of STh peptide remaining in the culture supernatant, confluent T84 cells were stimulated with (1) STh (3×10^{-7} M), (2) STh remaining in the culture supernatant after 18 h incubation with T84 cells and (3) STh incubated at 37°C without cells for 18 h. cGMP was monitored by radioimmunoassay. Values represent the mean ± S.D., with each experiment repeated twice and each well assayed in duplicate. *: $P>0.5$ when compared with 1.

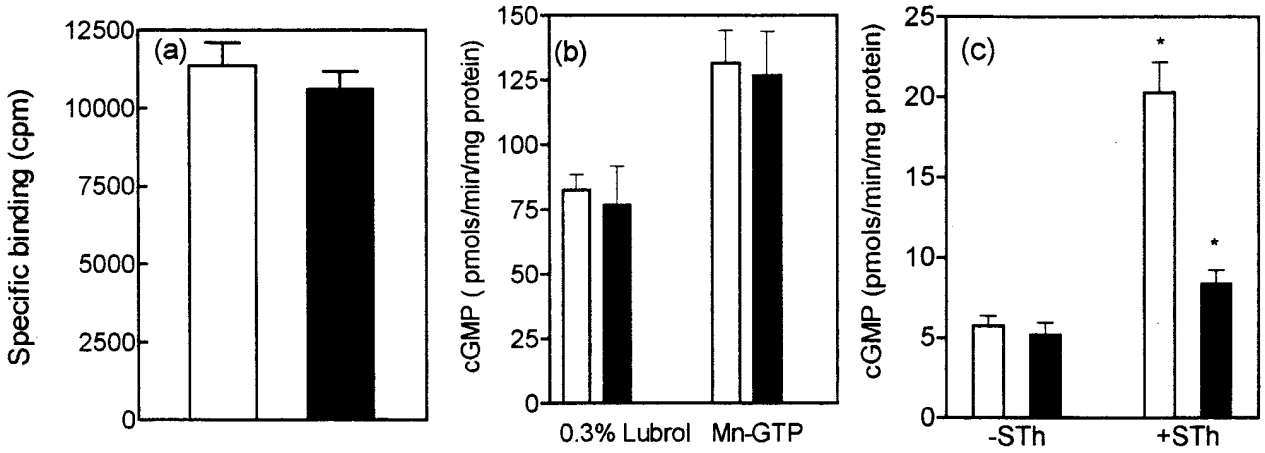

Fig. 2. GCC activity in desensitized cells. Confluent monolayers were preincubated either in the absence (open bars) or presence (closed bars) of STh (3×10^{-7} M) for 18 h, prior to membrane preparation. Membrane protein (50 μg) was used for binding assays or in vitro guanylyl cyclase assays. A: Membrane protein was incubated with ^{125}I-labeled STY72F (100 000 cpm) for 1 h in the absence or presence of STh (10^{-7} M) unlabeled STh peptide, and receptor associated radioactivity monitored following filtration. Values represent the mean ± S.D. of triplicate determinations with each assay repeated twice. B: Membranes were treated with 0.3% Lubrol-PX for 10 min prior to addition of MgGTP, or treated with MnGTP independently. Values represent the mean ± S.D. of duplicate determinations with each experiment repeated twice. C: Membrane protein was treated with STh (10^{-6} M) and cGMP produced was monitored. Values represent the mean ± S.D. of duplicate determinations with each experiment repeated twice. *: $P<0.001$.

from control and desensitized cells with Lubrol-PX (0.3%) and then monitored guanylyl cyclase activity. As shown in Fig. 2B, detergent treated membranes prepared from desensitized cells demonstrated no significant reduction in guanylyl cyclase activity ($P>0.7$). Membranes prepared from desensitized cells also did not show any difference in guanylyl cyclase activity in the presence of MnGTP, another non-specific activator of membrane associated guanylyl cyclase [13]. These results indicate that the general guanylyl cyclase catalytic activity of the receptor is retained on desensitization. It is pertinent to mention here that in T84 cells, the only membrane associated guanylyl cyclase activity observed is that of GCC [8].

We then investigated the sensitivity of GCC to STh stimulation in membranes prepared from control and desensitized cells. As shown in Fig. 2C, basal activities in both membrane preparations remained similar. However, STh-stimulatable guanylyl cyclase activity was significantly reduced to nearly 50% of control values, in desensitized cells ($P<0.001$), and STh addition only marginally increased cGMP production over basal values. This indicates that an important contribution to cellular refractoriness is receptor desensitization in terms of a reduced sensitivity of the receptor to STh.

A reduction in the accumulation of cGMP in desensitized cells could also be contributed by an increased rate of degradation of cGMP in desensitized T84 cells. A large family of PDEs are found in various cell types and while some are specific for cAMP or cGMP, others are dual specific and degrade both cGMP and cAMP with near equal efficiency [17–19]. A number of specific inhibitors are available for the various classes of PDEs, which allow the identification of a specific PDE responsible for cAMP or cGMP degradation. We therefore stimulated desensitized and control cells with STh, in the presence and absence of the general PDE inhibitor, IBMX, as well as inhibitors for the Ca^{+}-calmodulin-dependent PDE (phenothiazine), the cGMP-inhibited PDE (milrinone), the cAMP-specific PDE (Ro 20-1724 and zaprinast (cGMP-specific PDE) [17]. The results shown in Fig. 3 indicate that restimulation of desensitized cells in the presence of IBMX restored cGMP accumulation close to levels observed in control cells. Interestingly, only zaprinast, a specific inhibitor of the cGMP-binding, cGMP-specific (Type V) PDE, when added to desensitized cells prior to ST restimulation, could restore cGMP levels to those observed with IBMX, clearly indicating that the increased activity of this specific Type V PDE was responsible for the efficient degradation of cGMP in desensitized cells. No specific inhibitor is available for the cGMP-stimulated PDE, but our results with zaprinast suggest that a major contribution to enhanced cGMP degra-

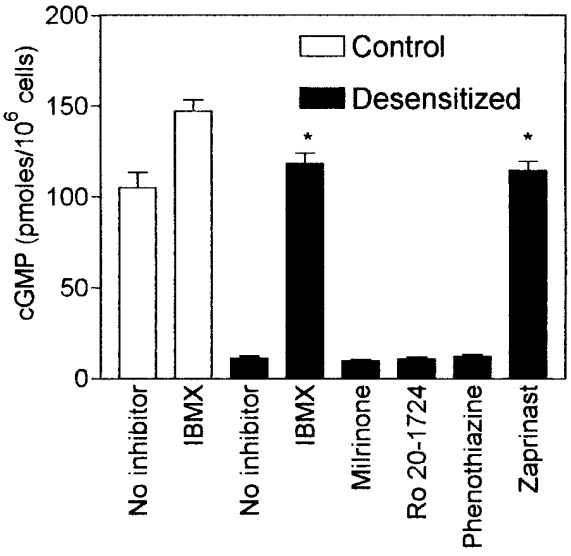

Fig. 3. Role of phosphodiesterases in inducing refractoriness to STh in T84 cells. Confluent monolayers were preincubated without or with STh (3×10^{-7} M) for 18 h. Cells were washed, incubated with no phosphodiesterase inhibitor, 1 mM IBMX or specific inhibitors as indicated (50 μM) for 30 min and restimulated with STh. cGMP levels were monitored and values represent the mean ± S.D. of triplicate experiments with each well treated in duplicate. *: $P>0.5$.

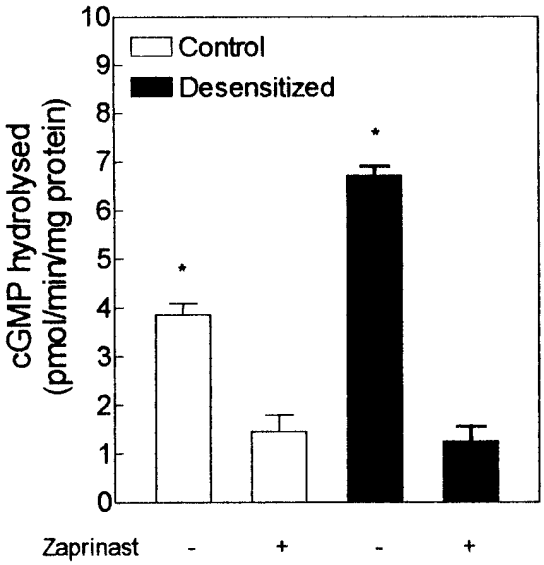

Fig. 4. In vitro phosphodiesterase activity in desensitized cells. Monolayers of T84 cells were incubated in serum-free medium either in the absence or presence of STh (3×10^{-7} M) for 18 h at 37°C. Cells were washed with serum-free medium and lysed as described in experimental procedures. Cell extracts prepared from desensitized and control cells were then used to perform in vitro PDE assays, in the presence or absence of 10 μM zaprinast. Values represent the mean of duplicate determinations of three experiments ± S.D. *: $P<0.001$.

dation in desensitized cells is through the Type V PDE. To our knowledge, this is the first report on the contribution of enhanced PDE activity towards inducing refractoriness in any cGMP-mediated system, and also the first report on the role of the Type V PDE in regulating cGMP degradation in a cGMP responsive cell in this manner.

The increased PDE activity observed in vivo should be detectable in cell extracts prepared from desensitized cells. We therefore prepared cell homogenates from control and desensitized cells and measured PDE activity in the extracts. As shown in Fig. 4, the activity observed in desensitized cell extracts is 2-fold higher than in control cells. This albeit modest increase in activity could nevertheless be significant in vivo, when coupled with reduced receptor activity in terms of synthesizing cGMP (Fig. 2). The enhanced enzyme activity observed in desensitized cells was inhibited by zaprinast, confirming that the major PDE activity we have measured is contributed by the Type V cGMP-specific PDE (Fig. 4).

4. Discussion

In this report, we demonstrate that the human colonic T84 cell line is refractory to prolonged exposure to the ST peptides, and this refractoriness is a result of receptor desensitization and the increased activity of a cGB-PDE. To our knowledge, the only other well documented report on the hyperactivation of a PDE inducing refractoriness in a cell is the observation that prolonged exposure of follicle stimulating hormone to Sertoli cells reduces the ability of these cells to respond to the hormone in terms of cAMP accumulation [7]. This refractoriness is brought about by the activation of a specific cAMP PDE, the Type IV PDE [21,22], and increased activity was detected in crude lysates prepared from cells. Hyperactivation in the case of the Type IV enzyme is through the increased transcription of the mRNA for this enzyme, and possibly also phosphorylation [22]. A second example indicates a rapid regulation of the cAMP-specific PDE by forskolin and isoproterenol in astroglial cells, where the increase in enzyme activity in the cytosol of cells is also of the order of 2-fold, as we have seen in the case of the Type V PDE [23], through a mechanism involving changes in the phosphorylation of the Type IV enzyme.

Till date, the human cDNA for the Type V enzyme has not been cloned, and the only available cDNA for this enzyme represents the enzyme present in the bovine lung [24]. Whether this bovine cDNA has homology to the human gene is not known, and we are presently investigating such a possibility. This should allow us to investigate regulation of the enzyme activity at the level of transcription. The bovine enzyme has consensus sites for phosphorylation by cAMP-dependent kinase [24]. Changes in the phosphorylation of the Type V enzyme could also contribute to the increased PDE activity observed in desensitized cells, and it has been shown that phosphorylation of the Type V PDE by protein kinase A in vitro leads to an increase in the V_{max} [15] of the enzyme. Whether this occurs in T84 cells remains to be investigated.

We have shown here that there is a significant reduction in the STh-stimulatable guanylyl cyclase activity of the receptor following exposure to ST peptide, with a marked reduction in sensitivity to STh, if one considers basal activities in control and desensitized cells. However, in whole cells, STh-stimulated cGMP levels seem to approach control values when desensitized cells were restimulated in the presence of IBMX and zaprinast (Fig. 3). This suggests low receptor desensitization in intact cells, which could be due to reactivation of the receptor by additional cellular machinery. The receptor guanylyl cyclases appear to be desensitized by a mechanism of dephosphorylation of the receptor, perhaps through the involvement of a specific phosphatase [25,26]. In the case of the receptor for the atrial natriuretic factor, receptor internalization has also been observed in certain cells [27]. More recently, there is evidence of a cGMP-mediated reduction in the level of the receptor mRNA in cells exposed to the atrial natriuretic peptide for prolonged periods of time [28]. Our report is the first to suggest that the ST receptor is desensitized but we do not find a significant degree of internalization of the receptor in T84 cells. This is in agreement with a recent report indicating efficient internalization and recycling of the receptor in T84 cells along with the ST peptide, without degradation of either the receptor or the peptide [29]. Whether the reduction in receptor sensitivity to STh that we have observed is correlated with a change in the phosphorylation of the receptor remains to be investigated. We have recently generated polyclonal antibodies to the extracellular domain of the receptor which should prove useful in such studies [30].

Stable toxin diarrheas have been observed to be transitory in vivo [20]. Thus, in the only valid in vivo assay for the ST peptides, the suckling mouse assay, fluid accumulation in the intestine of the suckling mouse is observed maximally 3 h following ST administration after which there is a gradual decline in the fluid content of the intestine [20]. We suggest that the enhanced degradation of the cGMP occurring in intestinal cells reported here, could contribute along with receptor desensitization, to the decline in fluid secretion, perhaps at

the level of chloride secretion. It remains to be seen whether the refractoriness that we observe in terms of cGMP production is reflected at the level of chloride secretion in desensitized cells. Our results indicate that the regulation of cGMP accumulation in the intestinal cell could be through its degradation rather than its synthesis by the receptor. This may be an essential requirement, since guanylin [31], the endogenous ligand for GCC, mediates its action through the same receptor. Hence a major decrease in ST receptor activity would curtail guanylin action in maintaining and regulating ion homeostasis in the intestine.

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