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Research Article

Intestinal salt and fluid secretion is stimulated by *Escherichia coli* heat-stable enterotoxins (ST) through activation of a membrane guanylate cyclase found in the intestine. Guanylin is an endogenous intestinal peptide that has structural similarity to the bacterial peptides. Synthetic preparations of guanylin or *E. coli* ST 5-17 stimulated Cl⁻ secretion in T84 cells cultured on semipermeable membranes as measured by increases in short circuit current (I_{sc}). The guanylin/ST receptors appeared to be on the apical surface of T84 cells, since addition of guanylin to the apical, but not basolateral, reservoir stimulated I_{sc}. Bumetanide added to the basolateral side effectively inhibited the I_{sc} responses of T84 cells to either guanylin or ST 5-17. Guanylin appeared to be about one-tenth as potent as ST in stimulating transepithelial Cl⁻ secretion. Guanylin and *E. coli* ST 5-17 both caused massive (> 1,000-fold) increases in cGMP levels in T84 cells, but guanylin was less potent than ST. Both peptides fully inhibited the binding of ¹²⁵I-ST to receptor sites on intact T84 cells. The radioligand binding data obtained with guanylin or ST 5-17 best fit a model predicting two receptors with different affinity for these ligands. The K_i values for guanylin were 19 ± 5 nM and 1.3 ± 0.5 μM, whereas the K_i values for ST 5-17 were 78 ± 38 pM and 4.9 ± ...]

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Guanylin Stimulation of Cl^- Secretion in Human Intestinal T_{84} Cells via Cyclic Guanosine Monophosphate

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Abstract

Intestinal salt and fluid secretion is stimulated by *Escherichia coli* heat-stable enterotoxins (ST) through activation of a membrane guanylate cyclase found in the intestine. Guanylin is an endogenous intestinal peptide that has structural similarity to the bacterial peptides. Synthetic preparations of guanylin or *E. coli* ST 5-17 stimulated Cl^- secretion in T_{84} cells cultured on semipermeable membranes as measured by increases in short circuit current (Isc). The guanylin/ST receptors appeared to be on the apical surface of T_{84} cells, since addition of guanylin to the apical, but not basolateral, reservoir stimulated Isc. Bumetanide added to the basolateral side effectively inhibited the Isc responses of T_{84} cells to either guanylin or ST 5-17. Guanylin appeared to be about one-tenth as potent as ST in stimulating transepithelial Cl^- secretion. Guanylin and *E. coli* ST 5-17 both caused massive ($> 1,000$ -fold) increases in cGMP levels in T_{84} cells, but guanylin was less potent than ST. Both peptides fully inhibited the binding of ^{125}I -ST to receptor sites on intact T_{84} cells. The radioligand binding data obtained with guanylin or ST 5-17 best fit a model predicting two receptors with different affinity for these ligands. The K_i values for guanylin were 19 ± 5 nM and 1.3 ± 0.5 μM , whereas the K_i values for ST 5-17 were 78 ± 38 pM and 4.9 ± 1.4 nM. We conclude that guanylin stimulated Cl^- secretion via the second messenger, cGMP, in T_{84} human colon cells. At least two guanylin receptors with different affinities for these ligands may exist in the cultured T_{84} cells. It may be postulated that guanylin is an endogenous hormone that controls intestinal Cl^- secretion by a paracrine mechanism via cGMP and that *E. coli* ST stimulates Cl^- secretion by virtue of an opportunistic mechanism through activation of guanylin receptors. (*J. Clin. Invest.* 1993. 91:2423–2428.)
Key words: guanylate cyclase • apical receptors • bumetanide • *E. coli* heat-stable enterotoxin • secretory diarrhea

Introduction

In recent years, attention has been focused on the cGMP signal transduction pathway as an important mechanism involved in the control of intestinal Cl^- secretion and thus salt and fluid transport. An intestinal membrane guanylate cyclase was recently identified by molecular cloning (1, 2). Expression of the specific cDNA encoding an intestinal guanylate cyclase in COS

cells resulted in the appearance of a form of guanylate cyclase in membranes that was activated by *Escherichia coli* heat-stable enterotoxin (ST).¹ The structure of this ST-activated guanylate cyclase was related to the atrial peptide-stimulated guanylate cyclases (1–3). *E. coli* ST is considered to be an important cause of secretory diarrhea resulting from the activation of intestinal guanylate cyclase. This bacterial peptide elicits both enhanced Cl^- secretion and a reduction in Na^+ absorption (4–6). *E. coli* ST-induced secretory diarrhea is associated with substantial morbidity and mortality, especially in the children of developing nations (7, 8).

The physiological significance of this ST-activated guanylate cyclase in the regulation of intestinal salt and fluid transport has been reemphasized because of the discovery of guanylin, an endogenous ligand for the intestinal guanylate cyclase (9). Guanylin was first isolated from rat jejunum with activation of the T_{84} human intestinal cell guanylate cyclase serving as the bioassay. It is a 15 amino acid peptide containing 4 cysteines and 2 disulfides that are required for biological activity. Guanylin is structurally similar to *E. coli* ST and competes with ^{125}I -ST for binding sites on intestinal T_{84} cells. In rat tissues, guanylin bioactivity was observed only in the intestine and kidney (9). A cDNA that was isolated from a rat intestine cDNA library contained an open reading frame that encodes a 115 amino acid precursor of guanylin (10). Guanylin that was purified from rat intestine represented the carboxy-terminal 15 amino acid portion of preproguanylin. A 600-nucleotide-long mRNA for guanylin was expressed throughout the intestine, but a gradient of guanylin mRNA was observed with the order of abundance being colon $>$ ileum $>$ jejunum $>$ duodenum. No guanylin mRNA was found in stomach or esophagus. It is likely that guanylin controls cGMP production in tissues other than intestine because kidney proximal tubules, testicular seminiferous tubules, hepatocytes, and airway epithelium of the opossum express the ST-activated guanylate cyclase (11–14).

In this study, we evaluated the effects of synthetic guanylin in comparison with synthetic *E. coli* ST on Cl^- secretion, cGMP production, and inhibition of the binding of ^{125}I -ST to receptors on T_{84} intestinal cells. The T_{84} human colon carcinoma cell line has been shown to express receptors for *E. coli* ST that are coupled to both the activation of guanylate cyclase and Cl^- secretion (15–17). T_{84} cells are considered to be a suitable model for intestinal crypt cells with respect to the several mechanisms controlling Cl^- secretion in the intestine (18–21). Guanylin stimulated Cl^- secretion, activated guanylate cyclase, and competed for binding sites labeled by ^{125}I -ST in cultured T_{84} cells. Guanylin appeared as efficacious but less potent than *E. coli* ST in all of these bioassays using T_{84} cells. The results of this study suggest that guanylin acts as a local

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regulator of intestinal fluid and electrolyte transport through a cGMP-guanylate cyclase signal transduction mechanism.

Methods

Cell culture. T₈₄ cells (passage 21 obtained from Dr. Jim McRoberts, Torrance, CA) were cultured using DME and Ham's F12 (1:1) containing 5% FBS, 60 µg penicillin, and 100 µg streptomycin per ml. T₈₄ cells were seeded at 150,000 cells/ml in plastic cultureware. These cells reached confluence in 3–5 d when cultured on plastic dishes. Subculturing was accomplished using trypsin and Ca/Mg-free PBS by standard methods. Falcon cell culture inserts of permeable cyclopore membranes, 25 mm diameter, 0.45 µm pore size (Fisher Scientific, St. Louis, MO) were first coated with 0.25 ml collagen (Bovine Type I, 1.3 mg/ml; Sigma Chemical Co., St. Louis, MO) for 16 h, while the filters were being sterilized by UV irradiation in a tissue culture hood. T₈₄ cells were seeded using 2.5–3.0 × 10⁶ cells per filter in 2-ml medium and cultured using the same medium described above for 7–14 d before using them to measure Cl[−] secretion in Ussing chamber experiments. The medium was changed 3 times per wk for T₈₄ cells on plastic or permeable supports.

Measurement of short circuit current in T₈₄ monolayers. T₈₄ cells raised on permeable filters were mounted in a custom-made Ussing chamber for measurement of Cl[−] secretion as previously described (22). The buffer was a Krebs-Ringer-bicarbonate solution, pH 7.4, containing 10 mM glucose for both the apical and basolateral reservoirs (5 ml volume). The Ussing apparatus was water jacketed to maintain the buffer temperature at 37°C. Both reservoir buffer solutions were mixed and oxygenated by bubbling 95% O₂/5% CO₂ through the medium. Short circuit current (I_{sc}) was measured continuously, and potential difference across the epithelium was measured intermittently. Resistance measurements were made by occasionally clamping the potential difference to a known voltage and measuring the current required to establish the potential. Resistance and conductance were calculated using Ohm's law. The I_{sc} observed with T₈₄ cells cultured on permeable filters has been shown to be caused by net secretion of Cl[−] across the T₈₄ monolayer when cells were treated with Cl[−] secretagogues such as VIP, PGE₁, *E. coli* ST, forskolin, or carbachol (16–21).

Assay of cGMP in T₈₄ cells. T₈₄ cells were cultured in 35-mm plastic dishes, and the cGMP levels were measured in control or agonist-stimulated cells by radioimmunoassay as previously described (11, 12, 22). In brief, confluent monolayers of T₈₄ cells in DME-Hepes, pH 7.4, were treated at 37°C with guanylin or *E. coli* ST or vehicle for 40 min. At the end of the incubation, the medium and cells were treated with 50 µl of 70% perchloric acid to denature proteins and extract cellular cGMP. The pH of the extract was adjusted to 7.0 with 10 N KOH and centrifuged to remove cell debris. The supernatant was used to measure total cGMP (cells plus medium) using a radioimmunoassay for cGMP.

Radioligand binding experiments. Iodination of synthetic *E. coli* ST 1-18 (NSSNYCCECCNPACTGCY, amino acid sequence—single letter code [Multiple Peptide Systems, San Diego, CA]) was accomplished using lactoperoxidase as previously described (11, 12). ¹²⁵I-ST was purified by reverse-phase HPLC using a C₁₈ column (3.9 mm × 30 cm, µBondapak; Water Associates, Milford, MA). The binding of ¹²⁵I-ST to receptors on T₈₄ cells was measured using about 50,000 cpm of ¹²⁵I-ST (12.7 fmol, 63 pM) per well of T₈₄ cells cultured in 24-well dishes. The medium was 0.2 ml of DME containing 15 mM 2-(*N*-morpholino) Mes, pH 5.5. After incubation for 60 min at 37°, the medium was aspirated and cells were washed two times with 1 ml of ice-cold medium. The cells were then solubilized with 1 N NaOH for measurement of radioactivity. Radioligand binding data comparing the effects of synthetic guanylin with synthetic *E. coli* ST 5-17 were analyzed with the Inplot computer program (GraphPAD Software for Sci., San Diego, CA). The concentrations at which specific binding of the radioligand at each binding site was inhibited by 50%, IC₅₀, were obtained by nonlinear regression of the untransformed competition binding data. A statistically significant better fit of the binding data was consistently

obtained with a two-site binding model as compared with a single-site model. The apparent equilibrium dissociation constants, K_i, for the competing ligands were calculated from the computed IC₅₀ values by the method of Cheng and Prusoff (23) using previously published estimates of the affinity of the radioligand, K_d, in these cells (15): K_i = IC₅₀/1 + (L/K_d) where L equals the radioligand concentration. It should be noted that the calculated IC₅₀ and K_i values are essentially identical because the concentration of radioligand used in these studies (63 pM) was a small fraction of the reported binding affinity of the radioligand, about 15 nM.

Peptide synthesis. Rat guanylin (PNTCEICAYAACTGC, amino acid sequence as designated by the single letter code) was prepared as described (9). Guanylin was synthesized by the solid-phase method with a peptide synthesizer (430A; Applied Biosystems, Foster City, CA) on Cys(4-CH₃Bzl)-OCH₂-Pam Resin using double coupling cycles to ensure complete coupling at each step. Coupling was effected with preformed symmetrical anhydride of *t*-butoxycarbonyl-amino acids (Applied Biosystems), and peptides were cleaved from the solid support in hydrogen fluoride, dimethylsulfide, anisole, and *p*-thiocresol (8:1:1:0.5, vol/vol/vol/wt) at 0°C for 60 min. Peptides were cyclized using dimethylsulfoxide as described by Tam et al. (24). Peptides were purified by successive reverse-phase chromatography on a 45 × 300-mm Vydac C18 column and on a 19 × 150-mm µBondapak C18 column, using a gradient of 10–30% acetonitrile in 0.5% trifluoroacetic acid.

E. coli ST 5-17 (CCECCNPACAGC) was prepared by the solid-phase method with a peptide synthesizer (430A; Applied Biosystems) on Cys(4-CH₃Bzl)-OCH₂-phenylacetamidomethyl resin. After coupling all the amino acids, the protected peptide resin (0.7 g) was treated with hydrogen fluoride/dimethylsulfide/anisole/*p*-thiocresol, 8:1:1:0.5, vol/vol/vol/wt, at 0°C for 60 min. The hydrogen fluoride was removed under reduced pressure, and the residue was washed with ethyl ether and ethyl acetate. ST 5-17 was extracted with 50% acetic acid (2 × 30 ml). The resulting solution was then diluted to 10% acetic acid. The crude reduced form of ST 5-17 was purified on low pressure C18 reverse-phase chromatography. The main fractions were collected and lyophilized. The residue was dissolved, and 500 ml of water and 20 ml of 0.5 M phosphate buffer (pH 8.2) was added. The solution was kept at room temperature with constant stirring and the pH was maintained at pH 8.2 until no free mercapto groups were detected. The resulting peptide solution was acidified to pH 2.5 and was purified by high pressure C18 reverse-phase chromatography. Structure of ST 5-17 was verified by electrospray mass spectrometry, gas-phase sequence analysis, and amino acid composition analysis.

Materials. Bumetanide, DME, and Hamm's F12 media were obtained from Sigma Chemical Co., and Na¹²⁵I was purchased from Dupont-New England (Boston, MA). FBS was obtained from Gibco Laboratories, (Grand Island, NY). Other reagent grade chemicals were purchased from Sigma Chemical Co. or Fisher Scientific.

Results

T₈₄ cells cultured on collagen-coated membranes were mounted into a modified Ussing chamber so that the effects of guanylin and ST on Cl[−] secretion could be measured by the changes in I_{sc}. These cells appear to secrete Cl[−] without any net transport of other ions, thus I_{sc} is proportional to the rate of Cl[−] secretion (16–21). Treatment of T₈₄ cells by adding 1 µM guanylin to the basolateral reservoir had little if any effect on I_{sc} (Fig. 1). However, addition of 1 µM guanylin to the buffer bathing the apical membrane elicited a marked and sustained increase in I_{sc}. Thus, guanylin receptors appear to be localized to the apical surface of T₈₄ cells cultured on permeable membranes.

The increased I_{sc} produced by 1 µM guanylin added to the apical side was rapidly reversed by the subsequent addition to

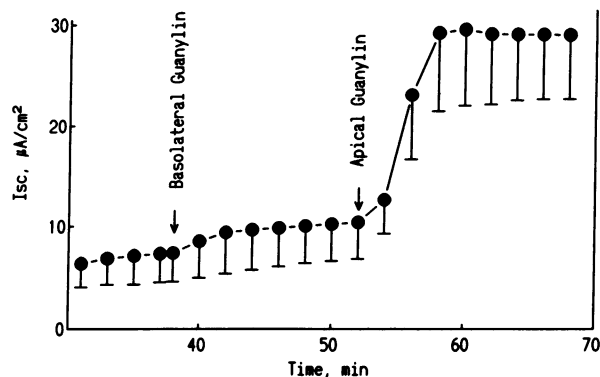


Figure 1. Guanylin stimulation of Isc in T_{84} cells by activation of apical receptors. T_{84} cells were cultured on collagen-coated membranes and mounted in an Ussing chamber as described in Methods. These data are the \pm SEM of 3 experiments. At the arrows, $1 \mu\text{M}$ guanylin was added to the basolateral reservoir followed by the addition of $1 \mu\text{M}$ guanylin to the apical reservoir. Zero time was when the cells were initially mounted in the chamber.

the basolateral reservoir of $100 \mu\text{M}$ bumetanide, an inhibitor of the Na-K-2Cl cotransporter (Fig. 2 A). Addition of $1 \mu\text{M}$ acetylcholine to the basolateral reservoir caused only a small increase in Isc after bumetanide treatment. Acetylcholine elicited a marked increase in Isc in control T_{84} cells when added after

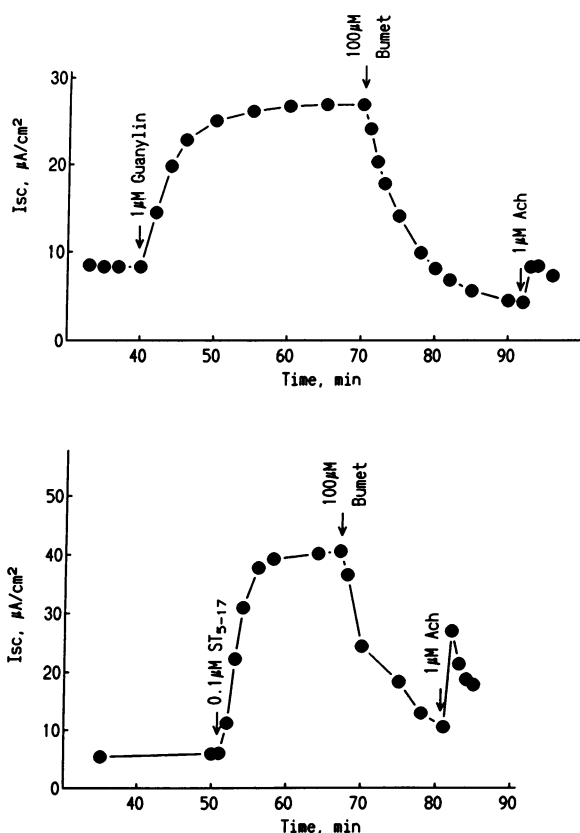


Figure 2. Effects of bumetanide on guanylin-stimulated Isc of T_{84} cells. See legend to Fig. 1 and Methods for details. These data are representative experiments of at least three separate experiments with guanylin and ST 5-17 added to the apical reservoir and bumetanide added to the basolateral reservoir.

the treatment of cell monolayers with either guanylin or ST (Isc peak response $> 130 \mu\text{A}/\text{cm}^2$, data not shown). Prior treatment of T_{84} cells with $1 \mu\text{M}$ guanylin substantially increased the Isc response to acetylcholine as compared with the responses of naive cells to acetylcholine (data not shown). Similar results were obtained when T_{84} cells were treated with 100 nM *E. coli* ST 5-17 followed by $100 \mu\text{M}$ bumetanide (Fig. 2 B). Synthetic ST 5-17 was used because this peptide has the minimum structure required to activate guanylate cyclase with a potency and efficacy equal to naturally occurring bacterial STs (9, 25). When $100 \mu\text{M}$ bumetanide was added before $1 \mu\text{M}$ guanylin, this peptide caused only a small increase in Isc (Fig. 3). These data show that guanylin-activated and ST-stimulated Isc in T_{84} cells are both dependent upon the bumetanide-sensitive Na-K-2Cl cotransporter localized in the basolateral membrane of T_{84} cells (16). Acetylcholine, which stimulates Cl^- secretion via a calcium/phosphoinositide signal transduction pathway in T_{84} cells (17, 20), also had little effect on Isc when bumetanide was present. $100 \mu\text{M}$ bumetanide did not completely block the Isc response to agonists, especially when bumetanide was added first (Fig. 3). Perhaps an additional Cl^- transport pathway exists to accomplish the basolateral membrane transport of Cl^- such as $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ exchangers.

The potencies of guanylin or ST were assessed using a cumulative concentration-response protocol for these preparations of rat guanylin and *E. coli* ST 5-17 (Fig. 4). Stimulation of Isc occurred at 10 nM guanylin, and subsequent addition of 100 or $1,000 \text{ nM}$ guanylin elicited proportionally greater increases in Isc. In comparison, *E. coli* ST 5-17 stimulated Isc at 1 nM , suggesting that this peptide was more potent than guanylin. The concentrations of guanylin or ST used in these experiments did not appear to elicit maximal responses, but these curves seem to be parallel with the potency of guanylin about one-tenth that of ST 5-17. The magnitude of Isc responses to $1 \mu\text{M}$ guanylin and 100 nM *E. coli* ST 5-17 were similar in T_{84} cells that had not been pretreated with lower concentrations of these agonists (data not shown).

The relative potencies of guanylin and *E. coli* ST 5-17 were also assessed by measuring the accumulation of cGMP in T_{84} cells exposed to these peptides. Using this bioassay, guanylin appeared to be less potent than *E. coli* ST 5-17 (Fig. 5). These data suggest that guanylin and ST 5-17 have approximately equal efficacies for activation of guanylate cyclase. The remark-

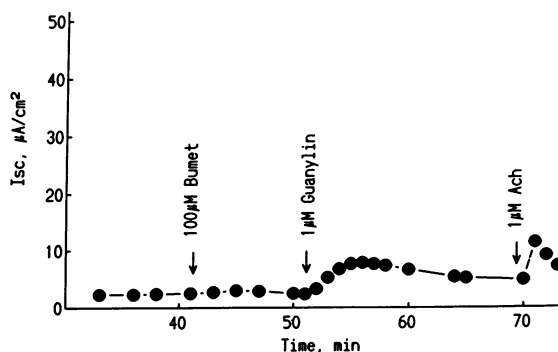


Figure 3. Bumetanide pretreatment inhibits the Isc response to guanylin. These data are a representative experiment of at least three separate experiments. Bumetanide was added to the basolateral and guanylin to the apical reservoir.

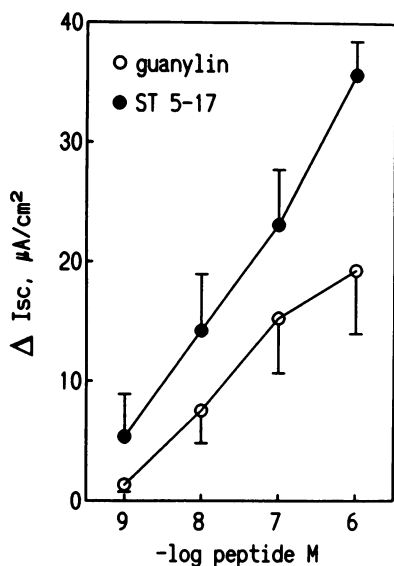


Figure 4. Comparison of guanylin and *E. coli* ST 5-17 potencies in the stimulation of Isc in T₈₄ cells. Synthetic rat guanylin or ST 5-17 peptides were added to the apical reservoir at 10–15-min. intervals. The lowest concentrations shown above were added first, followed by successive additions of the next highest concentrations. These data are the mean of five experiments with ST 5-17 and four experiments with rat guanylin. The horizontal bars are 1/2 SEM for each point.

able magnitude of the increase in cGMP production elicited by these agonists should be emphasized. The cGMP levels in this experiment are plotted on a log scale in Fig. 5. 1 μ M *E. coli* ST 5-17 caused a 4,140-fold increase in cGMP, whereas 10 μ M guanylin elicited a 1,490-fold increase in cGMP. Concentrations of guanylin greater than 10 μ M were not used because it consumed excessive amounts of this peptide.

The effects of guanylin and ST 5-17 on ¹²⁵I-ST binding to intact T₈₄ cells were evaluated in inhibition-binding assays in order to determine the relative affinities of these peptides for the ST receptor. As shown in Fig. 6, radioligand binding was fully inhibited by both peptides in a concentration-dependent manner. Inhibition of ¹²⁵I-ST binding occurred over a wide range of competing ligand concentrations, suggesting the presence of two classes of binding sites labeled by ¹²⁵I-ST with different affinities for the two competing ligands. This observation was supported by computer-assisted curve fitting, which revealed a significantly better fit of the inhibition binding data

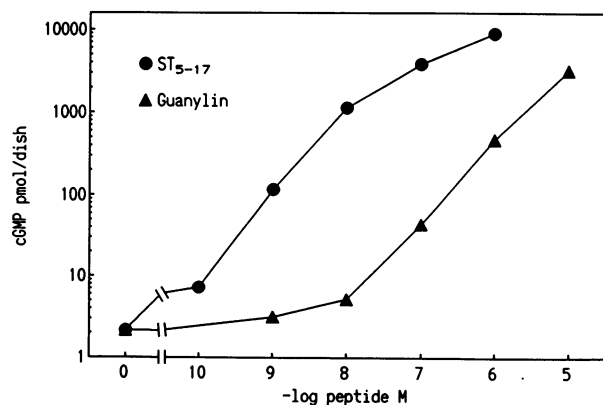


Figure 5. Comparison of cGMP accumulation responses to guanylin and *E. coli* ST 5-17. T₈₄ cells cultured in 35-mm dishes were treated with the indicated concentrations of peptide agonists for 60 min. in the presence of 1 mM 3-Isobutyl-1-methylxanthine (IBMX). These data are a representative experiment of duplicate dishes at each concentration of guanylin (\blacktriangle) or ST 5-17 (\bullet).

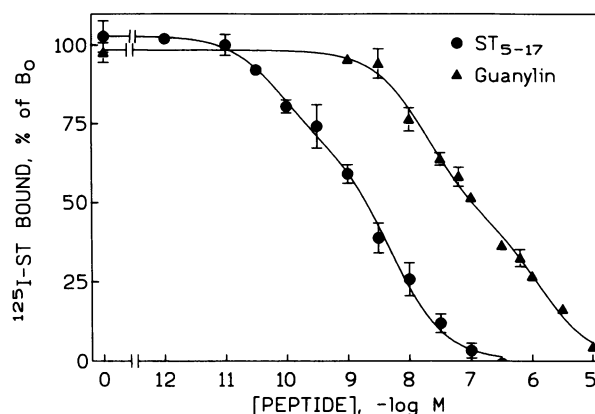


Figure 6. Inhibition of ¹²⁵I-ST 1-18 binding by guanylin and ST 5-17. Binding of ¹²⁵I-ST 1-18 to intact T₈₄ cells was determined in the presence of the indicated concentrations of guanylin (\blacktriangle) or ST 5-17 (\bullet) as described in Methods. The values shown are the composite data (\pm SEM) from four experiments performed in duplicate and are expressed as the percent of binding in the absence of competing ligand. The curves are the computer-derived best fits of the data to a two-site model. The K_i values are given in the text.

to a two-site model vs. a one-site model for both guanylin and ST 5-17 ($P < 0.01$ in both cases). Guanylin inhibited ¹²⁵I-ST binding with K_i values of 19 ± 5 nM (mean \pm SE) and 1.3 ± 0.5 μ M with 55 \pm 5% of the sites of the high affinity class. Inhibition of binding by ST 5-17 was characterized by K_i values of 78 ± 38 pM and 4.9 ± 1.4 nM with 34 \pm 7% of the binding to the high affinity site.

Discussion

Several key observations provided information suggesting that the intestinal membrane guanylate cyclase located in apical membranes of enterocytes may be controlled by an endogenous ligand. Discovery that *E. coli* ST was a potent activator of intestinal guanylate cyclase and that cGMP could influence intestinal fluid secretion was the first evidence for an endo-ST (5, 6). The observation that ST-stimulated guanylate cyclase was also found in opossum kidney proximal tubules and other epithelia of this species provided additional rationale that this enzyme would be regulated by an endogenous ligand instead of a bacterial peptide (11–14). Molecular cloning of rat and human cDNAs encoding a putative ST receptor protein with structural similarity to the atrial peptide receptor/guanylate cyclases further supported the proposal that an endo-ST existed (1, 2). Purification of the peptide, guanylin, from rat intestine using the T₈₄ human intestinal cell cGMP response as a bioassay has provided a candidate for the endogenous hormone that regulates intestinal Cl[−] secretion (9). Data presented in this manuscript are consistent with the hypothesis that guanylin serves this function in the intestine because synthetic guanylin activates guanylate cyclase and stimulates Cl[−] secretion in cultured human intestinal T₈₄ cells.

Guanylin stimulated Cl[−] secretion, as reflected by the increased Isc of T₈₄ cells, caused marked ($> 1,000$ -fold) increases in cGMP accumulation, and fully competed with ¹²⁵I-ST for binding sites on T₈₄ cells. The potency of guanylin for stimulation of cGMP production was about 1/100 of that observed with *E. coli* ST 5-17. However, the potency of guanylin in the

stimulation of Isc seemed appreciably closer to that of ST 5-17 (~ 10-fold different). The relative binding affinities of guanylin or *E. coli* ST 5-17 for ¹²⁵I-ST binding sites generally agreed with the cGMP bioassay data. These differences in apparent potency could be due to differences in assay conditions because T₈₄ cells were cultured on collagen-coated filters for Cl⁻ secretion measurements and cultured in plastic dishes for cGMP responses or ¹²⁵I-ST binding assays. The expression of guanylin receptors (i.e., guanylate cyclase-C, [1, 2]) may be influenced, leading to different types and/or densities of apical receptors in T₈₄ cells cultured on permeable substrates. It is unlikely that these potency differences are due to using a rat peptide in human intestinal cells, since rat and human guanylin peptides are highly conserved (10, 26–28). In the carboxyl-terminal 15 amino acids (i.e., amino acids 101–115 of preproguanylin) only one substitution was observed at position 102 between rat and human peptides. Moreover, the potencies of rat and human guanylin 101–115 for increases in cGMP levels of T₈₄ cells were not different (26). The potency of human guanylin to increase Isc in the rat colon (26) was similar to the potency of rat guanylin on Isc of T₈₄ cells in this study. In both instances, threshold increases in Isc were observed at 10 nM guanylin. Another consideration is that the secreted form of guanylin may be a longer peptide than the 15 amino acid peptide that was purified from rat jejunum (9). An acid-labile bond between Asp 100 and Pro 101 would have yielded the 15 amino acid peptide upon boiling in acetic acid. Perhaps proteolytic processing of proguanylin occurs before secretion using an upstream site such as the arginine at position 93 or 94, which is conserved in rat, human, and mouse guanylin precursor sequences (10, 26–28). A much longer peptide would be generated by cleavage at the lysine pairs found at amino acids 54, 55 for rat and mouse or positions 37, 38 found in human or mouse preproguanylin (10, 26–28). The potencies of longer forms of guanylin that may be derived by cellular processing of the precursor peptide should be compared with guanylin 101–115 and ST before concluding that bacterial STs are inherently more potent than guanylin. If ST peptides are more potent than guanylin, perhaps related to the additional pair of cysteines and the resulting disulfide bond in ST (25), the increased affinity of ST for guanylin receptors would help to explain their powerful pathophysiological effects in vivo (7, 8). Whether STs are “super” guanylinomimetic agonists is a postulate worthy of further investigation.

The finding that ¹²⁵I-ST appears to label two binding sites that have different affinities for guanylin or *E. coli* ST 5-17 raised the question of the relative contribution of these putatively different receptors in the cellular responses to agonists. What contribution to the Cl⁻ secretion response of T₈₄ cells might occur as a result of these agonists binding to either the high or low affinity receptors? Perhaps both receptors are required to elicit the Isc response to either guanylin or *E. coli* ST 5-17. Both receptors could be guanylate cyclases, or only one may have this enzymatic activity. Recent experiments evaluating the binding of ¹²⁵I-ST to rat intestinal brush border membranes provided data consistent with two receptors existing with different affinities for ST (29). Thus, heterogeneity of guanylin receptors may occur in target cells of the intestine. Presently, only one putative receptor for *E. coli* ST has been identified by molecular cloning (1–3). However, since the extracellular ligand-binding domains of the rat and human recep-

tors appear to be relatively poorly conserved (1, 2), perhaps these two receptors represent different isoforms of intestinal guanylin/ST receptors. Our data and those of other investigators (29) emphasize the possibility that guanylin/ST receptor heterogeneity may exist. It may be of interest that when the human guanylin/ST receptor was expressed in human embryonic kidney 293 cells, the cGMP response of these cells to human guanylin, derived from COS cells transfected with a guanylin cDNA, was relatively poor with respect to both potency and efficacy when compared with the cGMP response of T₈₄ cells to guanylin (28). For example, 0.1 μM guanylin caused an increase in the cGMP levels of T₈₄ cells that was equivalent (40–50-fold) to the cGMP response of 293 cells to 10 μM guanylin. Moreover, the IC₅₀ for guanylin inhibition of ¹²⁵I-ST binding to receptors on 293 cells was 0.1 μM, whereas the K_i for guanylin inhibition of ¹²⁵I-ST binding in T₈₄ cells was 0.02 μM for the high affinity site. It is possible that the expressed receptor responds differently to guanylin than does the guanylin/ST receptors found in T₈₄ cells. Another possibility is that COS cells transfected with guanylin cDNA may produce an intrinsically less potent guanylin, perhaps resulting from inappropriate disulfide pairing. Whether the control of intestinal Cl⁻ channels by guanylin occurs through a single guanylate cyclase receptor (i.e., GC-C) or by a more complex mechanism awaits further elucidation of the guanylin receptor/signal transduction pathway.

In summary, guanylin activated both guanylate cyclase and Cl⁻ secretion in T₈₄ human intestinal cells. Regulation of Cl⁻ secretion and thus fluid and salt secretion by the local release of guanylin at sites where this peptide can bind to its receptors on the apical membrane of intestinal crypt cells could be an important physiological mechanism. Activation of guanylin receptors by structurally similar peptides secreted by enteric bacteria reveals an opportunistic mechanism, which helps to explain the powerful pathophysiological effects of *E. coli* ST on intestinal salt and fluid secretion.

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