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Characterization of human uroguanylin: a member of the guanylin peptide family

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Kita, Toshihiro, Christine E. Smith, Kam F. Fok, Kevin L. Duffin, William M. Moore, Peter J. Karabatsos, James F. Kachur, F. Kent Hamra, Nykolai V. Pidhorodeckyj, Leonard R. Forte, and Mark G. Currie. Characterization of human uroguanylin: a member of the guanylin peptide family. *Am. J. Physiol.* 266 (*Renal Fluid Electrolyte Physiol.* 35): F342–F348, 1994.—Guanylin, a peptide homologue of the bacterial heat-stable enterotoxins (ST), is an endogenous activator of guanylate cyclase C (GC-C). We have initiated a search for other members of the guanylin peptide family and in the current study describe a "guanylin-like peptide" from human urine. Bioactivity was monitored by determining the effect of urine extracts on T84 cell guanosine 3′,5′-cyclic monophosphate (cGMP) levels. Purification yielded two bioactive peaks of peptides that, when sequenced by NH_2-terminal analysis, possessed 15 and 16 amino acids. The sequence of the smaller peptide represented an NH_2-terminal truncation of the larger peptide. We have termed the larger peptide human uroguanylin; it has the following amino acid sequence: NDDCELCVNVACTGCL. Human uroguanylin shares amino acid sequence homology with guanylin and ST. Synthetic uroguanylin increased cGMP levels in T84 cells, competed with ^{125}I-labeled ST for receptors, and stimulated Cl^- secretion as reflected by an increased short-circuit current. Thus we report the isolation from human urine of a unique peptide, uroguanylin, that behaves in a manner similar to guanylin and appears to be a new member of this peptide family.

guanosine 3′,5′-cyclic monophosphate; guanylate cyclase; chloride secretion; T84 cell; rat colon

THE MEMBRANE-BOUND FORMS of guanylate cyclase have been found to serve as receptors for specific peptide ligands and are thought to participate in the regulation of blood pressure as well as water and electrolyte homeostasis (8, 22). These receptors share several structural motifs, including an extracellular ligand-binding site, a transmembrane region, an intracellular ATP regulatory domain, and an intracellular catalytic site for the generation of guanosine 3′,5′-cyclic monophosphate (cGMP) (3, 8). The natriuretic peptide family of receptors with guanylate cyclase activity has been particularly well characterized. These receptors include guanylate cyclases A and B (GC-A and GC-B, respectively), also known as natriuretic peptide receptors A and B (8, 22). The two subtypes of natriuretic peptide receptor show different tissue distributions and exhibit selective affinities for the three different members of the natriuretic peptide family (18). GC-A has been shown to be quite sensitive to stimulation by atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) but is very insensitive to stimulation by C-type natriuretic peptide (CNP) (8, 13, 18). On the other hand, GC-B is potently stimulated by CNP and only slightly responsive to either ANP or BNP (13).

An additional form of membrane guanylate cyclase is GC-C (intestinal guanylate cyclase), which is an apparent receptor for guanylin and bacterial heat-stable enterotoxins (ST) (2, 3, 17). This receptor is abundant in intestinal epithelial cells, where it serves as a target for ST to cause secretory diarrhea (16, 17). Recently, guanylin, a peptide composed of 15 amino acids, has been identified as an endogenous ligand for GC-C (2, 23, 24). Guanylin was initially purified from rat jejunum and proposed as a modulator of intestinal fluid and electrolyte homeostasis (2). Subsequent cloning of a guanylin cDNA from rat (16, 23) and human (4, 24) intestinal cDNA libraries has determined that this peptide is synthesized as part of an 115-amino acid precursor. The COOH-terminal region is highly conserved between human, rat, and mouse species, and it is this portion of the prohormone that contains the guanylin peptide sequence (12). In these studies, it was found that proguanylin was relatively inactive and required the cleavage of the COOH-terminal bioactive region (4, 16). Guanylin mRNA, as has been previously described for GC-C mRNA, is most abundant in the intestinal tract, with the ileum and colon possessing the greatest level of expression (12, 23, 24). However, guanylin expression has also been detected in extra-intestinal sites, including the kidney, uterus, and adrenal gland (16).

Because of the similarities of the natriuretic peptide guanylate cyclase system with the guanylin GC-C system (8), we have initiated a search for guanylin and potential members of this peptide family in sites other than the intestine. Previous evidence suggested that the kidney may be a fruitful site for such a search. Rat kidney has a modest amount of guanylin-like bioactivity

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on T84 cell cGMP levels (2) and detectable levels of guanylin mRNA (16). ST binding sites, which have also been reported to be localized in the proximal tubules of opossum kidney and ST, caused large increases in cGMP levels in opossum renal tissue (6). Thus we hypothesized that endogenous ligand(s) for GC-C may be produced by the kidney and excreted in the urine. Efforts were initiated to isolate endogenous peptide activators of GC-C from both human and opossum urine. The findings with the opossum are the subject of a separate manuscript (10). In the current study, we report the isolation and characterization of a novel 16-amino acid peptide from human urine with biological activity and structural similarities to guanylin. However, it is distinct from guanylin, and we contend that uroguanylin represents a new member of the guanylin family. We have designated this peptide as uroguanylin because urine was the source and because of its sequence similarity with guanylin, including the four conserved cysteine residues.

MATERIALS AND METHODS

Cell culture. T84 cells were obtained from the American Type Culture Collection at passage 52. Cells were grown to confluence in 24-well culture plates with a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin/ml, and 100 μg streptomycin/ml. Cells were used at passages 56–58.

cGMP determination. Confluent monolayers of T84 cells in 24-well plates were washed twice with 250 μl of DMEM containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), preincubated at 37°C for 10 min with 250 μl of DMEM containing 50 mM HEPES and 1 mM isobutylmethylxanthine, and incubated with agents or fractions for 30 min. The medium was aspirated, and the reaction was terminated by the addition of 0.5 ml of ice-cold 0.1 M HCl. Aliquots (150 μl total) were evaporated under a hot-air dryer and resuspended in 5 mM sodium acetate buffer, pH 6.4. The samples were subsequently measured for cGMP by radioimmunoassay as described previously (19).

Purification of uroguanylin. Five separate batches of adult male human urine samples, 5 l each, were collected and immediately placed on ice. The urine samples were applied to C_{18} Sep-Pak columns (Waters). The columns were washed with 10% acetonitrile/0.1% trifluoracetic acid (TFA)/H_2O and eluted with 40% acetonitrile/0.1% TFA/H_2O. The eluted peptide fraction was lyophilized, resuspended in 7 ml of distilled H_2O, and centrifuged at 20,000 *g* for 20 min at 4°C. The resulting supernatant was separated by gel filtration chromatography (Sephadex G-25, superfine, 2.6 × 94 cm). The fractions were bioassayed, and the active fraction was lyophilized. The sample was resuspended in 1 ml of 10% acetonitrile/0.1% TFA/H_2O and applied to a C_{18} semipreparative high-performance liquid chromatography (HPLC) column (Vydac, Hesperia, CA). The column was developed with the following linear gradient: 10% acetonitrile/0.1% TFA/H_2O to 40% acetonitrile/0.1% TFA/H_2O in 120 min at a flow rate of 3 ml/min. The active fraction was lyophilized and resuspended in 1 ml of 10% acetonitrile/0.1% TFA/H_2O. The sample was applied to a C_{18} analytical HPLC column (Vydac), and active peptides were eluted using the above gradient over 180 min at a flow rate of 1 ml/min. Two active fractions were separately lyophilized and reconstituted in 0.05 ml of 0.1% TFA H_2O. The samples were then separately applied to a C_8 microbore column (Applied Biosystems, Foster City, CA) eluted with an increasing gradient of 0.33% acetonitrile/min in 0.1% TFA/H_2O. Two separately purified peptides of each batch were then subjected to sequence analyses.

NH_2-terminal protein sequence analysis. Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An applied Biosystems model 470A gas-phase sequencer was employed for the degradations (11) using the standard sequencer cycle 03RPTH. The respective phenylthiohydantoin (PTH)-amino acid derivatives were identified by reverse-phase HPLC analysis in an on-line fashion employing an Applied Biosystems model 120A PTH analyzer fitted with a Brownlee PTH-C_{18} column. Reduction and pyridylethylation for cysteine residue identification were performed directly on the filter.

Electrospray mass spectrometry. The molecular weights of uroguanylin samples were determined by a previously described technique (2). Briefly, a triple quadrupole mass spectrometer equipped with an atmospheric pressure ion source was used to sample positive ions produced from an electrospray interface. Mass analysis of sample ions was accomplished by scanning the first quadrupole in increments of 1 atomic mass unit from 1,000 to 2,400 atomic mass units in ≈3 s and passing mass-selected ions through the second and third quadrupoles operated in the radio frequency-only mode to the multiplier. For maximum sensitivity, the mass resolution of the quadrupole mass analyzer was set so that ion signals were ≈2 atomic mass units wide at one-half peak height but the centroid of the ion signal still represented the correct mass of the ion.

Radioligand binding assay. ^{125}I-labeled ST-(5—18) was prepared by the Iodo-Gen method as previously described (7). T84 cell monolayers were washed two times with 1.0 ml/well of ice-cold binding assay buffer (Earle's medium containing 25 mM 2-*N*-morpholino ethanesulfonic acid, pH 5.5). The cells were incubated for 30 min at 37°C in 0.5 ml/well of binding assay buffer with ^{125}I-ST [100,000 counts/min (cpm) per well] and unlabeled peptides. Then the cells were washed four times with 1 ml of ice-cold binding assay buffer and solubilized with 0.5 ml of 1 M NaOH per well. This volume was transferred to tubes and assayed for radioactivity by a multigamma counter. Results are expressed as the percentage of cells specifically bound.

Measurement of I_{sc} in T84 monolayers. Analysis of the effect of human uroguanylin on I_{sc} in T84 cells was performed as previously described (5). Briefly, T84 cells raised on permeable filters were mounted in a custom-made Ussing chamber for measurement of Cl^- secretion. The buffer was a Krebs-Ringer bicarbonate solution, pH 7.4, containing 10 mM glucose. Both reservoir buffer solutions were mixed and oxygenated by bubbling 95% O_2-5% CO_2 through the medium. I_{sc} was measured continuously, and the potential difference across the epithelium was measured intermittently.

Measurement of I_{sc} in rat colon. Rat proximal colon tissue, consisting of only mucosa and submucosa, was mounted between two Ussing half-chambers and bathed on both sides in a manner similar to that previously reported (21). Electrical measurements were monitored with an automatic voltage clamp, and direct-connecting voltage- and current-passing electrodes were used to measure transepithelial potential difference and I_{sc}. Tissues were equilibrated under short-circuit conditions until I_{sc} had stabilized.

Chemical synthesis of uroguanylin. Uroguanylin was synthesized by the solid-phase method (20) on an Applied Biosystems model 430A peptide synthesizer and purified by reverse-phase C_{18} chromatography. The purity and the structure were

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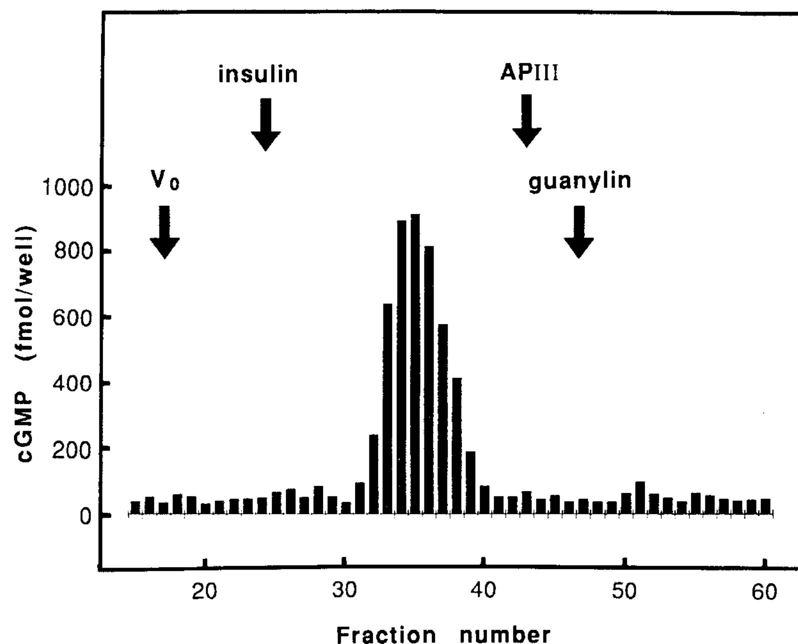

Fig. 1. Purification of uroguanylin from human urine by gel filtration chromatography. Extract of 5 l of human urine was applied to a 2.6 × 94-cm Sephadex G-25 (superfine) gel filtration column. Isocratic 50 mM ammonium acetate was used to elute peptides at a rate of 0.5 ml/min, and 5 ml of fractions were collected after 100 ml of initial elution. Molecular weight standards were separately assessed [void volume (V_0): blue dextran 200, insulin (mol wt 5,750), atriopeptin III (APIII; mol wt 2,550), rat guanylin (mol wt 1,516)]. All fractions were assessed in T84 cell cGMP accumulation bioassay.

verified by analytical HPLC, amino acid composition analysis, mass spectroscopy, and sequence analysis.

RESULTS

In initial experiments, the peptide fraction of human urine samples resulting from C_{18} Sep-Pak extraction was assayed for activity to increase cGMP levels in T84 cells. These preliminary experiments strongly suggested the presence of GC-C stimulatory activity. The urine extract was subjected to fractionation by gel filtration and a series of reverse-phase HPLC steps to produce a sufficiently pure preparation for the purpose of structural determination. Fractionation by G-25 gel filtration chromatography yielded a single major bioactive fraction that migrated on the column with an apparent size of 5,000 Da (Fig. 1). Subsequently, this active fraction was further purified by reverse-phase HPLC using a semipreparative C_{18} column, and the bioactivity was determined to reside in only one fraction eluting at 27.8% acetonitrile/0.1% TFA/H_2O (data not shown). Further purification by reverse-phase HPLC using a C_{18} analytical column yielded two active fractions that appeared to elute with peaks of substances that absorbed at 220 nm (Fig. 2). These two fractions were separately subjected to further characterization by microbore HPLC (C_8 column), and each fraction exhibited a single bioactive peak that absorbed at 220 nm (data not shown). The amino acid sequences of the two peaks were independently determined by the Edman degradation procedure. The sequences are NDDCELCVNVACTGCL and DDCELCVNVACTGCL for *peaks 1* and *2*, respectively. These two peptides are identical except that the peptide contained in *peak 1* possesses an additional amino acid (asparagine) at the NH_2-terminus. It is likely that *peak 2* is a degradation product of *peak 1*, probably a result of aminopeptidase action. Electrospray mass spectrometric analysis of the two fractions yielded observed molecular masses of 1,666.6 and 1,552.6 atomic mass units, respectively, for the peptides contained in *peaks 1* and *2*. These molecular weights correspond to the theoretical molecular weights derived from the sequences if two disulfide bonds link the four cysteines, and therefore indicate that the full sequences of these peptides were determined by NH_2-terminal protein sequence analysis.

Comparison of the sequence of *peak 1* with other proteins in the GenBank, National Biomedical Research Foundation, and SwissProt databases by computer-based search indicates that this sequence is a unique sequence. This search did reveal that human uroguanylin shares homology with guanylin and ST. The comparison between human uroguanylin, opossum uroguanylin, *Escherichia coli* ST, and human guanylin is shown in Fig. 3. The comparisons indicate that the four cysteine residues and the ACTGC COOH-terminal amino acid region of all four peptides are conserved. A unique feature of uroguanylin is the more acidic nature of the NH_2-terminal region, because the human peptide possesses two adjacent aspartic acid residues and the opossum peptide has a glutamate and an aspartate residue at these positions. Thus human uroguanylin appears to be a member of the guanylin/ST family of peptides.

Chemical synthesis of bioactive human uroguanylin (the 16-amino acid-containing peptide) was accomplished by directed folding of the peptide. The synthetic bioactive peptide possesses disulfide-linked bridges between the 4—12 and 7—15 amino acid positions as previously suggested for the disulfide links of guanylin (2, 24). Analysis of the biological activity of human uroguanylin was assessed by determining its effect on T84 cGMP levels, competition-binding studies with ^{125}I-ST as the radioligand in T84 cells, and stimulation of Cl^- secretion as reflected by increases in I_{sc} using T84 cells and rat colon.

Synthetic human uroguanylin caused a concentration-dependent increase in T84 cell cGMP (Fig. 4*A*). Human

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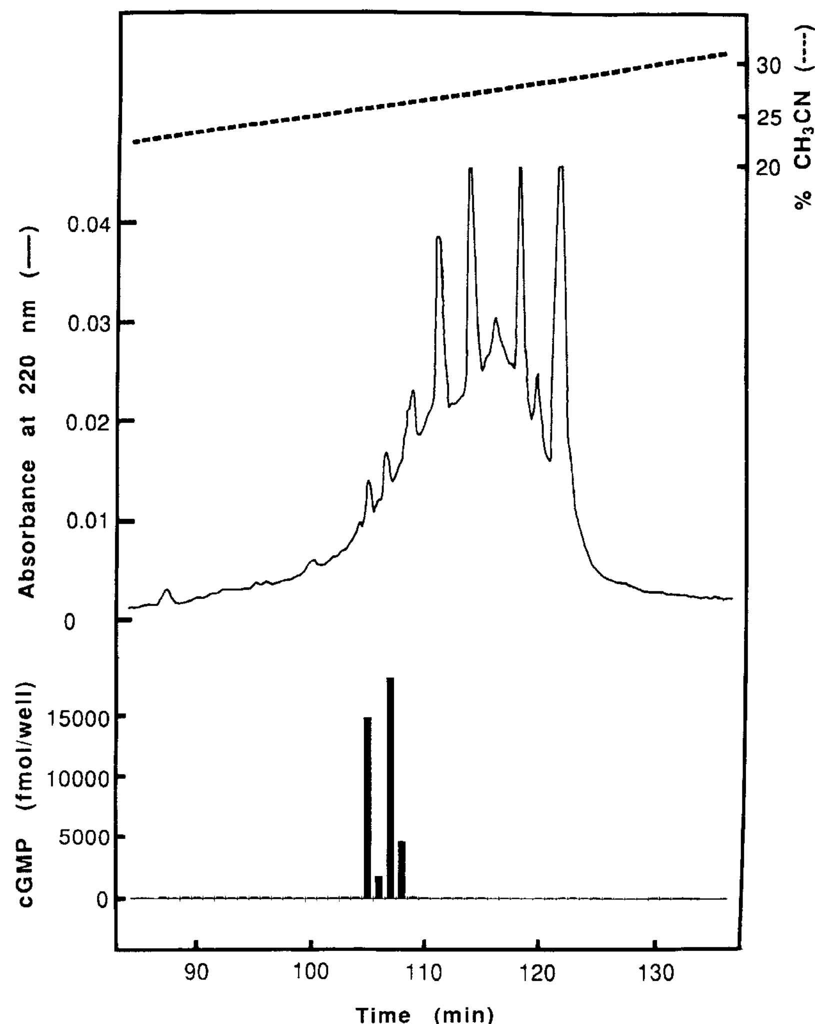

Fig. 2. Purification of uroguanylin from human urine by reverse-phase high-performance liquid chromatography (HPLC). Five liters of human urine extract was purified through the semipreparative reverse-phase HPLC, and active fraction was fractionated on an analytical C_{18} column (Vydac). A linear gradient of 10–40% acetonitrile, 0.1% TFA was developed at 1.0 ml/min over 3 h. One-minute fractions were collected and assayed for activity in T84 cell cGMP bioassay. This figure shows the biologically active region, with two peaks associated with changes in ultraviolet absorbance.

uroguanylin appeared to be more potent than human guanylin but less potent than ST for activation of GC-C in T84 cells. A similar profile of relative affinity was obtained using the competitive binding assay with ^{125}I-ST-(5—18) as the radioligand (Fig. 4*B*), but it should be noted that all of the peptides acted in a more potent manner in causing ^{125}I-ST-(5—18) displacement than in increasing cGMP levels. This difference in potencies for receptor binding and guanylate cyclase activation has been noted for the other members of the particulate guanylate cyclase family (8) and has recently been observed for cells transfected with human GC-C (9). The data indicate that these peptides all possess the ability to stimulate GC-C and share similar binding sites with varying degrees of relative affinities for the receptors in T84 cells.

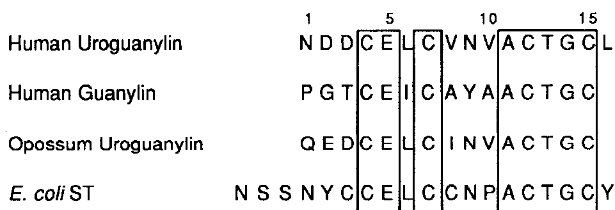

Fig. 3. Comparison of the structures of human uroguanylin, human guanylin, opossum uroguanylin, and *E. coli* heat-stable enterotoxin (ST). Identical amino acids are boxed. Human uroguanylin (position 1—15) is 53%, 80%, and 73% identical to human guanylin, opossum uroguanylin, and ST, respectively.

To assess the effect of human uroguanylin on well-characterized ST- and guanylin-sensitive transport functions, we assessed the effects of the peptide on I_{sc} of T84 cells and proximal rat colon. In these experiments, the measurement of I_{sc} is used as an indicator of transepithelial chloride secretion. Previous studies in these preparations have indicated that the change in I_{sc} elicited by ST and guanylin is mostly accounted for by an increase in chloride secretion (5). Human uroguanylin added to the apical reservoir of T84 cells mounted in Ussing chambers caused a marked and rapid increase in I_{sc} (Fig. 5*A*). Similarly, human uroguanylin added to the mucosal reservoir of rat colon mounted in an Ussing chamber also caused a sustained rise in I_{sc} (Fig. 5*B*).

DISCUSSION

The present study describes the purification and structural identification of a new peptide, isolated from human urine, that activates GC-C. The finding of a bioactive peptide in human urine extends our prior efforts to isolate and identify peptides in the opossum (10). We have termed this peptide human uroguanylin

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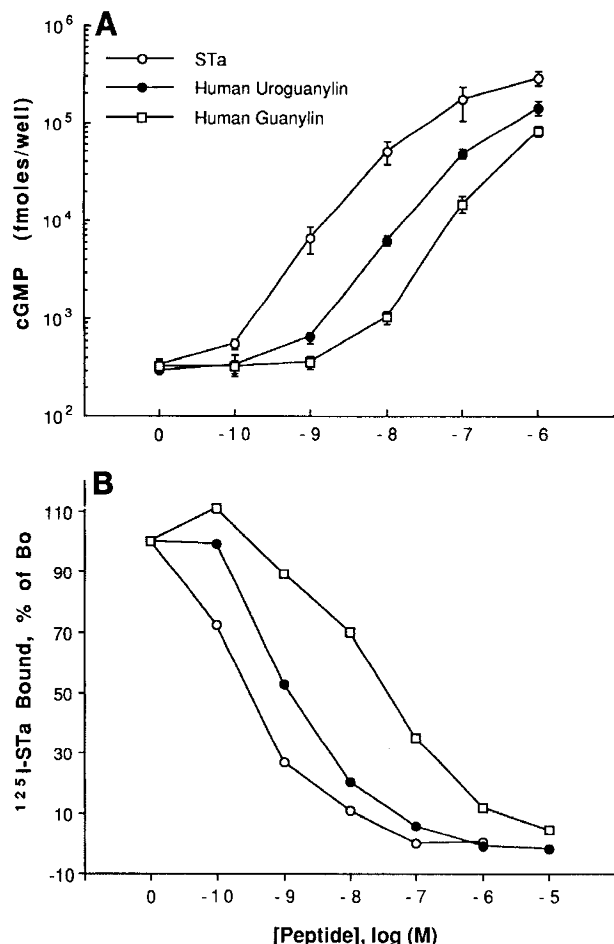

Fig. 4. *A*: concentration-response effect of synthetic human uroguanylin, human guanylin, and *E. coli* ST-(5—18) (STa) on cGMP levels in T84 cells. Cells were incubated with various concentrations of ligands for 30 min. Values are means ± SE (n = 4). *B*: displacement of ^{125}I-STa-specific binding from T84 cells by human uroguanylin, human guanylin, and STa. Cells were incubated for 30 min at 37°C with labeled STa and indicated concentrations of ligands. Specific binding (%) was determined by dividing specifically bound ^{125}I-STa at each ligand concentration by specifically bound ^{125}I-STa in absence of ligands. Each determination represents the mean of 4 wells examined.

because of its source, human urine, and because of its strong structural similarity to guanylin and opossum uroguanylin. Uroguanylin shares many biological properties with guanylin and ST. Uroguanylin stimulated cGMP accumulation and competed with ^{125}I-ST-(5—18) for binding sites on T84 cells. The more potent effect of uroguanylin on binding displacement than on the activation of GC-C is an unexplained observation, but it is characteristic of particulate guanylate cyclases, including GC-C (8, 9). In the T84 cell, this discrepancy may in part be explained by the presence of multiple receptors with different binding affinities. Indeed, our recent detailed characterization of ST and guanylin binding in T84 cells was consistent with a model predicting two receptors with different affinities for the peptide ligands. Uroguanylin also stimulated an increase in I_{sc} when added to the apical surface of both T84 cell monolayers and isolated proximal rat colon. These properties make uroguanylin an ideal candidate to serve as a paracrine/endocrine regulator of fluid and electrolyte homeostasis in tissues that express GC-C.

The observation that human urine contains uroguanylin but little or perhaps no guanylin is different from our results with opossum urine. We isolated both uroguanylin and a second peptide that is a putative opossum homologue of guanylin (10) from opossum urine. Species differences between human and opossum may account for this difference. It is also possible that guanylin was secreted in an inactive form. Recently, it was reported that the plasma form of guanylin from human renal patients is a much larger precursor that may have less intrinsic activity (14). We could find no evidence for the presence of a guanylin precursor. Attempts were made to activate the crude extract and larger-molecular-weight fractions from the G-25 column by acetic acid treatment, with no success (data not shown). This treatment has been shown to activate the guanylin precursor because of cleavage at an acid-labile bond (4, 16). These data strongly indicate that the major and perhaps the sole source of GC-C stimulatory activity in human urine is uroguanylin.

The finding of another member of the guanylin peptide family has parallels with other peptides and is particularly relevant to the natriuretic peptides. Both the guanylin peptides and the natriuretic peptides activate specific forms of particulate guanylate cyclase. ANP and BNP both potently activate GC-A, whereas CNP activates GC-B (13). These peptides appear to play an important role in the regulation of blood pressure and

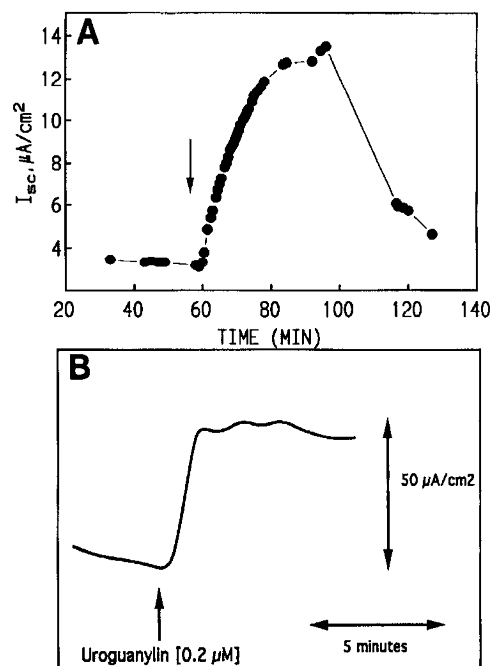

Fig. 5. Effect of synthetic human uroguanylin on short-circuit current (I_{sc}) of T84 cells or rat colon. *A*: stimulation of transepithelial Cl^- secretion in T84 cells by human uroguanylin. T84 cells cultured on collagen-coated filters were mounted in a modified Ussing chamber at *time 0*. After a steady baseline was achieved, 1 μM human uroguanylin was added to the apical reservoir. *B*: effect of human uroguanylin (0.2 μM) on I_{sc} across rat proximal colon after a mucosal addition. Response in both *A* and *B* is characteristic of results from 3 other experiments.

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