Opossum colonic mucosa contains uroguanylin and guanylin peptides

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Hamra, F. Kent, William J. Krause, Sammy L. Eber, Ronald H. Freeman, Christine E. Smith, Mark G. Currie, and Leonard R. Forte. Opossum colonic mucosa contains uroguanylin and guanylin peptides. *Am. J. Physiol.* 270 (*Gastrointest. Liver Physiol.* 33): G708–G716, 1996.—Uroguanylin and guanylin are structurally related peptides that activate an intestinal form of membrane guanylate cyclase (GC-C). Guanylin was isolated from the intestine, but uroguanylin was isolated from urine, thus a tissue source for uroguanylin was sought. In these experiments, uroguanylin and guanylin were separated and purified independently from colonic mucosa and urine of opossums. Colonic, urinary, and synthetic forms of uroguanylin had an isoelectric point of ~3.0, eluted from C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC) columns at 8–9% acetonitrile, elicited greater guanosine 3′,5′-cyclic monophosphate (cGMP) responses in T84 cells at pH 5.5 than pH 8, and were not cleaved and inactivated by pretreatment with chymotrypsin. In contrast, colonic, urinary, and synthetic guanylin had an isoelectric point of ~6.0, eluted at 15–16% acetonitrile on C_{18} RP-HPLC columns, stimulated greater cGMP responses in T84 cells at pH 8 than pH 5.5, and were inactivated by chymotrypsin, which hydrolyzed the Phe-Ala or Tyr-Ala bonds within guanylin. Uroguanylin joins guanylin as an intestinal peptide that may participate in an intrinsic pathway for cGMP-mediated regulation of intestinal salt and water transport. Moreover, uroguanylin and guanylin in urine may be derived from the intestinal mucosa, thus implicating these peptides in an endocrine mechanism linking the intestine with the kidney.

guanylate cyclase; T84 cells; guanosine 3′,5′-cyclic monophosphate; urine

UROGUANYLIN AND GUANYLIN are members of an emerging family of peptides that function as the physiological ligands for an intestinal isoform of membrane guanylate cyclase (GC-C) (7, 18; see Ref. 13 for review). Uroguanylin and guanylin stimulate GC-C activity, resulting in elevations of cellular guanosine 3′,5′-cyclic monophosphate (cGMP) (7, 18). All species of mammals and birds examined express GC-C-like receptor activity on the apical surface of enterocytes throughout the intestine (21, 22). The opossum kidney also expresses high levels of GC-C-like receptors located in the apical membranes of proximal tubular cells (14). Guanylin was first isolated from rat jejunum as a heat-stable, 15-amino acid peptide that activated GC-C in human intestinal T84 cells (7). Guanylin cDNAs encoding 115- to 116-amino acid precursors have been isolated from rat, human, and mouse intestine (19). Uroguanylin was initially purified as 13- to 15-amino acid peptides from opossum urine (18) and was named on the basis of its structural and functional similarities to guanylin and its isolation from urine. Uroguanylin was confirmed as a second member in the guanylin peptide family by the subsequent isolation of opossum guanylin (18) and the human and rat forms of uroguanylin (10, 20).

Before the discovery of guanylin and uroguanylin, the only peptide agonists for GC-C were the diarrhea-producing, heat-stable enterotoxins (STs) (11, 34). STs are produced by different strains of pathogenic, enteric microorganisms, including *Escherichia coli* (25). STs act as molecular mimics of guanylin and uroguanylin by binding to GC-C and stimulating dramatic increases in cellular cGMP accumulation (11, 13, 31). ST-stimulated cGMP production decreases sodium absorption and increases chloride secretion by enterocytes, which results in secretory, or "traveler's," diarrhea (11, 25, 31). Similar to STs, uroguanylin and guanylin stimulate transepithelial chloride secretion from T84 cells and intestinal tissues mounted in Ussing chambers by increasing cellular cGMP production (7, 18, 20). Thus previous studies of the mechanisms by which STs exert their pathological effects may provide insights into the physiological roles of uroguanylin and guanylin.

Guanylin mRNA is found throughout the intestine, with the highest levels of expression in the ileum and colon (20). Guanylin and/or its mRNA has been reported to occur in a heterogeneous population of intestinal cell types, including absorptive enterocytes (27), Paneth cells (9), enterochromaffin cells (4), and goblet cells (6). Northern analysis has also demonstrated lower levels of guanylin mRNA in the kidney, adrenal gland, and the uterus/oviduct (33). Moreover, proguanylin has been shown to circulate in the plasma of humans, demonstrating that guanylin may potentially regulate GC-C or other target receptors via an endocrine mechanism (23, 24, 30). Presently, uroguanylin has only been isolated from urine, and little is known regarding the tissues that may produce this peptide (10, 18, 20). The high levels of uroguanylin found in the urine of opossums, humans, and rats could be derived from the kidney and/or from other tissues via filtration of uroguanylin from the circulation. In the current study, we have isolated uroguanylin and guanylin peptides from the colonic mucosa of opossums. Several independent analytical techniques were used to identify the bioactive peptides. Thus intestinal mucosa is a potential tissue source for uroguanylin and guanylin found in urine (10, 18, 20).

MATERIALS AND METHODS

Purification of colon peptides. Full-length colons, including cecums, were removed from adult opossums (*Didelphis virginiana*), and the mucosae (150 g wet wt) were scraped from colonic muscle with the use of a glass microscope slide. Only healthy opossums with hard stools were used in these studies. The mucosae were divided into two batches, which were processed separately (75 g wet wt/batch). Each batch was suspended in 10 vol of 1 M acetic acid, heated at 100°C for 10 min with constant stirring, homogenized, and stored at −20°C. The homogenate was thawed and centrifuged at 10,000 *g* for 20 min, and the supernatant was made to 0.1% trifluoroacetic acid (TFA). The supernatants were processed with Waters Sep-Pak cartridges [octadecylsilane cartridges (C_{18})] as described previously (7, 18). Eluted fractions of the colon extract from C_{18} cartridges were dried in a Speed-Vac, resuspended in 10 ml of 25 mM ammonium acetate, pH 5.0, and centrifuged at 500 *g* for 10 min. From each batch, 8.0 ml of supernatant were applied to a 2.5 × 90 cm Sephadex G-25 column, and 10-ml fractions were collected for two successive runs. Fractions from this step and subsequent purification steps were assayed for bioactivity as described in *cGMP accumulation assay in T84 cells*. Bioactive fractions from each batch were combined after the Sephadex G-25 step. Final purification by isoelectric focusing on a Rotofor apparatus (Bio-Rad) and reverse-phase high-performance liquid chromatography (RP-HPLC) of individual uroguanylin-like or guanylin-like bioactive peptides was achieved using the same purification scheme as described previously for isolation of intestinal peptides from the opossum and rat (7, 18). An ampholyte range of pH 3.0 to 10.0 (Bio-Rad) was used for isoelectric focusing.

Purification of urine peptides. Uroguanylin and guanylin peptides were extracted from 4.5 liters of opossum urine and purified as previously described, except that an isoelectric focusing step was added after the semipreparative HPLC step (18). Active fractions were identified using the bioassay as described below. Separate columns and Rotofor apparatus materials were used during purification of urine peptides than were used for purification of the colon peptides.

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)-Ham's F-12 medium (1:1) containing 5% fetal bovine serum, 60 μg penicillin, and 100 μg streptomycin per milliliter as previously described (18).

cGMP accumulation assay in T84 cells. T84 cells were cultured in 24-well plastic dishes, and the cGMP levels were measured in control and agonist-stimulated cells by radioimmunoassay (18). Aliquots of column fractions and vehicle were suspended in 200 μl of each of two assay buffers: pH 8.0 buffer, consisting of DMEM, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 mM sodium bicarbonate, pH 8.0, and 1 mM 3-isobutyl-1-methylxanthine (IBMX); and pH 5.5 buffer, consisting of DMEM, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5, and 1 mM IBMX. Ammonium acetate and TFA were removed from test samples by drying fraction aliquots in a Speed-Vac before suspension in assay buffers. This was done to avoid changes in pH caused by the column reagents. T84 cells were washed twice with 200 μl of the respective pH 8.0 and pH 5.5 buffers before addition of reagents. These solutions of medium plus bioactive peptides were then added to T84 cells and incubated at 37°C for 40 min. After incubation, the reaction medium was aspirated, and 200 μl of 3.3% perchloric acid was added per well to stop the reaction and extract cGMP. The extract was adjusted to pH 7.0 with potassium hydroxide and centrifuged, and 50 μl of the extract was used to measure cGMP. For pH titration studies, DMEM, 20 mM HEPES, 1 mM IBMX was adjusted to pH 7.0, 7.5, 8.0, and 8.5 with NaOH, and DMEM, 20 mM MES, 1 mM IBMX was adjusted to pH 5.0, 5.5, 6.0, and 6.5 with NaOH.

Peptide-agonist concentration-response curves were analyzed with the computer program Prism (GraphPad Software, San Diego, CA). The concentrations at which peptide agonists elicited 50% of the maximal cGMP accumulation response (EC_{50}) were obtained by nonlinear regression of agonist-stimulated cGMP accumulation data.

Synthesis of uroguanylin, guanylin, and ST peptides. Synthetic uroguanylin-(2—15), EDCELCINVACTGC, and synthetic guanylin-(1—15), SHTCEICAFAACAGC, were synthesized by the solid-phase method with an Applied Biosystems 431A peptide synthesizer. *N*-(9-fluorenylmethoxycarbonyl) (FMOC)-protected amino acids activated with 2-(1H-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 ultraviolet absorbance of the released FMOC groups. The peptides were 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, ethanedithiol, and water (95:2.5:2.5, vol/vol) for 2 h at room temperature. The peptides were cyclized with the use of air oxidation. The acetamidomethyl groups on Cys^3 and Cys^{11} were removed with iodine. The peptides were 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 sequences of uroguanylin and guanylin were confirmed by protein sequencing on an Applied Biosystems 470A gas-phase protein sequencer. Amino acid composition analysis was performed to estimate peptide mass, after RP-HPLC purification.

E. coli ST-(5—17), CCELCCNPACAGC, was prepared as previously described by the solid-phase method with an Applied Biosystems 430A peptide synthesizer on Cys-(4-CH3Bzl)-OCH2-Pam resin, using double coupling cycles to ensure complete coupling at each step (6). The peptide was cyclized with the use of dimethyl sulfoxide, and its structure was verified by electrospray mass spectrometry, gas-phase sequence analysis, and amino acid composition analysis (7).

Synthesis, purification, and composition analysis of rat guanylin-(101—115), rat guanylin-(93—115), and human uroguanylin were performed by our previously described methods (7, 20).

Chymotrypsin digestion of peptides. Vehicle, synthetic peptides, or purified peptides were separated into aliquots in Microfuge tubes and dried in a Speed-Vac. Digestion reactions were started by resuspending the dried aliquots in 100 μl of 10 mM HEPES, pH 8.0, containing 0.15 U (3 μg solid) of bovine pancreatic α-chymotrypsin (Sigma, St. Louis, MO), either with or without 100 μM chymostatin (Sigma). Reactions were incubated at 34°C in a water bath for 1 h. After 1 h, the reaction tubes were placed on ice, 100 μM chymostatin was added to the reactions that had been incubated in the absence of chymostatin, and all the tubes were frozen at −80°C. After freezing, the reaction mixtures were dried in a Speed-Vac and then resuspended in 200 μl of DMEM containing 50 mM sodium bicarbonate (pH 8.0) and 1 mM IBMX for analysis in the T84 cell cGMP accumulation bioassay. The same experiments were repeated and confirmed with sequencing-grade bovine pancreatic chymotrypsin from a second source (Boehringer-Mannheim, Indianapolis, IN).

For NH_2-terminal sequence analysis of digested peptides, 400 pmol of synthetic opossum uroguanylin, synthetic opossum guanylin, and synthetic rat guanylin-(101—115) were incubated for 10 h at 34°C in a Microfuge tube containing 50 μl of 10 mM HEPES, pH 8.0, with either 20 or 200 pmol of bovine pancreatic α-chymotrypsin (sequencing grade, Boehringer-Mannheim).

RESULTS

Selective bioassay for uroguanylin and guanylin peptides. During the initial isolation of uroguanylin and guanylin peptides, we observed that a reduction in medium pH reduced the cGMP response elicited by guanylin in T84 cells. In contrast, uroguanylin was less sensitive to changes in pH. To further investigate these observations, the effects of medium pH on the cGMP responses elicited by 30 nM of the synthetic forms of opossum uroguanylin and guanylin in T84 cells were examined (Fig. 1). Uroguanylin caused a greater increase in cellular cGMP levels when assayed at pH 5 compared with pH 8. In contrast, guanylin caused only a doubling in cGMP accumulation above basal levels at pH 5, with the cGMP response increasing to 13-fold at pH 8 (Fig. 1). Results obtained with 3 nM *E. coli* ST-(5—17) were similar to those obtained with 30 nM uroguanylin (data not shown). Synthetic rat guanylin, like opossum guanylin, elicited greater cGMP responses in T84 cells at pH 8 than at pH 5.5 (Fig. 2). Moreover, the synthetic form of human uroguanylin was like opossum uroguanylin and *E. coli* ST-(5—17) in that each peptide stimulated greater levels of cGMP accumulation in T84 cells at acidic relative to alkaline pH (Fig. 2). These experiments defined pH conditions that were used to estimate the potencies of these peptide agonists. Uroguanylin was more potent at pH 5.5 (EC_{50}, 200 ± 50 nM, n = 5) than at pH 8 (EC_{50}, 1,900 ± 100 nM, n = 5) (Fig. 3). In contrast, guanylin was less potent at pH 5.5 (EC_{50}, 10.6 ± 4.2 μM, n = 3) than at pH 8 (EC_{50}, 0.68 ± 0.1 μM, n = 3). ST-(5—17) appeared similar in potency at both pH 5.5 (EC_{50}, 40 ± 10 nM, n = 3) and pH 8 (EC_{50}, 130 ± 60 nM, n = 3). Interestingly, the rank order of potency of ST > uroguanylin > guanylin at pH 5.5 changed to ST > guanylin > uroguanylin at pH 8 in the T84 cell bioassay. These results demonstrate that guanylin-selective and uroguanylin-selective pH conditions can be used to identify these peptides.

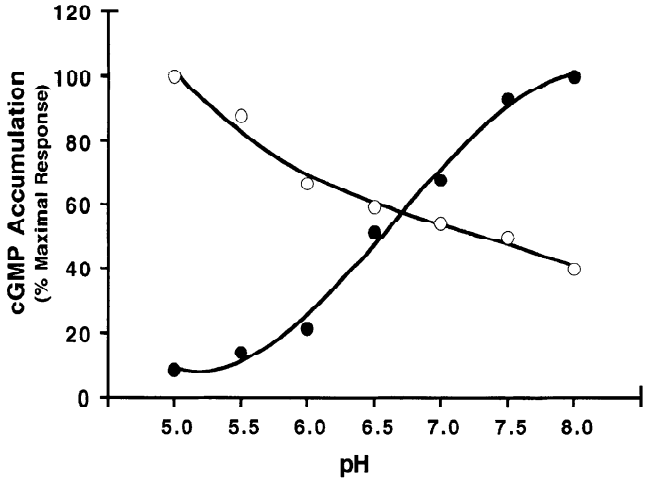

Fig. 1. Effects of medium pH on uroguanylin (○) and guanylin (●)-stimulated guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation in T84 cells. Vehicle, 30 nM synthetic opossum uroguanylin, and 30 nM synthetic opossum guanylin were suspended in buffered assay medium previously adjusted to pH values indicated, as described in MATERIALS AND METHODS. Levels of T84 cell cGMP accumulation (pmol/well, average of 3 wells) elicited by vehicle and peptides in this experiment when tested at pH 5.0 and pH 8.0, respectively, were as follows: basal (vehicle control) = 0.45 and 0.78, uroguanylin = 43.9 and 17.5, and guanylin = 0.85 and 10.0. Data are representative of 4 experiments with similar results.

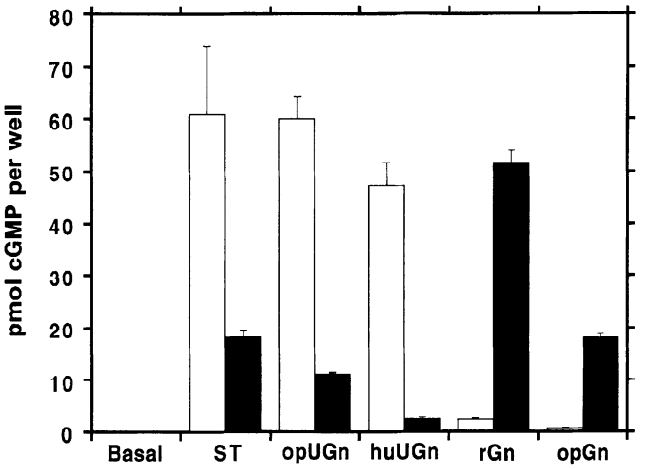

Fig. 2. Agonist-stimulated cGMP accumulation in T84 cells at pH 5.5 (open bars) and pH 8.0 (solid bars). Peptides and vehicle were suspended in HEPES and Dulbecco's modified Eagle's medium (DMEM) containing 50 mM sodium bicarbonate (pH 8.0), or 2-(*N*-morpholino)ethanesulfonic acid (MES) and DMEM at pH 5.5 (pH 5.5) for analysis in the T84 cell cGMP accumulation bioassay. Basal, vehicle control; ST, synthetic *E. coli* ST-(5—17); opGn, synthetic opossum guanylin; opUGn, synthetic opossum uroguanylin; huUGn, synthetic human uroguanylin; rGn, synthetic rat guanylin-(101—115). All peptides were tested at 30 nM except for *E. coli* ST-(5—17), which was tested at 3 nM. Error bars indicate standard error of the mean for 3 experiments.

Comparison of cGMP responses to endogenous and synthetic peptides. To demonstrate that the selective bioassay conditions using synthetic uroguanylin and guanylin could be extended to endogenous uroguanylin and guanylin, we isolated bioactive peptides from opossum urine, which contains greater levels of uroguanylin than guanylin (10, 18, 20). At each stage of purification before the isoelectric focusing step, the urine extracts elicited much larger cGMP responses in T84 cells at pH 5.5 compared with the cGMP responses at pH 8 (data not shown). The bioactivity profile obtained using preparative isoelectric focusing of the partially purified urine extract revealed a dominant peak of bioactivity migrating with a pI of about 3.0 (*peak 1*, Fig. 4). This pI is similar to that of the uroguanylin peptides previously isolated from urine, which contained two or three acidic amino acids and no basic residues (18). The bioactive components in *peak 1* also elicited greater cGMP responses in T84 cells when assayed at pH 5.5 than at pH 8. A small amount of

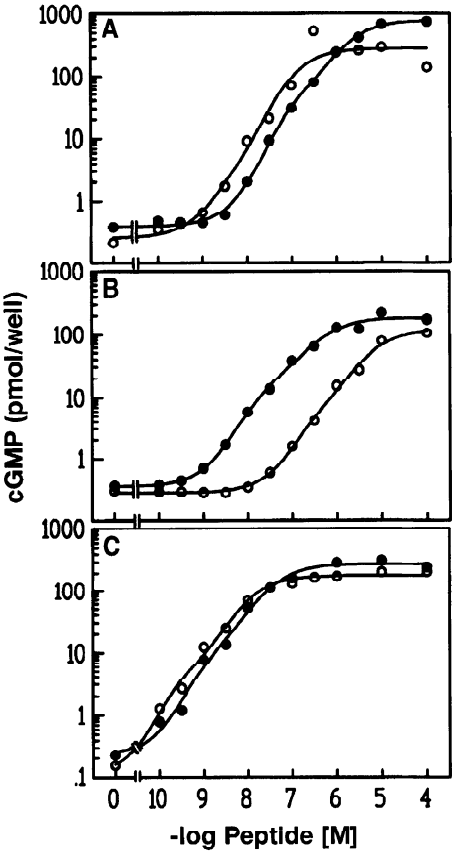

Fig. 3. Concentration-response curves for stimulation of cGMP accumulation in T84 cells by synthetic opossum uroguanylin (*A*), synthetic opossum guanylin (*B*), and synthetic *E. coli* ST-(5—17) (*C*). Peptides were suspended in MES, DMEM at pH 5.5 (pH 5.5) and in HEPES, DMEM adjusted to pH 8.0 with 50 mM sodium bicarbonate (pH 8.0). Data are representative of 3–5 experiments with each agonist at both pH 5.5 (○) and pH 8.0 (●).

guanylin-like activity was also observed migrating with a pI of 5.2 (*peak 2*, Fig. 4), similar to the pI of opossum guanylin, which contains one histidine (18). The guanylin-like peptide stimulated a fourfold increase in cellular cGMP when assayed at pH 8, with no detectable increase in cGMP at pH 5.5. These experiments confirmed that endogenous uroguanylin and guanylin peptides respond similarly to the synthetic forms of uroguanylin and guanylin when assayed for the ability to elicit cGMP responses at acidic vs. alkaline pH in T84 cells.

Isolation of uroguanylin and guanylin from colonic mucosa. Preliminary examination of extracts from colonic mucosa, full-length small intestinal mucosa, kidney, and plasma of the opossum revealed the presence of both uroguanylin-like and guanylin-like peptides in each of the extracts (data not shown). Because the amounts of extracted uroguanylin-like bioactivity in colonic mucosa appeared to be the greatest per gram wet weight of tissue, we further characterized the peptides from this tissue. Bioactive peptides were extracted from 150 g of opossum colonic mucosa and fractionated by gel-filtration chromatography. The profile of bioactivity obtained when column fractions were assayed at pH 5.5 revealed two distinct peaks of bioactivity that eluted within the internal volume of the Sephadex G-25 gel column (Fig. 5). When the column fractions were assayed at pH 8, a single peak of bioactivity was observed that was coincident with the *peak 2* that was found when the assay was conducted at pH 5.5. The uroguanylin-like peptides in *peak 1* elicited 27-fold increases in cellular cGMP accumulation when assayed at pH 5.5 and only fivefold increases in cellular cGMP at pH 8. In contrast, the guanylin-like peptides in *peak 2* stimulated greater levels of cGMP at pH 8 (100-fold) than when assayed at pH 5.5 (28-fold).

To further characterize the uroguanylin-like peptides, the components of *peak 1* were purified as previously described (7, 18). After each purification step, a dominant activity peak was observed that elicited greater cGMP responses at pH 5.5 than at pH 8. In addition, a pI of 3.0 was observed when the bioactive components from *peak 1* were fractionated by preparative isoelectric focusing (data not shown), consistent with *peak 1* containing an acidic, uroguanylin-like peptide (10, 18, 20). The chromatographic properties of *peak 1* were characterized further with the use of RP-HPLC. The peak fraction of bioactive peptides eluting from a C_{18} RP-HPLC column stimulated a 23-fold increase in cGMP when assayed at pH 5.5, compared with only an eightfold increase in cGMP at pH 8 (Fig. 6). Moreover, a characteristic uroguanylin elution profile (18) was observed, with the uroguanylin-like peptide(s) eluting at 8% acetonitrile from this C_{18} RP-HPLC column (Fig. 6). This elution pattern is

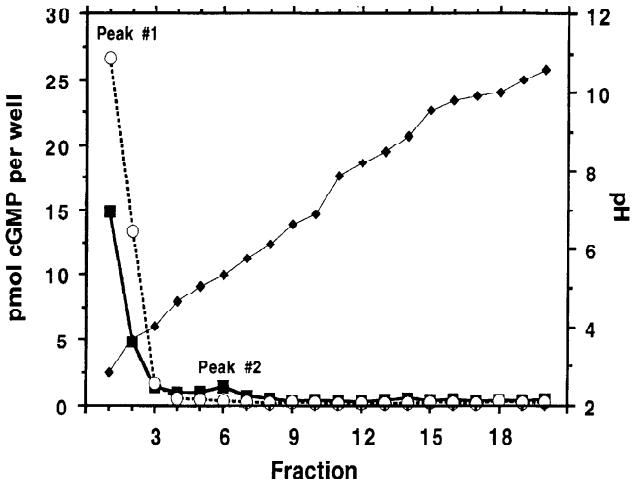

Fig. 4. Isoelectric focusing of peptides extracted from opossum urine. Peptides were extracted from 4.5 liters of opossum urine using C_{18} cartridges and purified as described in MATERIALS AND METHODS. Purified peptides were suspended in 50 ml water containing 0.8% ampholytes (pH range 3–10, Bio-Rad) and then fractionated on a preparative isoelectric focusing cell (Rotofor, Bio-Rad). Fractions (0.2% of fraction volume) were next assayed for the ability to elicit a cGMP response in T84 cells after suspension in MES, DMEM at pH 5.5 (○), and in HEPES, DMEM adjusted to pH 8.0 with 50 mM sodium bicarbonate (■). Right *y*-axis represents pH (◆) of Rotofor fractions obtained after isoelectric focusing of partially purified peptides. pH of the Rotofor fraction containing peak of bioactivity, as determined by T84 cell cGMP accumulation bioassay, was estimated as the isoelectric point (pI).

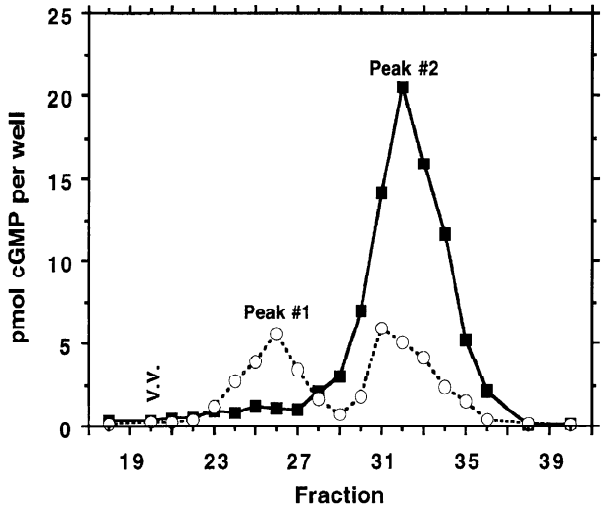

Fig. 5. Sephadex G-25, gel-filtration chromatography elution profile of uroguanylin-like and guanylin-like peptides extracted from colonic mucosa. Peptides were extracted from colonic mucosa using C_{18} cartridges and processed as described in MATERIALS AND METHODS for application to a 2.5 × 90 cm Sephadex G-25 gel column. Fractions (10 ml) were collected, and 1.0% of each fraction was assayed in MES, DMEM at pH 5.5 (○), and in HEPES, DMEM adjusted to pH 8.0 with 50 mM sodium bicarbonate (■) for stimulation of cGMP accumulation in T84 cells. Illustrated bioactivity profile is a representative experiment from 1 of 2 Sephadex G-25 column runs.

consistent with that previously observed for the 14- and 15-amino acid forms of opossum uroguanylin that were isolated from urine (18). When the putative uroguanylin peptide from colon was subjected to NH_2-terminal sequence analysis, no signal for amino acids was observed, indicating that this peptide may be blocked at the NH_2-terminal end, a phenomenon previously encountered when uroguanylin was purified from urine.

To evaluate the bioactive components contained in *peak 2* (Fig. 5), the same purification methods were utilized. When the components of *peak 2* were further purified by isoelectric focusing, both guanylin-like and uroguanylin-like peptides were resolved (*peaks 2A* and *2B*, Fig. 7). *Peak 2A* migrated with a pI of 3.0, similar to the pI of uroguanylin. *Peak 2A* also caused a greater cGMP response in T84 cells at pH 5.5 than it did at pH 8, which is consistent with this peptide being uroguanylin. It is likely that uroguanylin was not completely separated from guanylin in *peak 2* (Fig. 5) during Sephadex G-25 chromatography. This uroguanylin-like activity of *peak 2A* exhibited the same chromatographic properties as authentic uroguanylin when subjected to further analysis by RP-HPLC using C_{18} columns (data not shown). An insufficient quantity of this purified peptide prevented its identification by NH_2-terminal sequence analysis. The guanylin-like bioactivity peak that was resolved by isoelectric focusing migrated with a pI of 6.0, elicited 27-fold increases in T84 cell cGMP levels when assayed at pH 8, and caused only twofold increases in cGMP at pH 5.5 (*peak 2B*, Fig. 7). The pI of 6.0 is similar to the pI estimated for opossum guanylin (~5.2), which contains a histidine residue (18). *Peak 2B* was further purified by a series of C_{18} RP-HPLC steps (7, 18). The guanylin-like activity eluted at about 15% acetonitrile (Fig. 8), which is a characteristic property of guanylin (18). One percent of this peak elicited a 15-fold increase in cGMP accumulation when assayed at pH 8, but caused no detectable increase in cGMP levels in T84 cells at pH 5.5. Further purification by RP-HPLC followed by NH_2-terminal sequence analysis

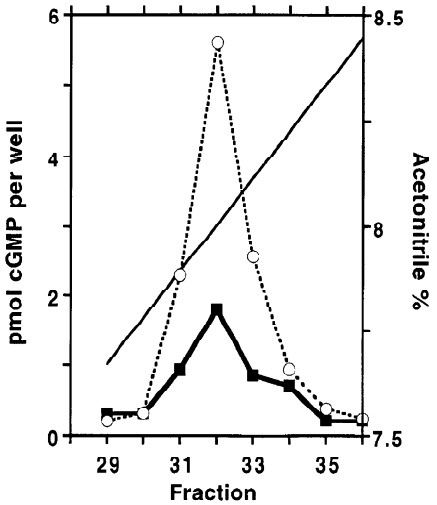

Fig. 6. T84 cell cGMP accumulation responses elicited by uroguanylin purified from opossum colonic mucosa. Fractions were bioassayed using MES, DMEM at pH 5.5 (○), and HEPES, DMEM, 50 mM sodium bicarbonate at pH 8.0 (■). Uroguanylin-like peptides (including the fractions of *peak 1* shown in Fig. 5) from 2 successive Sephadex G-25 column runs of colonic mucosal extracts were further purified as previously described (18) by a preparative isoelectric focusing step and 3 reverse-phase high-performance liquid chromatography (RP-HPLC) steps. Purified uroguanylin-like bioactivity shown is from fractions obtained after the third RP-HPLC step (thin solid line), which used acetonitrile and 10 mM ammonium acetate, pH 6.2, to purify peptides, using a 4.9 × 250 mm C_{18} column, as previously described (18).

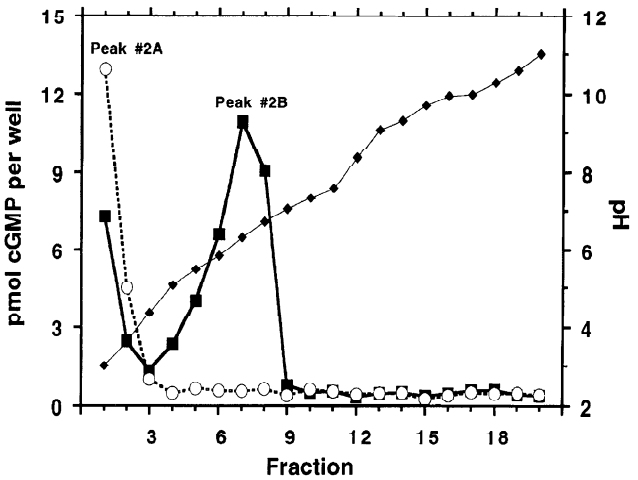

Fig. 7. Isoelectric focusing of peptides extracted from colonic mucosa. Fractions displaying predominately guanylin-like activity (including fractions comprising *peak 2* in Fig. 5) were pooled from 2 separate Sephadex G-25 column runs and fractionated by isoelectric focusing on the Rotofor apparatus, as described in Fig. 4 legend. Fractions (1.0% of fraction volume) were bioassayed for cGMP accumulation in T84 cells using pH 5.5 (○) and pH 8.0 (■) medium, as described in Fig. 4 legend. ◆, pH of Rotofor fractions.

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