Original Paper

Acta Anat 1995;153:210–219

W.J. Krause[a]
R.H. Freeman[b]
S.L. Eber[c, d]
F.K. Hamra[c]
K.F. Fok[c]
M.G. Currie[e]
L.R. Forte[c, d]

Departments of
Pathology and Anatomical Sciences,
Physiology, and
Pharmacology, School of Medicine,
Missouri University, Columbia, Mo.,
Harry S. Truman Memorial VA
Medical Center, Columbia, Mo., and
Searle Research and Development,
St. Louis, Mo., USA

Distribution of *Escherichia coli* Heat-Stable Enterotoxin/Guanylin/ Uroguanylin Receptors in the Avian Intestinal Tract

Abstract

Pathogenic strains of enteric bacteria secrete small heat-stable toxins (STs) that activate membrane guanylyl cyclase receptors found in the intestine. The intestinal peptide agonists, guanylin and uroguanylin, are structurally related to STs. Receptors for ^{125}I-ST were found throughout the entire length of the intestinal tract of all the birds examined. These receptors were restricted to intestinal epithelial cells covering villi and forming intestinal glands and were not observed in other strata of the gut wall. The most intense labeling of receptors by ^{125}I-ST occurred in the region of the microvillus border of individual enterocytes. There appeared to be a decrease in receptor density distally along the length of the small intestine, although labeling of receptors by ^{125}I-ST was observed throughout the small intestine and colon. Cellular cGMP accumulation responses to *Escherichia coli* ST and rat guanylin in the domestic turkey and duck were greater in the proximal small intestine compared to the distal small intestine or colon. Brush border membranes (BBM) isolated from the mucosa of proximal small intestine of turkeys exhibited agonist-stimulated guanylyl cyclase activity. The rank order potency for enzyme activation was *E. coli* ST > uroguanylin > guanylin. Competitive radioligand binding assays using ^{125}I-ST and turkey intestine BBM revealed a similar rank order affinity for the receptors that was exemplified by the K_d values of ST 2.5 n*M*, uroguanylin 80 n*M* and guanylin 2.6 µ*M*. It may be concluded that functional receptors for the endogenous peptides, guanylin and uroguanylin, occur in the apical membranes of enterocytes throughout the avian intestine. The receptor-guanylyl cyclase(s) of proximal small intestine were preferentially activated by uroguanylin relative to guanylin, but both endogenous peptides were less potent than their molecular mimic, *E. coli* ST.

Key Words
Enterocytes
Guanylyl cyclase
Cyclic GMP
Avian species

Introduction

Heat-stable enterotoxins (STs) are peptides produced by pathogenic strains of bacteria such as *Yersinia enterocolitica* or *Escherichia coli* that are a major cause of secretory diarrhea in children as well as in laboratory and domestic animals [Sack et al., 1975; Donta et al., 1977; Burgess et al., 1978; Giannella, 1981; Black et al., 1982]. The apical plasmalemma (microvillus border) of enterocytes lining the intestinal tract and forming the intestinal glands (crypts of

Received:
November 28, 1994
Accepted:
May 19, 1995

William J. Krause
Department of Pathology and Anatomical Sciences
School of Medicine
University of Missouri
Columbia, MO 65212 (USA)

Leberkühn) of man and several other mammalian species have been shown to contain specific, high-affinity binding sites for these peptides [Forte et al., 1989; Krause et al., 1990, 1994] . These STs share receptors with two recently described endogenous peptides: guanylin and uroguanylin [Currie et al., 1992; Hamra et al., 1993; Kita et al., 1994]. The ST/guanylin/uroguanylin receptor has been shown to be an intestinal isoform of membrane guanylyl cyclase (GC-C) that belongs to a family of cell surface proteins which catalyze the production of cyclic 3',5'guanosine monophosphate (cGMP) [Schulz et al., 1990; Forte and Currie, 1995]. It is by the increase in production of cGMP that STs, guanylin or uroguanylin influence cellular function. Increased intracellular levels of this second-messenger molecule can activate a cAMP-dependent protein kinase which stimulates intestinal epithelial cell Cl^- secretion [Forte et al., 1992; Tien et al., 1994]. The net effect of bacterial STs, guanylin, and uroguanylin in the mammalian intestinal tract is to promote Cl^- secretion and to inhibit Na^+ absorption [Field et al., 1978; Rao et al., 1981; Guandalini et al., 1982; Currie et al., 1992; Hamra et al., 1993]. We previously reported that $^{1-5}$I-ST labeled receptors on enterocytes throughout the intestinal tract of man and other mammals [Krause et al., 1994]. Proximal small intestine had the greatest apparent density of these receptor-guanylyl cyclases that serve as common receptors for bacterial STs and the endogenous peptide hormones, guanylin and uroguanylin.

Receptors for *E. coli* ST and ST-stimulated guanylyl cyclase activity have been reported to occur in brush border membranes isolated from chicken intestine [Katwa and White, 1992]. The present study examined the intestinal tract of several species of birds, to better define the distribution of the ST/guanylin receptors in enterocytes along both the length and vertical axis (villus/crypt unit) of the avian intestinal tract utilizing ^{125}I-ST as a radioligand for these receptors and cGMP responses to these peptide agonists in vitro [Forte et al., 1988; Krause et al., 1990, 1994]. We report here that all birds that were examined had ST/guanylin receptors localized to the enterocytes throughout the intestinal tract and that agonist-stimulated cGMP accumulation responses of intestinal mucosa were greatest in the proximal small intestine of turkeys and ducks. Brush-border membranes (BBM) isolated from the mucosa of turkey proximal small intestine exhibited guanylyl cyclase activity that was stimulated by ST > uroguanylin > guanylin in rank order potency. A similar order of relative affinities was observed in competitive radioligand binding assays using these BBMs suggesting that the receptors were preferentially activated by ST > uroguanylin > guanylin.

Materials and Methods

Receptor Autoradiography

Specimens of small intestine and colon were gathered from a variety of avian species (table 1) and frozen in liquid nitrogen as soon as possible after death. After freezing, the tissues were stored at −80 °C until used. The frozen specimens were sectioned at 14 μm in a cryostat maintained at −20 °C. Two cut sections were mounted onto opposite ends of gelatin-coated slides, air-dried and stored at −80 °C until used. Each slide was then incubated with 50 μl of Dulbecco's modified Eagle's medium (DMEM), pH 5.5, containing 0.5% bovine serum albumin (BSA) at 37 °C for 15 min as previously described [Forte et al., 1989; Krause et al., 1990, 1994]. To measure the total binding for this radioligand, 50 ul DMEM containing 1,000 cpm ^{125}I-ST/μl was added to one tissue section. The adjacent section was incubated with the same concentration of radioligand plus 1 μ*M* unlabeled ST to assess nonspecific binding of ^{125}I-ST. Additional sections also were incubated with 10 μ*M* of rat guanylin to determine if guanylin inhibited binding of ^{125}I-ST to intestinal receptors. ^{5}I-ST_{1-19} was chosen as the radioligand because iodination of Tyr 9 in guanylin appears to interfere with the biological activity of this radioligand resulting in poor binding of ^{125}I-guanylin to receptors on cultured T_{84} human intestinal epithelial cells. For this reason, a radiolabeled form of guanylin was not used. Thus, ST peptides currently are the best radioligands for identifying the tissue location of guanylin/uroguanylin receptors. Following an incubation of 15 min at 37 °C, the slides were washed with a gentle stream of cold phosphate-buffered saline (PBS) and then washed 3 additional times by placing them into 50 ml of ice-cold PBS for 5 min. The sections were air-dried prior to being coated with Kodak NTB-2 or NTB-3 emulsion, dried again, then sealed in light-tight boxes and stored at 4 °C for 3–4 weeks until developed. Following routine photographic development and fixation, the sections were coverslipped and examined by bright and dark field microscopy.

Cyclic GMP Accumulation Bioassay

Proximal, middle and distal segments of the small intestine as well as a segment of midcolon from domestic turkeys (Nicholas broad-breasted strain) and domestic ducks (Peking white strain) were dissected and mucosa prepared by scraping the intestinal mucosa free, and washing the tissue gently once in 0.9% NaCl and twice in DMEM, 20 m*M* HEPES, pH 7.4. To measure ST and guanylin stimulation of cGMP production, mucosal suspensions (60 mg wet weight) were placed in 0.2 ml DMEM (pH 7.4) containing 20 m*M* HEPES buffer (pH 7.4) at 4 °C. The tissue was incubated for 40 min at 37 °C with either 1 u*M E. coli* ST, 10 μ*M* rat guanylin or vehicle that was added to DMEM-HEPES containing 1 m*M* isobutylmethylxanthine (IBMX). Perchloric acid was then added to a final concentration of 3.3%, cells were centrifuged and the resulting supernatants neutralized with 1 *N* KOH. The supernatant solution was used to measure cGMP concentration by radioimmunoassay as reported previously [Forte et al., 1988].

Competitive Radioligand and Binding Assays

BBM were thawed and centrifuged at 16,000 *g* for 15 min, then resuspended in a solution containing 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, and 1 m*M* EDTA. The binding assay consisted of 35 μl of the same buffer, 20 ul ^{125}I-ST (50,000–100,000 cpm), 20 μl of peptide ligand in H_2O, and 25 ul of BBM (15–20 μg protein). Incubation was at 37 °C for 1 h. Then 3 ml of cold phosphate-buffered saline (PBS) was added and this solution filtered using Whatman GF/F filters

Table 1. Avian species examined for *E. coli* ST-specific binding sites in intestinal epithelial cells

Number of animals used[1]	Species	ST_{1-19}
3	Emu *(Dromaius novaehollandie)*	+++
2	Blue jay *(Cyanocitta cristata)*	++
2	Red-headed woodpecker *(Melanerpes erythrocephalus)*	++
2	Purple grackle *(Quiscalus quiscula)*	++
2	Starling *(Sturnus vulgaris)*	++
2	Bob-white quail *(Colinus virginianus)*	++
3	Ring-necked pheasant *(Phasianus colchicus torquatus)*	++
6	Domestic chicken (Barred rock/leghorn cross)	++
8	Domestic turkey (Nicholas broad breasted)	++
2	Mourning dove *(Zenaidura macroura)*	++
2	Domestic pigeon (Feral)	++
2	Coot *(Fulica americana)*	++
2	Wood duck *(Aix sponsa)*	++
3	Ring-necked duck *(Aythya collaris)*	++
2	Northern shoveller *(Anas clypeata)*	++
2	Mallard duck *(Anas platyrhynchos)*	++
2	American green-winged teal *(Anas crecca)*	++
2	Blue-winged teal *(Anas discors)*	++
6	Domestic duck (Peking white)	++
2	Giant Canada goose *(Branta canadensis maxima)*	++
2	Wilson's snipe *(Capella gallinago delicata)*	++
3	Woodcock *(Philohela minor)*	++

Intensity of silver grains: +++ = strong; ++ = moderate.

[1] Total number of animals used for both in vitro autoradiography and cGMP accumulation bioassays of intestinal mucosa.

(25 mm diameters) and a vacuum manifold. The filters were washed twice with 3 ml cold PBS. Each filter had been soaked prior to use with 0.1% polyethylenimine and washed with 3 ml PBS before filtering the BBM reaction mixture. Each filter was then placed into glass tubes and radioactivity measured by gamma scintillation spectrometry. The radioligand binding data were analyzed using the Inplot computer program to estimate K_d and B_{max} values (Graph Pad Software for Science, San Diego, Calif., USA). The data were fit to a single-site model in these experiments.

Isolation of Intestinal BBM

The proximal one-half of the small intestine from an adult turkey was washed in 0.9% NaCl, cut open longitudinally and mucosa scraped free with a microscope slide. The mucosa was homogenized in 7.5 vol of a buffer containing 300 m*M* *D*-mannitol, 5 m*M* EGTA, 12 m*M* Tris-HCl, pH 7.5, per gram of mucosa with a Polytron homogenizer according to the method of Biber et al. [1981]. The isolation of a fraction enriched in BBM was carried through the P4 stage of purification using a one-precipitation step with 12 m*M* $MgSO_4$. The BBMs were stored frozen at −80 °C prior to use. Protein content was measured by the method of Bradford [1979].

Measurement of Guanylyl Cyclase Activity

Enzyme activity was measured in an assay volume of 100 µl containing 10 µg BBM protein, 50 m*M* HEPES, pH 7.6, 0.5 m*M* isobutylmethylxanthine (IBMX), 1 m*M* ATP, 10 m*M* creatine phosphate, 5 units creatine phosphokinase, 1 m*M* GTP and 5 m*M* $MgCl_2$. Incubation was for 15 min at 37 °C. The reaction was stopped by adding 100 µl of 6% perchloric acid. Then each reaction mixture was neutralized with 10 *N* KOH and centrifuged to remove the potassium perchlorate precipitate. A 25-µl portion was removed to use for the estimation of cGMP by radioimmunoassay as previously described [Forte et al., 1988]. The data are expressed as pmol cGMP formed per ug protein per 15 min. Agonists or vehicle were added at the indicated concentration and assayed in duplicate.

Preparation of Intestinal Extracts and Bioassay

About 200 g of mucosa isolated from the small intestine and colon of turkeys was suspended in 2 liters of 1 *M* acetic acid, heated at 100 °C for 10 min and then homogenized with a Polytron as previously described for opossum intestinal mucosa [Hamra et al., 1993]. The homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant was made in 0.1% trifluoroacetic acid (TFA). Extracts were isolated using C18 cartridges as previously described [Hamra et al., 1993]. Bioactive peptides that eluted with 40% acetonitrile and 0.1% TFA in H_2O were chromatographed on a 2.5 × 90 cm Sephadex G-25 column as previously described. The bioassay for guanylin/uroguanylin-like peptides was carried out by removing 0.5 ml from each 10-ml column fraction, drying in a Speed-Vac and resuspending each fraction in 200 µl of DMEM. This sample was added to one well of a 24-well culture plate containing confluent T_{84} cells. Incubation was for 40 min at 37 °C and cellular cGMP was measured by RIA as previously described [Forte et al., 1988; Hamra et al., 1993]. The conditions for culture of T_{84} cells were as previously described [Hamra et al., 1993].

Synthesis of E. coli *ST 5-17 and Guanylin*

Rat guanylin (PNTCEICAYAACTGC) and *E. coli* ST5-17 (CCELCCNPACAGC) were synthesized by the solid-phase method as previously described [Currie et al., 1992].

Preparation of ^{125}I-ST

The iodination of *E. coli* ST (NSSNYCCELCCNPACTGCY, Multiple Peptide Systems, San Diego, Calif., USA) was carried out using the lactoperoxidase procedure described previously [Forte et al., 1988, 1989]. Purification of ^{125}I-ST was achieved using high-performance liquid chromatography with a C18 column under reverse-phase conditions also as described earlier [Forte et al., 1988, 1989]. $Na^{125}I$ was purchased from DuPont NEN, Wilmington, Del., USA, as the carrier-free radionuclide (14–17 µCi/µg). Lactoperoxidase was purchased in a solid-state form from Biorad Laboratories, Richmond, Calif., USA. *E. coli* ST, GTP, ATP, creatine phosphate, and creatine phosphokinase were purchased from the Sigma Chemical Company, St. Louis, Mo., USA. Other reagents and materials were obtained from various suppliers.

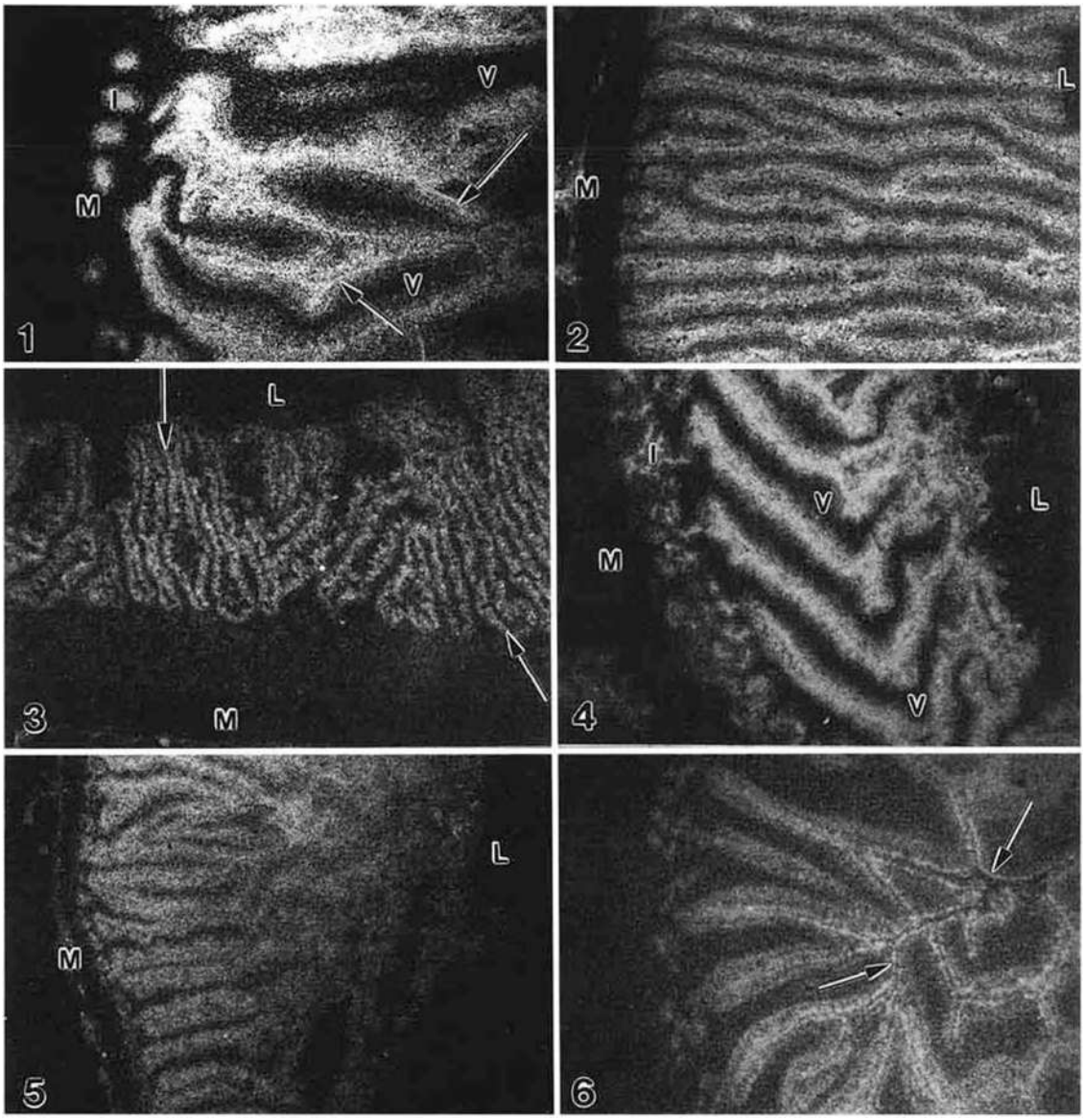

Fig. 1. A portion of intestinal mucosa from the proximal small intestine of an emu. The enterocytes covering villi (V) and forming the intestinal glands (I) express ST/guanylin receptors. Elements of the lamina propria and muscularis mucosae (M) show little if any receptor labeling. Note the intense labeling in the region of the microvillus border (arrows) by ^{125}I-ST. Dark field. ×297.

Fig. 2. A region of small intestinal wall from the red-headed woodpecker illustrating the even distribution of receptors labeled by ^{125}I-ST in enterocytes of the intestinal mucosa. The lumen of the small intestine (L) is shown at the extreme right; the muscularis externa (M) at the far left. Dark field. ×119.

Fig. 3. A portion of small intestine from the purple grackle also shows a relatively even distribution of ^{125}I-ST-labeled receptors among enterocytes (arrows) of the intestinal mucosa. The lumen (L) is shown near the top; the muscularis externa (M) near the bottom of the photomicrograph. Dark field. ×119.

Fig. 4. ^{125}I-ST-receptor density in the intestinal mucosa of the blue-jay, unlike the red-headed woodpecker and purple grackle, appears greater in enterocytes covering villi (V) than in enterocytes forming intestinal glands (I). The intestinal lumen (L) is oriented to the right; the muscularis externa (M) to the left of the photomicrograph. Dark field. ×119.

Fig. 5. ^{125}I-ST-receptor density in enterocytes lining the small intestine of a domestic chicken. The intestinal lumen (L) is to the right; the muscularis externa (M) is to the left. Dark field. ×119.

Fig. 6. A segment of colon taken from a mallard duck illustrates greater ^{125}I-ST-receptor density in enterocytes lining the luminal surface. Note that the most intense labeling occurs in the microvillus border (arrows). Dark field. ×119.

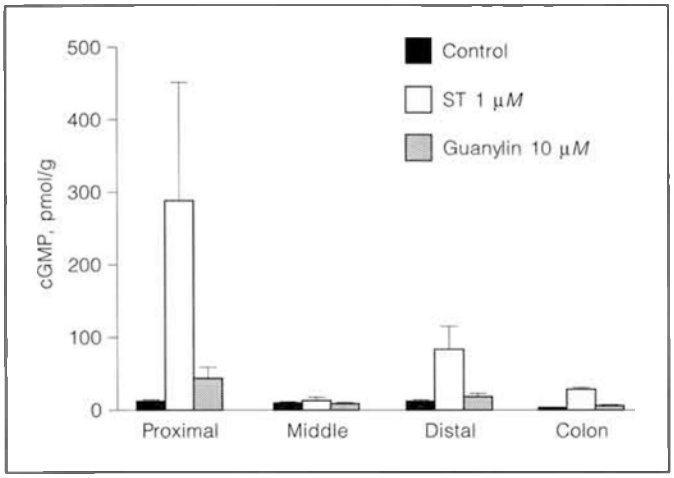

Fig. 7. cGMP production by the intestinal mucosa taken from different segments of turkey small intestine and colon following stimulation by 1 μ*M E. coli* ST and 10 μ*M* rat guanylin. The data are the mean of three experiments. Small intestine – proximal, middle and distal segments.

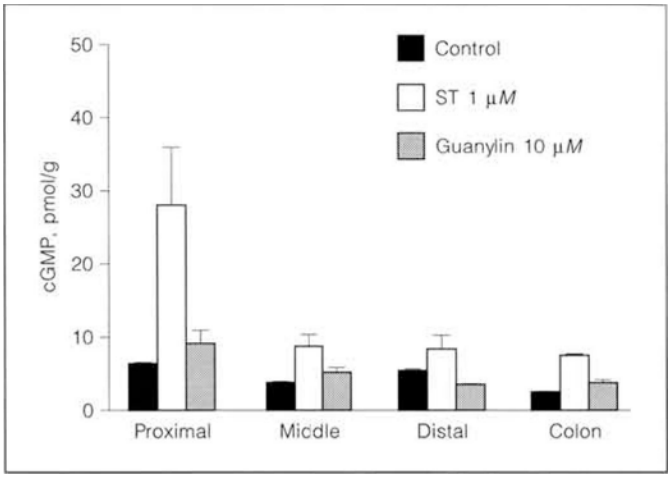

Fig. 8. cGMP production by the intestinal mucosa taken from different segments of Peking duck small intestine and colon following stimulation by 1 μ*M* *coli* ST and 10 μ*M* rat guanylin. The data are the mean of two experiments. Small intestine – proximal, middle and distal segments.

Results

Distribution of ST/Guanylin Receptors

Examination of the distribution of receptors labeled with ^{125}I-ST from various segments of the small intestine and colon of a number of avian species showed that high-affinity ^{125}I-ST-binding sites were present and confined to the intestinal epithelium throughout the length of the intestinal tract (table 1; fig. 1–6). ^{125}I-ST binding sites were not observed in other layers of the gut wall (lamina propria, muscularis mucosae, submucosa, muscularis externa or serosa). The greatest intensity of ^{125}I-ST binding occurred along the microvillus (striated) border of intestinal epithelial cells. Adjacent sections that were incubated with the same concentration of radioligand plus 1 μ*M* of either unlabeled ST or 10 μ*M* of rat guanylin effectively inhibited ^{125}I-ST binding suggesting that common receptors were labeled by ^{125}I-ST.

Along the vertical axis (the villus/crypt unit) of the small intestine in the birds that were examined, ^{125}I-ST binding to receptors appeared to be evenly distributed among intestinal epithelial cells (enterocytes) covering villi and forming the intestinal glands (fig. 2, 3, 5). In some birds, such as the blue jay, receptor density appeared greatest in enterocytes covering villi and lining the intestinal lumen (fig. 4). Receptor density of enterocytes lining the surface of the colon in the mallard duck (fig. 6) and Canada goose (data not shown) also appeared to be greater than in those enterocytes forming the intestinal glands. Receptor density along the longitudinal axis of the small intestine following in vitro receptor autoradiography appeared to be greater in the proximal region in most species.

To evaluate further the possibility that ST/guanylin receptors are more abundant in proximal small intestine, different segments of the intestinal tract of domestic turkeys and ducks were used to measure guanylin and *E. coli* ST-stimulated guanylyl cyclase activity. Agonist-mediated activation of the membrane guanylyl cyclase was measured by the increased cGMP content of mucosa exposed to these peptides. Treatment of the intestinal mucosa with 1 μ*M* ST elicited a large increase in cGMP levels in the proximal small intestine of turkeys with a much reduced cGMP accumulation response to ST occurring in the distal small intestine and colon (fig. 7). ST stimulated cGMP production by the intestinal mucosa to much higher levels than did 10 μ*M* guanylin in all segments. Likewise, cGMP accumulation responses to ST in the intestinal mucosa of the domestic (Peking) duck were much greater in the proximal small intestine as compared to the middle or distal small intestine and colon (fig. 8). In comparison to ST, 10 μ*M* guanylin was considerably less effective in stimulating cGMP accumulation in the mucosa of small intestine or that of the colon in ducks. The magnitude of cGMP responses in proximal intestine of turkeys was substantially greater than that observed in ducks.

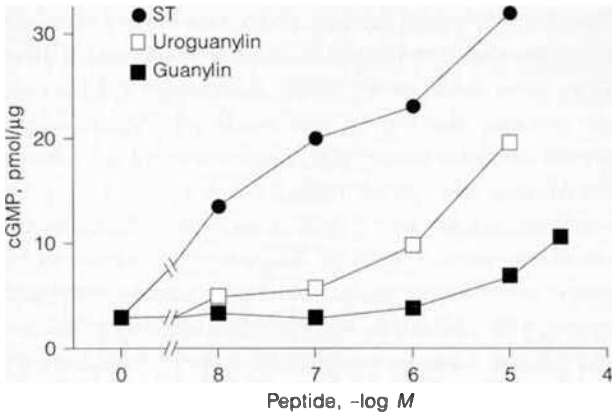

Fig. 9. Guanylyl cyclase activity of brush border membranes isolated from turkey small intestine. The data shown in this figure are representative of two such experiments with each point assayed in duplicate. Synthetic peptides, ST_s opossum uroguanylin and rat guanylin were used in these experiments.

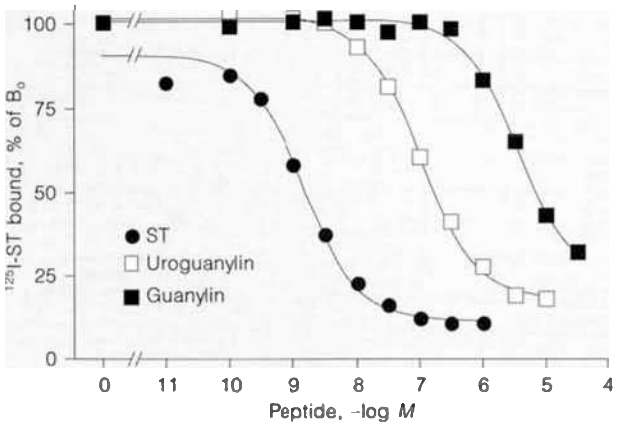

Fig. 10. Comparison of the relative affinities of ST, uroguanylin and guanylin for receptors in turkey intestine. These data are the mean of two experiments with each point assayed in triplicate for each experiment. The radioligand was ^{125}I-ST (*E. coli* STD). Bo = Total bound ^{125}I-ST in the absence of competing ligand.

To characterize further the properties of the guanylyl cyclase receptors in turkey intestine, isolated BBM from the mucosa of proximal small intestine were used. Agonist-stimulated guanylyl cyclase activities were measured to compare the relative potencies and efficacies of *E. coli* ST, rat guanylin and opossum uroguanylin. These peptides exhibited a rank order of potencies with ST > uroguanylin > guanylin (fig. 9). A similar rank order of agonist potencies has been reported for the human GC-C isoform that is expressed in T_{84} colon carcinoma cells [Hamra et al., 1993; Kita et al., 1994]. Thus, turkey small intestine, like the human receptor-guanylyl cyclase, has a BBM receptor-guanylyl cyclase that appears to prefer uroguanylin relative to guanylin. It should be noted that ST is considerably more potent than either uroguanylin or guanylin. Our supplies of these peptide agonists were insufficient to use concentrations higher than 10–30 µ*M* in this assay so that maximal stimulation of the guanylyl cyclase was not achieved in these experiments.

Evaluation of the pharmacological properties of the BBM receptors was extended using competitive radioligand binding assays to measure the affinities of the receptors for *E. coli ST*, opossum uroguanylin and rat guanylin (fig. 10). All three peptides inhibited the binding of ^{125}I-ST to an apparently common set of binding sites (B_{max} = 6.13 pmol/mg protein) on turkey intestine BBM. The K_i values for these peptides were: ST 2.5 n*M*, uroguanylin 80 n*M* and guanylin 2.6 u*M*. Thus, ST had a 40-fold higher apparent affinity for these receptors than did uroguanylin, which had an approximate 32-fold higher affinity than guanylin. These data are consistent with the relative potencies of ST > uroguanylin > guanylin as activators of the BBM guanylyl cyclase.

Guanylin and uroguanylin peptides are found in the mammalian intestine [Currie et al., 1992; Hamra et al., 1993, 1995]. To examine whether these bioactive peptides also occur in avian intestine, we prepared an extract of turkey intestinal mucosa and subjected this extract to gel filtration chromatography (fig. 11). A broad peak of bioactivity eluted in the internal volume of this Sephadex G-25 column. These fractions activated the human T_{84} cell intestinal guanylyl cyclase (GC-C) that was used for the bioassay of turkey intestinal agonists. The dominant peak of bioactive fractions eluted before either uroguanylin or guanylin, suggesting that these putative peptides from turkey intestine may be longer forms of guanylin and/or uroguanylin or have different structures from the peptides isolated from mammalian intestine or urine [Currie et al., 1992; Hamra et al., 1993, 1995]. Therefore, intestinal mucosa of turkeys contains bioactive agonists that stimulate cGMP production in human intestinal cells. It is likely that these substances are peptides that are structurally similar to uroguanylin, guanylin and ST peptides, the known agonists for this class of receptors.

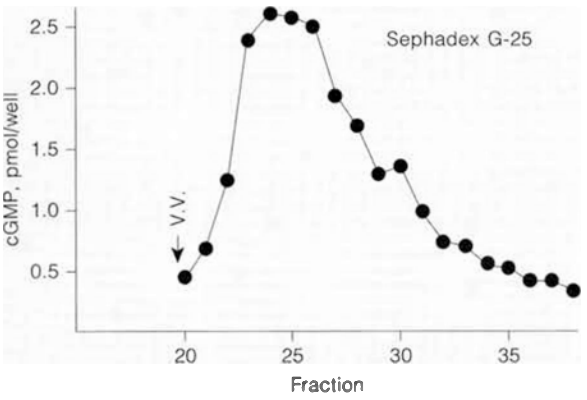

Fig. 11. Guanylin/uroguanylin-like activity isolated from the intestinal mucosa of turkeys. The bioassay is the cGMP accumulation response of culture T_c cells to 200 µl of each fraction from a 2.5×90 cm Sephadex G-25 column. vv = Void volume.

Discussion

Receptors for *E. coli* ST had previously been identified in purified BBM isolated from small intestine of the chicken [Katwa and White, 1992]. The current study extends those observations and demonstrates that the ST/guanylin/uroguanylin receptor is localized to enterocytes of the chicken intestine as well as those cells lining the intestinal tract of a number of other avian species. Like man and other mammalian species examined to date, these receptors are localized to the enterocytes lining the intestinal lumen and forming the intestinal glands. As in the avian species investigated in the present study, the greatest receptor density in enterocytes of mammals occurs in the region of the microvillus border [Krause et al., 1990, 1994]. There is an apparent decrease in receptor density distally along the longitudinal axis of the small intestine of some avian species as demonstrated by in vitro receptor autoradiography. A similar decrease has been observed in man and other mammalian species using this methodology [Krause et al., 1994]. *E. coli* ST/guanylin-stimulated guanylyl cyclase activity, as indicated by an increased accumulation of cellular cGMP, also suggests that the receptor-guanylyl cyclase density is greatest in the proximal small intestine as compared to distal regions of small intestine or colon in the turkey and duck. Similar observations have been made using these techniques which showed decreasing gradients of receptor density along the longitudinal axis of the intestinal tract of some mammalian species [Krause et al., 1994]. Although substantial interspecies variation may occur in birds and mammals, a general conclusion can be drawn from these experiments indicating that proximal small intestine has the highest levels of ST/guanylin/uroguanylin receptor-guanylyl cyclases relative to other regions of small or large intestine.

With regard to the vertical axis (the villus/crypt unit) of the avian small intestine, ST/guanylin receptor density appeared relatively evenly distributed among enterocytes covering villi and in those enterocytes forming intestinal glands of the majority of birds that were examined. This observation is in contrast to that found in the human small intestine as well as several other mammalian species in which the receptor density is greatest in enterocytes covering the basal one-half of villi and in those lining the proximal one-half of the intestinal glands (crypts of Liberkühn) [Cohen et al., 1992; Li and Goy, 1993; Krause et al., 1994].

The ST/guanylin/uroguanylin receptor belongs to the guanylyl cyclase family of proteins that differ markedly in their selectivity and activation by ligands. Included in this group are atrial natriuretic peptides and nitric oxide which are endogenous activators for two particulate forms (GC-A and GC-B) and a soluble (GC-S) form of guanylyl cyclase, respectively [Drewett and Garbers, 1994]. All these enzymes catalyze the production of cGMP and by this mechanism influence cellular function. The ST/guanylin/uroguanylin receptor, an intestinal cytoskeletal-associated form of membrane guanylyl cyclase (GC-C) is selectively activated by the STs [Field et al., 1978; Guerrant et al., 1980; Schulz et al., 1990; Forte and Currie, 1995] as well as by guanylin [Currie et al., 1992] and the newly discovered peptide, uroguanylin [Hamra et al., 1993; Kita et al., 1994]. Guanylin and uroguanylin have 15 or 16 amino acids, are produced in the intestine and appear to serve as endogenous regulators for the intestinal form of guanylyl cyclase, GC-C. Enterotoxigenic strains of bacteria cause secretory (i.e. travelers) diarrhea by producing molecular mimics of guanylin and/or uroguanylin; thereby activating the GC-C receptors in enterocytes. Guanylin, uroguanylin and *E. coli* STs bind to the N-terminal, extracellular domain of this cell surface receptor and activate a C-terminal, intracellular catalytic domain causing increased cellular levels of cGMP. Although the physiological role of guanylin and uroguanylin in the avian intestinal tract is unknown at present, both peptides stimulate chloride secretion in enterocytes of mammalian species [Currie et al., 1992; Forte et al., 1993; Hamra et al., 1993; Cuthbert et al., 1994; Kita et al., 1994]. Thus, guanylin and uroguanylin may regulate ion transport and fluid secretion in both the mammalian and avian intestinal tract by

this signaling mechanism. Other epithelial tissues may also be targets for these peptides. Receptor-guanylyl cyclase responsiveness to ST occurs in kidney, liver and testes [Forte et al., 1988, 1989; Krause et al., 1990; Laney et al., 1992]. Thus, in addition to their intestinal paracrine function, these peptides may also function as hormones.

Receptors for these peptides have now been demonstrated in both mammals and birds suggesting that the regulation of enterocyte function by guanylin and uroguanylin through the cGMP second messenger mechanism is a fundamental signaling pathway that appeared early in vertebrate evolution. It is of interest that intense receptor labeling by ^{125}I-ST occurs in the distal small intestine and the colon of some birds, yet these regions of the gut had very small cGMP responses to either ST or guanylin. The explanation for the receptors in the colon and distal small intestine of these species being less responsive to these agonists is unclear, particularly in light of the fact that guanylin mRNA levels are most abundant in the mammalian colon [Wiegand et al., 1992a, b; Li and Goy, 1993]. Our observation that ^{125}I-ST-labeled receptors appear to be abundant in the avian colon, which in the turkey and duck had little or no cGMP responses to either ST or guanylin, is consistent with the possibility that ST receptors (i.e. binding proteins) exist in the avian distal small intestine and colon, which are not guanylyl cyclases. A similar conclusion was made from our recent studies of these receptors in the mammalian colon [Krause et al., 1994]. Similarly, cultured IEC-6 intestinal cells have been shown to have specific, high affinity binding sites for ^{125}I-ST, but these cells express no GC-C mRNA and are devoid of ST-stimulated guanylyl cyclase activity [Mann et al., 1993]. A 56-kD protein, isolated from the small intestine of rats, bound ^{125}I-ST but exhibited no guanylyl cyclase activity [Hakki et al., 1993]. This protein may be a proteolytic fragment of GC-C with the C-terminal catalytic portion of the receptor missing. However, these preliminary findings suggest that other receptor proteins may occur in the distal small intestine and/or colon of both birds and mammals, which bind ^{125}I-ST with high affinity, but have no guanylyl cyclase activity. A similar cell-surface protein occurs which binds atrial peptides with high affinity but lacks the intracellular catalytic domain found in the GC-A and GC-B guanylyl cyclases [Drewett and Garbers, 1994]. These atrial peptide binding proteins have been described as clearance receptors and may function to sequester atrial peptides from the extracellular fluid. Perhaps a clearance type of receptor exists for peptides of the guanylin/uroguanylin/ST family thus explaining the relatively high binding of ^{125}I-ST in distal small intestine and colon of birds and mammals [Krause et al., 1990, 1994].

In the colon and distal small intestine of mammals, activation of the receptor-guanylyl cyclase by bacterial ST peptide agonists results in the stimulation of salt and water secretion through activation of transepithelial chloride secretion and inhibition of sodium absorption [Field et al., 1978; Guandalini et al., 1982; Rao et al., 1981]. The receptors localized in the proximal small intestine appear to be involved in the cGMP-mediated stimulation of bicarbonate secretion from enterocytes lining the duodenum [Guba et al., 1995]. It is of interest that the greatest cGMP responses to ST and guanylin occurred in the proximal small intestine of turkeys and ducks similar to previous findings in the opossum and raccoon [Krause et al., 1994]. Because the ST/guanylin/uroguanylin receptor is a membrane guanylyl cyclase, this finding suggests that the receptor density is higher in the duodenum when compared to the distal small intestine or colon of these representative species of birds and mammals. One speculation is that the physiological effects of guanylin and uroguanylin on intestinal transport in birds may be similar to that previously reported for mammalian intestine involving the cGMP-mediated control of chloride, sodium and bicarbonate transport described above. Our finding that the receptor-guanylyl cyclase in the BBM of turkey duodenum was activated by peptide agonists with a rank order potency of ST > uroguanylin > guanylin is also consistent with previous reports that the human form of intestinal GC-C has a similar rank order potency of the cGMP response to these peptides [Hamra et al., 1993; Kita et al., 1994]. The relative affinities of these peptides to inhibit the binding of ^{125}I-ST to turkey intestine BBM receptors of ST > uroguanylin > guanylin agrees with the relative potencies of these peptides as activators of the guanylyl cyclase in the BBM. While the intestinal mucosa of turkeys contained bioactive peptides that stimulated cGMP accumulation in human intestinal T_{84} cells, it is not yet known whether both guanylin and uroguanylin are present in that intestinal extract. Guanylin was first isolated from rat jejunum and its mRNA is most abundant in the colon and ileum of rat and human intestine [Currie et al., 1992; Wiegand et al., 1992a, b; Li and Goy, 1993]. Uroguanylin was first isolated from opossum urine, but recent experiments have identified uroguanylin and guanylin in the colon of opossums [Hamra et al., 1993, 1995]. A uroguanylin-like peptide has also been described in the rat duodenum [Li et al., 1994]. It may be postulated that birds have two related peptides that are similar in structure to the mammalian forms of guanylin and uroguanylin that have been identified thus far in a few mammals [Forte and Currie, 1995]. Our data obtained using the turkey intestinal BBM suggest that the receptor-guanylyl cyclase in the proximal small intestine of

this species may be either uroguanylin-selective relative to guanylin or that the structures of the avian forms of uroguanylin and guanylin are sufficiently different from the mammalian peptides as to make such an interpretation speculative. Clearly, the identification of peptide agonists in the avian intestine that serve to regulate this membrane guanylyl cyclase signaling pathway will provide additional insights into the cellular mechanisms operating within the intestinal tract of birds.

In conclusion, receptors for ^{125}I-ST, guanylin, and uroguanylin occur throughout the full length of the intestinal tract in all of the avian species examined in this study. Receptor sites were restricted to the enterocytes lining the small and large intestine. Receptor autoradiography and cGMP accumulation assays revealed a decrease in receptor-guanylyl cyclase abundance from the proximal to distal small intestine and to colon. The receptor guanylyl cyclase of proximal small intestine BBM was preferentially activated by ST > uroguanylin > guanylin. A similar rank order of affinities for these BBM receptors was observed using the competitive radioligand binding assay. Extracts of turkey intestinal mucosa had bioactive peptides that activated the human T_{84} cell guanylyl cyclase, GC-C. Like mammalian intestine, avian species have apical membrane receptors for guanylin and uroguanylin and bioactive peptides that could regulate these receptors by autocrine and/or paracrine mechanisms.

Acknowledgments

All animals were collected under license. We would like to thank the United States Fish and Wildlife Service, the Missouri Department of Conservation, the Australian Department of Agriculture and the Gaylord Laboratory for assistance during this study. We would also like to thank the following individuals for providing specimens used in the study: T. Daily, Dr. J.D. Firman, Dr. L.H. Fredrickson, P. Magee, M. Petria and M. Shanon. We gratefully acknowledge M. Lind and Kristin Nelson for their preparation of the manuscript. This study was supported by NIH Institutional Biomedical Research Support Grant RR07053 awarded by the Research Council of the University of Missouri, Columbia Campus and the Medical Research Service, Department of Veterans Affairs, USA.

References

Biber, J., B. Stieger, W. Haase, H. Murer (1981) A high yielded preparation for rat kidney brush border membranes: Different behaviour of lysosomal markers. Biochem Biophys Acta *647:* 169–176.

Black, R.E., K.H. Brown, S. Becker, M. Yunus (1982) Longitudinal studies of infectious disease and physical growth of children in rural Bangladesh. I. Patterns of morbidity. Am J Epidemiol *115:* 305–314.

Bradford, M.M. (1979) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72:* 248–254.

Burgess, M.N., R.J. Bywater, C.M. Cowley, N.A. Mullan, D.M. Newsome (1978) Biological evaluation of a methanol soluble, heat-stable *E. coli* enterotoxin in infant mice, pigs, rabbits, and calves. Infect Immun *221:* 526–531.

Cohen, M.B., E.A. Mann, C. Lau, S.J. Henning, R.A. Giannela (1992) A gradient in expression of the *Escherichia coli* heat-stable enterotoxin receptor exists along the villus-to-crypt axis of rat small intestine. Biochem Biophys Res Commun *186:* 483–490.

Currie, M.G., K.F. Fok, J. Kato, R.J. Moore, F.K. Hamra, K.L. Duffin, C.E. Smith (1992) Guanylin: An endogenous activator of intestinal guanylate cyclase. Proc Natl Acad Sci USA *89:* 947–951.

Cuthbert, A.W., M.E. Hickman, L.G. MacVinish, M.J. Evans, W.H. Colledge, R. Ratcliff, P.W. Seale, P.P.A. Humphrey (1994) Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice. Br J Pharmacol *112:* 31–36.

Donta, S.T., R.B. Wallace, S.C. Whipp, J. Olarte (1977) Enterotoxigenetic *Escherichia coli* and diarrheal disease in Mexican children. J Infect Dis *135:* 482–485.

Drewett, J.G., D.L. Garbers (1994) The family of guanylyl cyclase receptors and their ligands. Endocr Rev *15:* 135–162.

Field, M., L.H. Graf, W.J. Laird, P.L. Smith (1978) Heat-stable enterotoxin of *Escherichia coli:* In vivo effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. Proc Natl Acad Sci USA *75:* 2800–2804.

Forte, L.R., M.G. Currie (1995) Guanylin: A peptide regulator of epithelial transport. FASEB J *9.* 643–650.

Forte, L.R., W.J. Krause, R.H. Freeman (1988) Receptors and cGMP signaling mechanism for *E. coli* enterotoxin in opossum kidney. Am J Physiol *225:* F1040–F1046.

Forte, L.R., W.J. Krause, R.H. Freeman (1989) *Escherichia coli* enterotoxin receptors: Localization in opossum kidney, intestine and testis. Am J Physiol *257:* F874–F881.

Forte, L.R., W.J. Krause, R.H. Freeman (1993) Guanylin bioactivity in human intestinal and opossum kidney cells; in Brown BI., PRM Dobson (eds): Advances in Second Messenger and Phosphoprotein Research. New York, Raven Press, vol 28, pp 133–138.

Forte, L.E., P.K. Thorne, S.L. Eber, W.J. Krause, R.H. Freeman, S.H. Francis, J.D. Corbin (1992) Stimulation of intestinal Cl tranport by heat-stable enterotoxin: Activation of cAMP-dependent protein kinase by cGMP. Am J Physiol *263.* C607–C615.

Giannella, R.A. (1981) Pathogenesis of acute bacterial diarrheal disorders. Ann Rev Med *32:* 341–357.

Guandalini, W., M.C. Rao, P.L. Smith, M. Field (1982) cGMP modulation of ileal ion transport: In vitro effects of *Escherichia coli* heat-stable enterotoxin. Am J Physiol *243:* G36–G41.

Guba, M., U. Seidler, M. Kuhn, W. Forssmann, M. Classen (1995) Luminal guanylin is a strong stimulant of duodenal bicarbonate secretion in the rat. Gastroenterology *108:* A287.

Guerrant, R.L., J.M. Hughes, B. Chang, D.C. Robertson, F. Murad (1980) Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli:* Studies of tissue specificity, potential receptors, and intermediates. J Infect Dis *141:* 220–228.

Hakki, S., D.C. Robertson, S.A. Waldman (1993) A 56 kDa binding protein for *Escherichia coli* heat-stable enterotoxin isolated from the cytoskeleton of rat intestinal membranes does not possess guanylate cyclase activity. Biochim Biophys Acta *1152:* 1–8.

Hamra, F.K., L.R. Forte, S.L. Eber, N.V. Pidhorodeckyj, W.J. Krause, R.H. Freeman, D.T. Chin, J.A. Tomkins, K.F. Fok, C.E. Smith, K.L. Duffin, N.R. Siegel, M.G. Currie (1993) Uroguanylin: Structure and activity of a second endogenous peptide that stimulates guanylate cyclase. Proc Natl Acad Sci USA *90:* 10464–10468.

Hamra, F.K., W.J. Krause, S.L. Eber, R.H. Freeman, L.R. Forte (1995) Guanylin and uroguanylin: Endogenous peptides adapted for the regulation of intestinal guanylate cyclase. FASEB J *9:* A6.

Katwa, L.C., A.A. White (1992) Presence of functional receptors for the *Escherichia coli* heat-stable enterotoxin in the gastrointestinal tract of the chicken. Infect Immun *60:* 3546–3551.

Kita, T., C.E. Smith, K.F. Fok, K.L. Duffin, W.M. Moore, P.J. Karabatsos, J.F. Kachur, F.K. Hamra, N.V. Pidhorodeckyj, L.R. Forte, M.G. Currie (1994) Characterization of human uroguanylin: Member of the guanylin peptide family. Am J Physiol *266:* F342–F348.

Krause, W.J., G.L. Cullingford, R.H. Freeman, S.L. Eber, K.C. Richardson, K.F. Fok, M.G. Currie, L.R. Forte (1994) Distribution of heat-stable enterotoxin/guanylin receptors in the intestinal tract of man and other mammals. J Anat *184:* 407–417.

Krause, W.J., R.H. Freeman, L.R. Forte (1990) Autoradiographic demonstration of specific binding sites for *E. coli* enterotoxin in various epithelia of the North American opossum. Cell Tissue Res *260:* 387–394.

Laney, D.W., E.A. Mann, S.C. Dellon, D.R. Perkins, R.A. Giannella, M.B. Cohen (1992) Novel sites for expression of an *Escherichia coli* heat-stable enterotoxin receptor in the developing rat. Am J Physiol *263:* G816–G821.

Li, Z., M. Campa, M.F. Goy (1994) Is there another guanylin in the GI tract? Identification of a possible homologous peptide in duodenum. Gastroenterology *106:* A822.

Li, Z., M.F. Goy (1993) Peptide-regulated guanylate cyclase pathways in rat colon: In situ localization of GCA, GCC and guanylin mRNA. Am J Physiol *265:* G394–G402.

Mann, E.A., M.B. Cohen, R.A. Giannella (1993) Comparison of receptors for *Escherichia coli* heat-stable enterotoxin: Novel receptor present in IEC-6 cells. Am J Physiol *264:* G172–G178.

Rao, M.C., S.A. Ovellano, M. Field, D.C. Robertson, R.A. Giannella (1981) Comparison of the biological actions of three purified heat-stable enterotoxins: Effects on ion transport and guanylate cyclase activity in rabbit ileum in vitro. Infect Immun *33:* 165–170.

Sack, D.A., M.H. Merson, J.G. Wells, R.B. Sack, G.K. Morris (1975) Diarrhea associated with heat-stable enterotoxin-producing strains of *Escherichia coli*. Lancet *ii:* 239–241.

Schultz, S., C.K. Green, P.S.T. Yuen, D.L. Garbers (1990) Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell *63:* 941–948.

Tien, X.-Y., T.A. Brasitus, M.A. Kaetzel, J.R. Dedman, D.J. Nelson (1994) Activation of the cystic fibrosis transmembrane conductance regulator by cGMP in the human colonic cancer cell line, Caco-2. J Biol Chem *269:* 51–54.

Wiegand, R.C., J. Kato, M.G. Currie (1992a) Rat guanylin cDNA: Characterization of the precursor of an endogenous activator of intestinal guanylate cyclase. Biochem Biophys Res Commun *175:* 812–817.

Wiegand, R.C., J. Kato, M.D. Huang, K.F. Fok, J.F. Kachur, M.G. Currie (1992b) Human guanylin: cDNA isolation, structure and activity. Fed Eur Biochem Soc Lett *311* 150–154.