Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10464–10468, November 1993
Medical Sciences

Uroguanylin: Structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase

(guanylin/urine/cyclic GMP/chloride secretion)

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Communicated by James O. Davis, August 11, 1993

ABSTRACT **The intestinal hormone guanylin and bacterial heat-stable enterotoxins (STs) are members of a peptide family that activates intestinal membrane guanylate cyclase. Two different peptides that activate the human intestinal T84 cell guanylate cyclase have been purified from urine and intestinal mucosa of opossums (*Didelphis virginiana*). The highly acidic peptide, QEDCELCINVACTGC, was named uroguanylin because it was isolated from urine and shares 53% identity with guanylin. A second peptide, SHTCEICAFAACAGC, was purified from urine and intestinal mucosa. This alanine-rich peptide was 47% identical to uroguanylin and 73% identical to human guanylin, suggesting that it may be an opossum homologue of guanylin. Synthetic uroguanylin-(2–15) (i.e., EDCELCINVACTGC) was 10-fold more potent than synthetic rat guanylin, but both peptides were less potent than *Escherichia coli* ST in the T84 cell cGMP bioassay. Uroguanylin-(2–15) and guanylin inhibited ^{125}I-ST binding to T84 cell receptors in competitive radioligand binding assays. Transepithelial Cl^- secretion was stimulated by 1 μM uroguanylin, indicated by an increase in the short circuit current of T84 cells. Thus, uroguanylin is another paracrine hormone in the emerging peptide family that activates intestinal membrane guanylate cyclase. The second peptide may be the opossum form of guanylin, or perhaps it is still another member of this peptide family. The presence of uroguanylin and guanylin in urine and receptors in proximal tubules suggests that these peptides may also originate from renal tissue and may regulate kidney function.**

Secretory diarrhea caused by intestinal microorganisms in humans and domestic animals is a major public health problem (1). Enteric bacteria, including *Escherichia coli, Yersinia enterocolitica*, and *Vibrio cholerae*, cause diarrhea by secreting small heat-stable enterotoxins (STs) that bind to and activate an intestinal isoform of membrane guanylate cyclase (2–4). Guanosine 3′,5′-cyclic monophosphate (cGMP) is the second messenger molecule that mediates the parallel stimulation of Cl^- secretion and inhibition of Na^+ absorption elicited by STs (5, 6). The stimulation of intestinal Cl^- secretion occurs via cGMP-regulated phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) protein located in the apical membrane of crypt cells (7, 8). Cystic fibrosis patients have no ion transport response to *E. coli* ST (9). Refractoriness to ST occurs because the mutant CFTR either functions improperly or is not localized to apical membranes where it serves as a Cl^- channel (10, 11).

Guanylin is a recently discovered endogenous ST-like peptide hormone that was originally isolated from rat jejunum (12). Guanylin/ST-like bioactivity was also found in extracts of rat kidney (12). Molecular cloning of intestinal cDNAs from rats, humans, and mice revealed that the purified form of guanylin was a 15-residue carboxyl-terminal peptide of a 115-residue precursor polypeptide (13–16). Guanylin shares either 7 or 8 amino acid residues with the STs secreted by different strains of *E. coli* that cause secretory diarrhea. Moreover, synthetic preparations of guanylin or ST-(5–17) stimulated Cl^- secretion and cGMP accumulation responses in human T84 intestinal cells. Guanylin also competed with ^{125}I-labeled ST (^{125}I-ST) for high-affinity binding sites on T84 cells (12, 17). It was suggested that guanylin may be an endogenous ST-like agonist that serves as a paracrine hormone for regulation of intestinal salt and water transport (12). Guanylin may function *in vivo* to regulate the phosphorylation state of the CFTR in Cl^--secreting cells by cGMP-mediated control of intestinal protein kinases (18).

Because receptors for ST are abundant in the kidney of the opossum (19, 20) and guanylin-like bioactivity was observed in extracts of rat kidney (12), it was suggested that guanylin may also be a renal hormone. The receptors for ST occur on the apical membranes of proximal tubule cells in the opossum (20–22), thus an endogenous ST-like agonist could be secreted from renal cells into the proximal tubule filtrate and appear in urine. In support of this hypothesis, two distinctly different peptides were purified from opossum urine, and both peptides were found to activate the guanylate cyclase of human intestinal T84 cells. One of these peptides may be the putative opossum homologue of guanylin, since it has a similar amino acid sequence. The second, a highly acidic peptide, is 53% identical to guanylin and 67% identical to *E. coli* ST. This peptide was named uroguanylin because it was isolated from opossum urine. Preliminary elements of this work have been reported (23).

MATERIALS AND METHODS

Purification of Guanylin Peptides. Urinary and intestinal peptides were purified separately. Urine was collected daily from opossums (*Didelphis virginiana*) housed in metabolism cages and was stored at −20°C. After thawing, the urine was centrifuged at 10,000 × *g* for 20 min. The supernatant was made 0.1% in trifluoroacetic acid (TFA) before further use. Full-length intestines were removed from six adult opossums. The mucosa was scraped from intestinal muscle by using a glass microscope slide, suspended in 10 vol of 1 M acetic acid, heated for 10 min at 100°C, homogenized and

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Abbreviations: ST, heat-stable enterotoxin; CFTR, cystic fibrosis transmembrane conductance regulator; TFA, trifluoroacetic acid; RP-HPLC, reverse-phase HPLC.

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stored at −20°C. The homogenate was thawed and centrifuged at 10,000 × g for 20 min, and the supernatant was made 0.1% in TFA. These supernatants were processed with Waters Sep-Pak cartridges [octadecylsilane (ODS) cartridges; C_{18}] that had been washed with 20 ml of 100% acetonitrile/0.1% TFA followed by 20 ml of 0.1% TFA in H_2O before 50 ml of extract was applied to the cartridge. The cartridge was then washed with 10 ml of 0.1% TFA, and bioactive peptides were eluted with 8 ml of 40% (vol/vol) acetonitrile/0.1% TFA in H_2O. The eluted fractions were lyophilized, resuspended in 16 ml of 50 mM ammonium acetate, pH 5.0, and then centrifuged at 500 × g for 10 min, and 7.5 ml was applied to a 2.5 cm × 90 cm Sephadex G-25 column. Ten-milliliter fractions were collected, and those with activity in the T84 cell cGMP bioassay were combined, lyophilized, and resuspended in 50 ml of 0.1% TFA and applied to a C_{18} cartridge. A step gradient with 6 ml each of 5%, 10%, 15%, 20%, 25%, 30%, 35%, and 60% acetonitrile, all vol/vol in H_2O containing 0.1% TFA, was used to elute the peptides. The active fractions eluted primarily at 20% and 25% acetonitrile/0.1% TFA, and these fractions were pooled and lyophilized. Intestinal extracts were resuspended in 50 ml of distilled H_2O containing 0.8% ampholytes, pH range 3–10 (Bio-Rad), and applied to a preparative isoelectric focusing cell (Rotofor; Bio-Rad). Each extract was focused by using 12 W of constant power for 60 min, and fractions were collected as described by the manufacturer. Two peaks of bioactivity were observed, at pI ≈ 3.0 and pI ≈ 5.2. Urinary extracts were not purified by isoelectric focusing because the extracts were highly acidic and disrupted the pH gradient.

Final purification of the active peptides was accomplished by a series of four reverse-phase (RP)-HPLC steps as previously described (12).

Cell Culture. T84 cells (passage 21 obtained from Jim McRoberts, Harbor–University of California, Los Angeles, Medical Center, Torrance, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) containing 5% fetal bovine serum, and 60 μg of penicillin and 100 μg of streptomycin per ml as previously described (17, 18). Permeable membranes (Falcon cell culture inserts of Cyclopore membranes, 25 mm diameter, 0.45 μm pore size, Fisher Scientific) were first coated with 0.25 ml of collagen (bovine type I, 1.3 mg/ml; Sigma) for 16 hr, while the filters were being sterilized by UV irradiation. T84 cells were seeded at 2.5–3.0 × 10^6 cells per filter as previously described (17, 18).

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 (17, 18).

Assay of cGMP in T84 Cells. T84 cells were cultured in 24-well plastic dishes, and the cGMP levels were measured in control or agonist-stimulated cells by radioimmunoassay (17, 18). In brief, confluent monolayers of T84 cells in DMEM containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) at pH 7.4 and 1 mM isobutylmethylxanthine were treated at 37°C with agonists or vehicle for 40 min. Then cells were treated with 200 μl of 30% perchloric acid to extract cGMP. The pH of the extract was adjusted to 7.0, the extract was centrifuged, and the supernatant was used to measure cGMP.

Radioligand Binding Experiments. Iodination of synthetic *E. coli* ST-(1–19) (NSSNYCCELCCNPACTGCY; Multiple Peptide Systems, San Diego) was accomplished by using lactoperoxidase, and the product was purified as previously described (17). The binding of ^{125}I-ST to receptors on T84 cells was measured using 50,000 cpm of ^{125}I-ST per well of T84 cells cultured in 24-well dishes. The medium was 0.2 ml of DMEM containing 15 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 5.5. After incubation for 60 min at 37°C, the medium was aspirated and the cells were washed two times with 1 ml of ice-cold medium. The cells were then solubilized with 1 M NaOH for measurement of radioactivity.

Peptide Synthesis. Rat guanylin (PNTCEICAYAACTGC) and *E. coli* ST-(5–17) (CCELCCNPACAGC) were prepared as previously described by the solid-phase method with an Applied Biosystems 430A peptide synthesizer on Cys(4-CH_3Bzl)-OCH_2-Pam resin, using double coupling cycles to ensure complete coupling at each step (12). Peptides were cyclized by using dimethyl sulfoxide as described by Tam *et al.* (24). Peptide structures were verified by electrospray mass spectrometry, gas-phase sequence analysis, and amino acid composition analysis.

Synthetic uroguanylin-(2–15), EDCELCINVACTGC, was synthesized by the solid-phase method with an Applied Biosystems 431A peptide synthesizer. Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids activated with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate were added to Fmoc-Cys(trityl)-Wang resin (Nova Biochem). Coupling efficiencies were monitored by the UV absorbance of the released Fmoc groups. The peptide was cleaved from the resin and the side chains were deprotected, except for the acetamidomethyl groups on Cys-3 and Cys-11, by incubation in TFA, ethane dithiol, and water (95:2.5:2.5, vol/vol) for 2 hr at room temperature. The peptide was cyclized by using air oxidation. The acetamidomethyl groups on Cys-3 and Cys-11 were removed with iodine. The peptide was desalted with a 12-ml Whatman ODS-3 solid-phase extraction device and purified to a single peak by C_{18} RP-HPLC (acetonitrile/ammonium acetate). The sequence of uroguanylin was confirmed by protein sequencing on an Applied Biosystems 470A gas-phase protein sequencer.

Materials. DMEM and Ham's F12 media and Sephadex G-25 gel were obtained from Sigma and Na^{125}I was purchased from DuPont/NEN. Fetal bovine serum was obtained from GIBCO. Other reagent-grade chemicals were purchased from Sigma or Fisher Scientific.

RESULTS AND DISCUSSION

Isolation of ST-Like Peptides from Urine. Three liters of urine was used as starting material for the first purification experiments. When the bioactive fractions were subjected to the RP-HPLC step using a semipreparative C_{18} column (acetonitrile/TFA), a single broad peak of bioactivity eluted from the C_{18} column at 21–22% acetonitrile (data not shown). Further separation of this fraction on RP-HPLC using an analytical-scale C_{18} column (acetonitrile/ammonium acetate) resolved two distinct bioactive components (Fig. 1). Each of these components was subjected separately to further purification by RP-HPLC using a C_{18} analytical column (acetonitrile/TFA) and a C_8 microbore (Vydac) column (acetonitrile/TFA). The two bioactive fractions from the microbore RP-HPLC were subjected to gas-phase amino-terminal sequence analysis. The component contained in peak 2 (see Fig. 1) yielded a 13-residue peptide with the sequence DCELCINVACTGC. Electrospray mass spectrometry revealed $[M + H]^+$ at m/z 1339.5, which was consistent with the calculated monoisotopic molecular weight of this peptide (1338.5). Peak 1 contained two peptides, 14 and 15 residues in length. The structure of the 15-residue peptide was QEDCELCINVACTGC and the 14-residue form of this peptide lacked the glutamine residue at the amino terminus. The mass spectrum of this fraction contained $[M + H]^+$ signals at m/z 1488.4 and 1596.6, which was consistent with the calculated monoisotopic molecular weights (1487.4 and 1595.6) of the 14- and 15-residue peptides predicted from the sequence analysis. Either glutamine or lysine at the amino terminus of the 15-residue peptide would be consistent with the data from mass spectrometry. The peptide that was isolated from

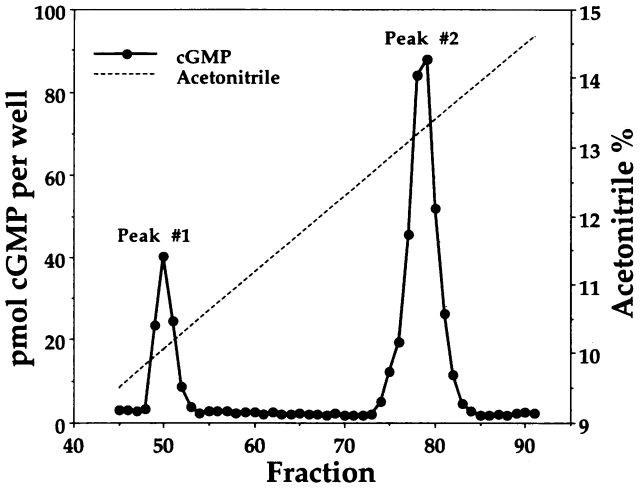

FIG. 1. Purification of uroguanylin peptides from urine by RP-HPLC. Urine extracts were purified through the semipreparative RP-HPLC step (no. 4) and the active fractions were combined, dried, and dissolved in 5% acetonitrile to inject onto a 3.9 mm × 30 cm C_{18} column (μBondapak; Waters). The elution was isocratic for 5 min before a linear gradient of 5–25% acetonitrile in 10 mM ammonium acetate, pH 6.2, was used to elute peptides at a rate of 1 ml/min over a 3-hr period, and 185 fractions were collected. This figure shows only the region of the chromatogram where biological activity, as assessed in the T84 cell cGMP accumulation bioassay, was observed.

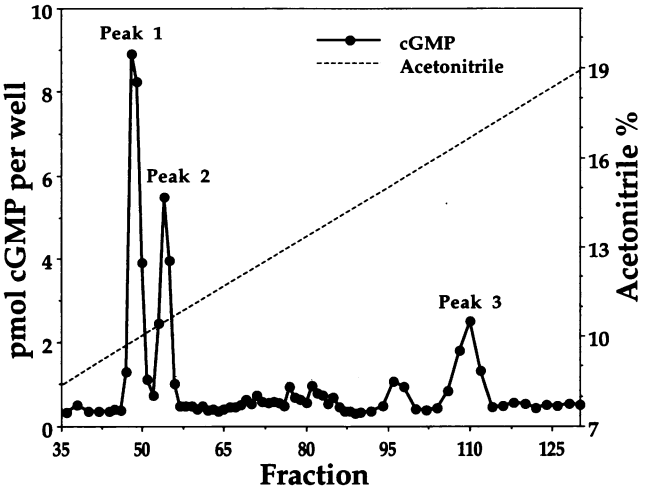

FIG. 3. Purification of the opossum homologue of guanylin from urine. An 8-liter batch of urine was purified through the third step and the active fractions were separated on a 7.8 mm × 30 cm C_{18} column (semipreparative scale, μBondapak; Waters). A linear gradient of 10–30% acetonitrile containing 0.1% TFA was developed at 3 ml/min over 3 hr. A total of 180 fractions were collected and assayed for activity in the T84 cell cGMP bioassay. The fractions in the second peak, which was eluted at 22% acetonitrile/0.1% TFA, were combined and chromatographed on an analytical scale C_{18} column exactly as described in the legend to Fig. 1 and the bioactivity of eluted fractions plotted here was assessed in the T84 cell cGMP bioassay.

opossum urine was designated as uroguanylin (Fig. 2). Uroguanylin is 67% identical to *E. coli* ST and 53% identical to rat guanylin (underlined amino acids). Conserved regions between uroguanylin and ST or guanylin include the carboxyl-terminal ACTGC motif, the relative positions of the four cysteine residues, and the glutamate residue following the first cysteine. Most of the ST peptides, however, have six cysteine residues and a uniformly conserved proline residue, whereas the guanylin peptides have four cysteines and an alanine residue instead of proline (2–4, 12–14). The possibility exists that uroguanylin may have been derived from bacteria, which could have contaminated the urine samples. We believe this to be unlikely in view of the distinct structural differences found in uroguanylin compared with bacterial ST peptides. The presence of receptor/guanylate cyclases in the brush border membranes of opossum kidney cortex also argues for the existence of an endogenous ligand, such as uroguanylin, to activate those receptors (19–22). However, the proof that uroguanylin is derived from opossum tissue will require isolation of the cDNA for this peptide.

When 8 liters of urine was used as a starting batch, a second bioactive fraction was isolated by semipreparative RP-HPLC. This fraction, which was eluted following the main peak that had yielded the uroguanylin peptides described above (data not shown), was subjected to further purification by RP-HPLC using a C_{18} analytical column (acetonitrile/ammonium acetate). Three separate fractions were resolved as shown by their activity in the T84 cell cGMP bioassay (Fig. 3). Peak 3 was subsequently purified and the amino acid sequence was determined. Surprisingly, this alanine-rich peptide was distinctly different from uroguanylin. This 14-amino acid peptide, HTCEICAFAACAGC, appeared to be an opossum homologue of guanylin. It was 79% identical to rat or human guanylin but only 50% identical to uroguanylin. Thus, opossum urine contains at least two different ST-like peptides that activate T84 human intestinal guanylate cyclase.

Isolation of ST-Like Peptides from Intestine. Bioactive peptides were then purified from opossum intestinal mucosa. In these experiments, preparative isoelectric focusing was added after the third step. Interestingly, two peaks of bioactivity were resolved by this method, with pI ≈ 3.0 and pI ≈ 5.2 (Fig. 4). The pI ≈ 5.2 fraction of active peptides was purified to homogeneity by using the RP-HPLC methods utilized above. Upon sequencing, the pI ≈ 5.2 fraction yielded the 15-residue peptide SHTCEICAFAACAGC, which is the same as the guanylin-like peptide above that was purified from urine except that serine occurs at the amino terminus (Fig. 2). The pI ≈ 3.0 fraction isolated from intestine may be uroguanylin. This fraction has not been purified to homogeneity and subjected to sequence analysis. Thus, intestinal mucosa contains a guanylin-like peptide and additional peptides with pI ≈ 3.0 that stimulate the intestinal guanylate cyclase.

Since the final purification of uroguanylin required C_8 microbore RP-HPLC to provide homogenous peaks of UV-absorbing peptides and all of the purified peptides were used in the analyses, a peptide was synthesized corresponding to the linear sequence EDCELCINVACTGC [uroguanylin-(2–15)] to test its potency and efficacy when T84 cells are used as a model bioassay. Sequential oxidation methods were used to provide disulfide bonds from Cys-3 to Cys-11 and Cys-6 to

Uroguanylin$_{op}$				Q	E	D	C	E	L	C	I	N	V	A	C	T	G	C								
E. coli ST	N	S	S	N	Y	C	C	E	L	C	C	N	P	A	C	T	G	C	Y							
"Putative" Guanylin$_{op}$				S	H	T	C	E	I	C	A	F	A	A	C	A	G	C								
Guanylin$_{rat}$				P	N	T	C	E	I	C	A	Y	A	A	C	T	G	C								

FIG. 2. Comparison of the primary structures of opossum uroguanylin and guanylin to *E. coli* ST and rat guanylin. The primary structures of *E. coli* ST and rat guanylin were taken from refs. 2 and 12, respectively.

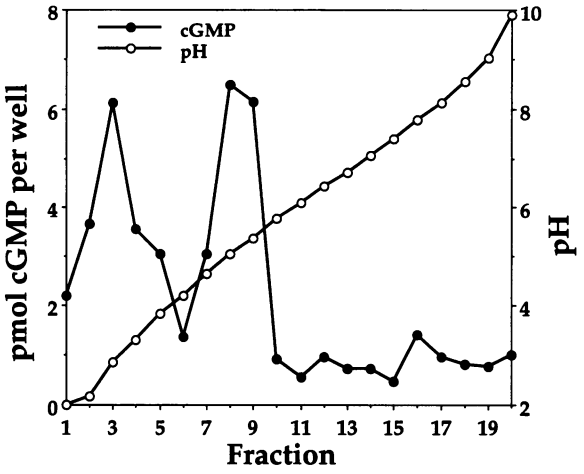

FIG. 4. Separation of guanylin/ST-like peptides by preparative isoelectric focusing. The intestinal mucosa of six opossums was used to purify active peptides through step 3. The active peptides were fractionated by preparative isoelectric focusing and each fraction was assayed by using the T84 cell cGMP bioassay.

Cys-14. After purification of the active polypeptide to a single peak by RP-HPLC, the bioactivity of uroguanylin-(2–15) was compared with that of *E. coli* ST-(5–17) and rat guanylin. Uroguanylin-(2–15) stimulated cGMP accumulation in T84 cells (Fig. 5). Its potency appeared to be about 10-fold greater than that of guanylin, and the concentration–response curves were parallel, indicating that uroguanylin-(2–15) was a full agonist. *E. coli* ST-(5–17) was about 10-fold more potent than uroguanylin-(2–15) and 100-fold more potent than guanylin. Both uroguanylin-(2–15) and guanylin inhibited the binding of ^{125}I-ST to receptor sites on T84 cells (Fig. 6). Uroguanylin-(2–15) had an ≈10-fold higher affinity for these binding sites than did guanylin, but ST-(5–17) had a higher affinity than either of the endogenous ligands. When T84 cells were raised on semipermeable filters and mounted in modified Ussing chambers, it was observed that uroguanylin stimulated short circuit current, I_{sc}, which in these cells is proportional to the rate of transepithelial Cl^- secretion (Fig. 7). The I_{sc} response to 1 μM uroguanylin-(2–15) was less than that found with 1 μM ST-(5–17), consistent with the lower potency of uroguanylin compared with ST-(5–17) observed in both the cGMP and radioligand-binding bioassays.

It appears that intestinal guanylate cyclase may be regulated by at least two different peptide hormones, which could be made in the intestine and secreted luminally. One or both

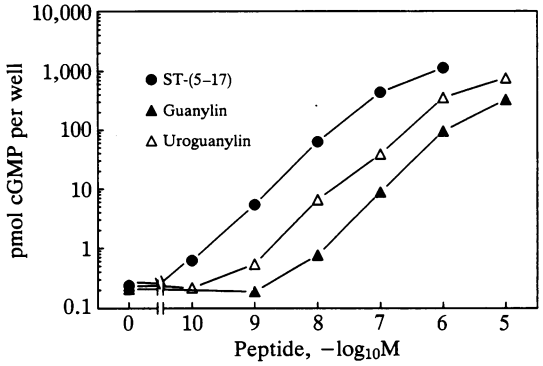

FIG. 5. Stimulation of cGMP accumulation in T84 cells by uroguanylin, guanylin, and *E. coli* ST-(5–17). Synthetic preparations of opossum uroguanylin, rat guanylin, and *E. coli* ST-(5–17) were tested for their potency and efficacy in activation of cGMP accumulation in T84 cells. This is a representative experiment that has been repeated with the same results.

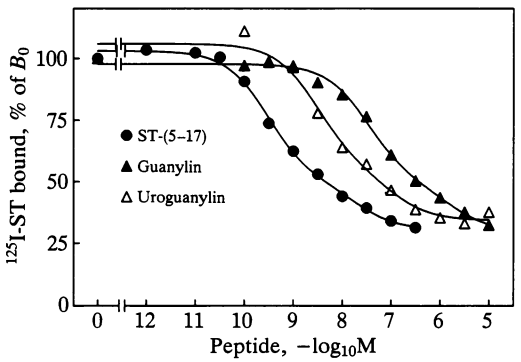

FIG. 6. Inhibition of ^{125}I-ST binding to receptors on T84 cells by uroguanylin, guanylin, and *E. coli* ST-(5–17). Synthetic preparations of these peptides were tested for their affinity as competitors with ^{125}I-ST for binding to receptors. B_0, amount bound in the absence of competitor. These data are the mean of two experiments.

of these bioactive peptides may be made by the kidney as well, since both were isolated from urine. The acidic peptide was named uroguanylin because it was originally isolated from urine and is similar in primary structure to guanylin. It is also apparent that uroguanylin and the putative opossum homologue of guanylin are different gene products. The discovery of uroguanylin suggests that further diversity may exist in peptide ligands as well as in their receptors. It is likely that, as with atrial peptides and their receptors (25), at least two different selective receptors exist for uroguanylin and guanylin.

The alanine-rich peptide (pI ≈ 5.2) that was isolated from urine and intestine shares 11 amino acids (i.e., 73% identity) with rat or human guanylin peptides (12–16). Other differences between these putative forms of guanylin represent conservative changes, since the pI 5.2 opossum peptide is 93% similar to rat or human guanylin. Thus the bioactive domains of guanylin hormones appear to be highly conserved between these species. Another peptide could exist that is more closely related to guanylin than is the pI ≈ 5.2 peptide, which has been tentatively classified as the opossum form of guanylin. Uroguanylin contains two additional acidic amino acids and does not contain the aromatic amino acids that occur in all guanylin peptides examined thus far (12–16). These differences in structure between uroguanylin and guanylin suggest that different receptors may exist, which have selectivity for uroguanylin versus guanylin. The recep-

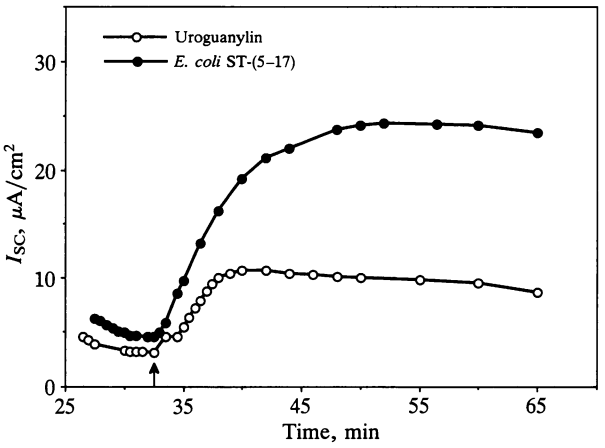

FIG. 7. Stimulation of short circuit current in T84 cells by uroguanylin and *E. coli* ST-(5–17). The peptides were added at 1 μM to the apical reservoir at the time designated by the arrow. Data are the mean of three experiments with uroguanylin and two experiments with ST.

tor/guanylate cyclase in T84 cells appeared to be selective for uroguanylin, because this peptide was about 10-fold more potent than guanylin in stimulating cGMP accumulation or in inhibiting ^{125}I-ST binding. Moreover, rat kidney has guanylin-like bioactivity (12) and an mRNA that hybridizes to guanylin cDNA (15). These findings and the occurrence of guanylin and uroguanylin in opossum urine imply that one or both of these peptides are made by the kidney and secreted into the filtrate, where they appear in urine. Thus, uroguanylin and/or guanylin may be renal hormones that could regulate kidney function through activation of receptor/guanylate cyclases localized on the apical membranes of tubular cells (19–22).

In summary, the opossum has two distinctly different peptides, uroguanylin and a peptide that is similar to guanylin. A reasonable assumption is that these closely related peptides play a role in the regulation of intestinal and/or renal electrolyte transport. The determination of the cellular distribution of these two peptides should prove exciting, with the possibility that in some instances they share sites of production and in other instances the pattern of their distribution may be independent. It will be particularly interesting to examine their distribution in the different regions of the intestine and along the crypt–villus axis. Similarly, the distribution of the two peptides along the length of the nephron should yield valuable information important to understanding their function. Furthermore, it appears that pathogenic enteric bacteria cause secretory diarrhea by releasing a molecular mimic of guanylin and uroguanylin which activates a common set of receptor/guanylate cyclases. The biochemical result of this molecular mimicry is an activation of guanylate cyclase, followed by an increase in intracellular cGMP, which serves as a second messenger that regulates the phosphorylation of CFTR molecules, thus activating Cl^- secretion. Evidence has been presented that cGMP may activate a cAMP-dependent protein kinase as one possible mechanism for regulation of this transport pathway (18). The discovery of a second member of the guanylin peptide family, termed uroguanylin, indicates that this paracrine/endocrine system may have a significant role in the control of epithelial cell function in the kidney as well as the intestine and opens the field for further studies of this peptide family in other epithelia.

We express our appreciation to Judy Richey for typing this manuscript and to Matthew Buss and Helena Hillman for their assistance. This research was supported by the Medical Research Service, Department of Veteran's Affairs, and by a Weldon Springs Grant from the University of Missouri.

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