Marked increase of guanylin secretion in response to salt loading in the rat small intestine

TOSHIHIRO KITA, KAZUO KITAMURA, J UNICHIRO SAKATA, AND TANENAO ETO
First Department of Internal Medicine, Miyazaki Medical College, Miyazaki 889-1692, Japan

Kita, Toshihiro, Kazuo Kitamura, J unichiro Sakata, and Tanenao Eto. Marked increase of guanylin secretion in response to salt loading in the rat small intestine. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G960–G966, 1999.—Guanylin and uroguanylin are intestinal peptides that stimulate guanylate cyclase C and cause chloride secretion. These peptides show topological instability due to two disulfide bonds. The disulfide bonds were reduced and S-carboxymethylated to cleave the bonds and obtain stable and sole derivatives. We established a new and reliable RIA system for the stable derivatives from both peptides. With the use of this system, the response of the peptides to salt loading of the rat small intestine was evaluated. The lumen of the small intestines of Sprague-Dawley rats was perfused in vivo with Krebs-Ringer solution containing different concentrations of salt or mannitol. Mature guanylin, proguanylin, and mature uroguanylin were found in the perfusate in the basal state. The highest salt loading (200 mM NaCl for 20 min) increased the guanylin secretion about threefold (1.9 ± 0.2 vs. 5.4 ± 0.5 pmol/min), with the effect lasting for 60 min. The uroguanylin secretion was less affected. Hyperosmolar mannitol also caused a significant but smaller increase of guanylin secretion. Increased guanylin could lead to increased salt and water secretion of the intestine; thus members of the guanylin family have potential roles in the regulation of water and salt metabolism in the small intestine.

uroguanylin; radioimmunoassay; chloride secretion; reductive S-carboxymethylation

GUANYLATE CYCLASE C (GCC) is present predominantly throughout the intestine and is known to be a receptor for heat-stable enterotoxins (4, 9). When they bind to GCC, heat-stable enterotoxins cause a marked increase in intracellular cGMP and activate chloride secretion, subsequently resulting in acute secretory diarrhea (5, 10). The 15- to 16-amino acid peptides guanylin and uroguanylin were recently identified from rat intestinal extract and human urine, respectively. These peptides are believed to be endogenous ligands for GCC (2, 15). Both guanylin and uroguanylin mRNA are expressed throughout the intestine, and observations from immunohistochemical and in situ hybridization studies strongly suggest that guanylin and uroguanylin are secreted into the intestinal lumen (18, 20, 26, 29, 30). If guanylin and uroguanylin are secreted into the intestinal lumen, they may act to stimulate GCC and participate in the regulation of chloride secretion. However, no information is available regarding the secretion of guanylin and uroguanylin into the lumen of the small intestine. In this study, we clarified the basal secretion rate of these two peptides and the molecular forms of the secreted peptides.

Evidence has accumulated that the guanylin family of peptides is involved in salt and water homeostasis. The plasma or urine concentration of guanylin or uroguanylin was found to be elevated in patients with renal and heart diseases (3, 14, 25). The uroguanylin concentration in human urine was increased after a high-salt diet (13). A low-salt intake downregulates the guanylin signaling pathway in the rat distal colon (19). Guanylin and uroguanylin can cause relatively weak but significant natriuresis in rats and mice (6, 11). In addition, guanylin may contribute to sodium handling in the intestinal tract. Increased chloride secretion to the lumen of the intestine induced by the guanylin family simultaneously increases the flow of water and sodium to the lumen to preserve the ion balance. Increased guanylin secretion may thus increase sodium secretion to the lumen or, alternatively, depress sodium absorption through the intestinal tract (28). In the present study, the reactions of guanylin and uroguanylin to acute sodium loading were examined to clarify the potential participation of these peptides in the sodium handling of the intestine.

To determine the guanylin and uroguanylin concentrations in biological samples, we established a novel and reliable RIA system, since guanylin and uroguanylin show unique but unfavorable topological stereoisomerism resulting from the existence of two disulfide bonds in a 15- to 16-amino acid sequence (1, 27). Each member of this set of isoforms has not only different physical properties but also different types of immunogenicity and bioactivity (24). Chemically synthesized guanylin and uroguanylin show dual peaks on reverse phase (RP)-HPLC. More importantly, one isoform, with one peak on RP-HPLC, can rapidly transform to another isoform in solution (1). This feature of the peptides causes serious confusion in the measurement of the concentration and bioactivity of the peptides. Therefore, we established a new RIA system for these peptides in which the disulfide bonds of the peptides are opened by reduction and stabilized by the use of S-carboxymethylation. Reduced and S-carboxymethylated (RCM) guanylin or uroguanylin generated from both standard peptides and biological samples clearly showed a single peak on RP-HPLC, and whole amounts of the peptides in biological samples were correctly measured by this system.

MATERIALS AND METHODS

Peptides. Custom-synthesized rat RCM [Tyr0]guanylin and RCM [Tyr0]uroguanylin (YDCELCINVACTGC) and other peptides were purchased from Peptide Institute (Osaka,
Japan). The correct synthesis was confirmed by amino acid analysis and sequencing. Each peptide shows a single peak on RP-HPLC with over 95% purity.

RIA procedure for guanylin and uroguanylin. Rat RCM [Tyr]-guanylin and -uroguanylin (15 mg each) were separately conjugated with bovine thyroglobulin (25 mg) with the use of 5% glutaraldehyde and used as antigens (16). The antiserum for rat guanylin and rat uroguanylin were raised in New Zealand White rabbits as previously described (22). Rat RCM [Tyr]-guanylin and rat RCM [Tyr]-uroguanylin were radioiodinated by the lactoperoxidase method (21). The 125I-labeled peptides were purified by RP-HPLC, and, in the case of guanylin, only a guanylin moniodinated at position Tyr0 was used.

The same assay buffer used in the adrenomedullin RIA system (16) was used for this RIA procedure. The incubation mixture consisted of 0.1 ml of diluted sample or standard (rat RCM [Tyr]-guanylin or rat RCM [Tyr]-uroguanylin), 0.1 ml of antiserum diluent (final dilution of 1:150,000 for guanylin and 1:30,000 for uroguanylin), and 0.1 ml of tracer solution (18,000 cpm). Incubation was carried out for 24 h. The bound and free ligands were separated by incubation with 0.05 ml of second antibody (goat anti-rabbit γ-globulin) with 0.75 ml of 20% polyethylene glycol for 2 h and centrifugation. The radioactivity in the precipitate was counted in a gamma counter (ARC-600, Aloka, Tokyo, Japan). All procedures were performed at 4°C. Samples were assayed in duplicate.

Rat small intestinal perfusion. Eight-week-old male specifically pathogen-free Sprague-Dawley rats (n = 27) were purchased from Charles River (Atsugi, Japan). The rats were housed in a temperature- and humidity-controlled environment and maintained on standard rat chow (Nihon CLEA CE-2; 145 µmol sodium/g) and tap water ad libitum for at least 1 wk before the experiment.

The rats were anesthetized with pentobarbital sodium (50 mg/kg) and placed on a heating table (37°C) and then underwent laparotomy. Incisions were made in the middle portion of the duodenum and the end of the ileum. The lumen of the small intestine was rinsed gently with physiological saline to remove fecal contents. The duodenum and the end of the ileum were cannulated for luminal perfusion. Luminal perfusion was started with modified Krebs-Ringer bicarbonate solution at a rate of 5 ml/min. After a 10-min equilibration period, every 10 min perfusate was collected on ice for 100 min. The composition of the modified Krebs-Ringer bicarbonate solution was (in mM) 50 NaCl, 26 NaHCO3, 5 KCl, 1 MgSO4·7H2O, 0.03 Na2EDTA, 11 dextrose, and 2.4 CaCl2·2H2O (pH 7.4). The buffer solution was aerated with 95% O2-balance CO2 and maintained at 37°C. After a 20-min control period, rats were perfused with Krebs-Ringer bicarbonate solution containing various concentrations of salt (122, 170, and 200 mM NaCl) or mannitol (257 mM) for 20 min and then perfused again with ordinary modified Krebs-Ringer bicarbonate solution for another 60 min. Immediately after collection, the samples (50 ml each) were manually applied to C18 Sep-Pak columns (Waters, Milford, MA), which were pre-equilibrated with 0.1% trifluoroacetic acid (TFA)-H2O. The columns were washed with 10 ml of 0.1% TFA-H2O, eluted with 10 ml of 60% acetonitrile-0.1% TFA-H2O, and lyophilized.

Osmolarities of the solutions for the perfusion experiment were directly measured by cryoscopic methods. With the use of standard curves, the osmolarity of the solution containing mannitol was exactly matched with the highest salt solution.

RMC method for biological samples. The lyophilized sample was reconstructed with 0.5 ml of H2O and centrifuged at 5,000 rpm for 10 min at 4°C (MRX-151, Tomy, Tokyo, Japan). Fifty microliters of the supernatant were placed in an Eppendorf tube, mixed with reaction buffer, and adjusted to a final concentration of 0.5 M Tris·HCl (pH 8.0), 2 mM EDTA, and 25 mM dithiothreitol (DTT) (total volume was 125 µl). The tube was flushed with nitrogen, capped, and placed in a 37°C water bath for 60 min. The solution was cooled to room temperature, and 12.5 µl of 1 M iodoacetic acid were added. After 20 min in the dark, 12.5 µl of concentrated acetic acid were added to acidify the solution (final volume was 150 µl). The solution was immediately diluted 10 times with the assay buffer and then subjected to the RIA for guanylin and uroguanylin.

The tissue extracts were prepared as described previously (16), with minor modifications. The lyophilized samples were reconstructed with distilled H2O and adjusted to a concentration of 1 g wet tissue/ml. One hundred microliters of tissue extract containing 125I-labeled rat guanylin, which was radioiodinated by the lactoperoxidase method and purified by RP-HPLC, were converted using the same ratio of reagents and then analyzed by RP-HPLC. Every 1-min fraction was collected; the radioactivity of the fractions was monitored by the gamma counter.

Characterization of guanylin and uroguanylin in the perfusate. Two Sprague-Dawley rats were perfused with modified Krebs-Ringer bicarbonate solution for 60 min. The perfusate was extracted with a Sep-Pak C18 column and lyophilized as described in Rat small intestinal perfusion. The sample was reconstructed with 1 ml of H2O and briefly centrifuged. Five hundred microliters of the supernatant were treated by the same method to gain the RCM peptides. The solution was centrifuged at 9,000 rpm for 5 min, and the resulting supernatant was applied to a C18 HPLC column. The column was developed with the following linear gradient: 10% acetonitrile-0.1% TFA-H2O to 60% acetonitrile-0.1% TFA-H2O in 60 min at a flow rate of 1 ml/min. Every 1-min sample (1 ml each) was collected, and 5 µl aliquots of the samples were assayed by the RIA.

Samples (n = 24) from the perfusion study, which represent initial and peak secretions induced by the highest salt and mannitol loading, were reconstructed with 0.5 ml of H2O; 0.1 ml of each sample was then treated by the same RCM method and separately applied to the HPLC column. Corresponding 1-min samples were collected and lyophilized. The samples were reconstructed with RIA buffer and subjected to the RIA.

Statistics. Results are expressed as means ± SE. A repeated-measures ANOVA was used to assess the significance of changes. After evaluation with ANOVA, Student’s t-test was performed for the salt or mannitol loading group and the corresponding control group. P < 0.05 was taken to indicate a significant difference.

RESULTS

Figure 1 shows the RP-HPLC profiles of synthetic rat guanylin (Fig. 1A) and RCM rat guanylin (Fig. 1, B and C). Authentic rat guanylin exists as a mixture of two compounds, but the RCM reaction converts these peptides to a single compound that was eluted at a slightly later position by RP-HPLC compared with authentic rat guanylin. Various incubation times and ratios (DTT to iodoacetic acid) were examined to obtain the appropriate method for RCM reaction. Two suitable methods are indicated in Fig. 1: a 1-h incubation with DTT at 37°C (Fig. 1B) or an overnight incubation with DTT at 4°C (Fig. 1C) followed by a 20-min incubation with...
iodoacetic acid at room temperature. These methods cause the complete conversion of guanylin to RCM guanylin. The subsequent amino acid analysis revealed that peak 1 in Fig. 1 is the completely converted guanylin and peak 2 is the partially converted guanylin. The ratio of DTT to iodoacetic acid (1:4) is crucial in this reaction to avoid the formation of partially converted guanylin. This method achieved an identical complete conversion in human guanylin and human and rat uroguanylin (data not shown). To determine whether the RCM reaction was completed in biological samples, we employed the same method using 125I-labeled rat guanylin. The radioactive peak of 125I-labeled rat guanylin was clearly shifted after the RCM reaction (Fig. 2 A). The same peak shifts were observed in the RCM reactions coexisting with various biological materials (Fig. 2 B). 125I-labeled rat uroguanylin also showed a peak shift in the same conditions but in an opposite direction (data not shown, see the elution positions of the standard peptides in Fig. 4).

Figure 3 shows standard curves of RIAs. Half-maximal inhibitions of radiiodinated ligand binding by guanylin and uroguanylin were observed at 20–30 fmol/tube. The minimum detection quantity of guanylin or uroguanylin was 1 fmol/tube, and we usually used 8–250 fmol/tube as a working range. The antisera did not show cross-reaction with authentic forms of guanylin and uroguanylin (Table 1). The antisera recognize and measure both mature guanylin and proguanylin and uroguanylin and prouroguanylin, respectively. The antisera for rat RCM uroguanylin showed little cross-reaction (up to 5%) with rat RCM guanylin at high concentrations. However, this interference usually did not cause problems within the working range. The other antisera for rat RCM guanylin did not show any cross-reaction to rat RCM uroguanylin (Table 1), so dual measurements for guanylin and uroguanylin can be corrected in measurements with the RIA for uroguanylin when needed.

The characterization of rat intestinal perfusate was performed by RP-HPLC combined with the two RIAs (Fig. 4). Immunoreactive guanylin consisted of two peaks, in which peak 1 emerged at a position identical to that of RCM rat guanylin. Peak 2 eluted at a position close to that of biologically inactive proguanylin. In contrast to guanylin, immunoreactive uroguanylin
showed only a single peak at the position of RCM uroguanylin. In preliminary experiments, initial secretion rates of guanylin and uroguanylin with isosmolar Krebs-Ringer solution (122 mM NaCl, osmolarity of 305 mosM) were 1.84 ± 0.23 and 1.39 ± 0.18 pmol/min (n = 5), respectively. However, the secretion rate of guanylin gradually increased after a 30-min perfusion (data not shown). We surveyed the optimal concentration of NaCl to obtain a stable secretion rate for guanylin and finally selected a relatively low concentration of NaCl (50 mM) as a control solution (osmolarity of 190 mosM). The basal secretions of both guanylin and uroguanylin in the perfusate from rat small intestinal tract maintained relatively stable levels during the experiment (Fig. 5). The amount of secreted immunoreactive guanylin was almost twice that of uroguanylin, which is consistent with the characterization of the perfusate (Figs. 4 and 5). After an equilibration period, the 20-min loading of high-salt solution (osmolarity of 450 mosM) induced a marked increase of guanylin secretion. This increase occurred after a 10-min loading and lasted another 60 min. The peak of increased secretion in the high-salt group reached almost three times that of the control group (5.4 ± 0.5 vs. 1.9 ± 0.2 pmol/min, respectively, at 50 min). Hyperosmolar solution containing 257 mM mannitol and 50 mM NaCl (osmolarity of 450 mosM) also caused a significant but lesser increase of guanylin secretion. Interestingly, the reaction of uroguanylin to the highest salt loading was limited to a slight increase compared with the reaction of guanylin (1.46 ± 0.06 vs. 0.92 ± 0.10 pmol/min at 50 min), and uroguanylin did not react with mannitol loading. Using the RP-HPLC and the RIA system, we performed characterizations of basal and peak fractions of the secreted guanylin stimulated by the highest salt or mannitol loading. This characterization clearly revealed that both stimulations similarly increased mature guanylin and proguanylin secretion (Fig. 6). Figure 7 shows the dose-response relationship of salt loading and guanylin or uroguanylin response, in which the maximal rate of increase compared with initial secretion was plotted against the different concentrations of NaCl used for the stimulation. Guanylin secretion was increased dose dependently, but uroguanylin secretion seemed to reach a plateau at isosmolar solution (122 mM NaCl).

**DISCUSSION**

The present paper describes a new RIA system for rat guanylin and uroguanylin. To our knowledge, this is the first report to describe an RIA combined with the RCM reaction being applied to small peptides. The RCM reaction is commonly used for peptide sequence analyses (12). By consulting a previous study of γ-immunoglobulin (8), we optimized the conditions for the RCM reaction in the guanylin family of peptides. The disul-
Basal secretions. and monitored by RIA. Fractions of each group were separately fractionated by RP-HPLC. [50 mM (control, initial secretion was plotted against different concentrations of NaCl. Maximal rate of increase compared with

Fig. 7. Dose-response relationship of salt loading and guanylin or uroguanylin response. Maximal rate of increase compared with initial secretion was plotted against different concentrations of NaCl [50 mM (control, n = 6), 122 mM (n = 4), 170 mM (n = 5), and 200 mM (n = 6)]. *P < 0.05, **P < 0.01 compared with the corresponding initial secretions.

Uroguanylin response. Maximal rate of increase compared with the corresponding initial secretions.

Fig. 6. Characterization of basal and peak fractions of the guanylin stimulated by high salt (200 mM NaCl) and mannitol (257 mM mannitol + 50 mM NaCl) loading. Initial (n = 6) and peak (n = 6) fractions of each group were separately fractionated by RP-HPLC and monitored by RIA. **P < 0.01 compared with the corresponding basal secretions.

Flexible disulfide bonds of the peptides are easily opened by a relatively mild reduction using DTT. This may reflect flexible disulfide bonds in guanylin, which transform between two isomers in solution (17). Prolonged incubation (e.g., 4 h) at high temperature (e.g., 50°C) destroyed the sample, regardless of the amount of the peptides (data not shown). The ratio of DTT to iodoacetic acid (1:4) is also crucial for this reaction to avoid the formation of partially converted guanylin. In a previous report, a DTT-to-iodoacetamide ratio of 1.2 was used (12), but this ratio of the reagents led to a significant amount of incompletely converted guanylin formation. The RCM reaction was also completed in extracts of biological materials (Fig. 2). The final recovery rates of RCM guanylin were slightly higher in extracts from RCM guanylin were slightly higher in extracts from small intestine compared with those in a peptide-only solution and extracts from plasma. The cause of this phenomenon is unclear. Some protective proteins for peptides (data not shown). The ratio of DTT to iodoacetamide ratio of 1.2 was used (12), but this ratio of the reagents led to a significant amount of incompletely converted guanylin formation. The RCM reaction was also completed in extracts of biological materials (Fig. 2). The final recovery rates of RCM guanylin were slightly higher in extracts from small intestine compared with those in a peptide-only solution and extracts from plasma. The cause of this phenomenon is unclear. Some protective proteins for guanylin may exist in the extracts.

A simple method is usually better than a complicated procedure in an RIA, since a more complicated method may entail more problems. However, the unique features of the guanylin family oblige a novel solution for correct measurements. The topological stereoisomerism of the guanylin family, in which each isomer has different physical properties, immunogenicities, and bioactivities (1, 24, 27), may prevent complete cross-reactions to antibodies. An antibody that recognizes one isomer may not react with another isomer. To make matters more complex, one isomer quickly transforms to another isomer during incubation in an RIA, so that a complete separation of the isomers is basically impossible. We decided to open the disulfide bonds, which are the origin of topological stereoisomerism, and make a single compound. The RCM reaction worked well with guanylin and uroguanylin, and the established RIA showed high sensitivity because of the artificial modification for the peptides. This new RIA system should contribute to the study of guanylin and related peptides.

Using this RIA system, we characterized guanylin and uroguanylin in perfusate from rat small intestine. Immunoreactive guanylin contained two peaks that eluted at the position of RCM guanylin and near the position of proguanylin in RP-HPLC. Interestingly, immunoreactive uroguanylin consisted of only a single peak, which emerged at the position of RCM uroguanylin (Fig. 3). Coupled with the previously reported bioassay (15), we have already completed the isolation and sequencing of bioactive guanylin and uroguanylin in the perfusate, where almost the same amounts of bioactive guanylin-14 (NTCEICAYAUCTGC) and uroguanylin-15 (TDECELICNVACTGC) were yielded (unpublished data). This observation, together with the same elution positions for RCM guanylin or RCM uroguanylin on RP-HPLC, strongly suggested that immunoreactive guanylin (peak 1) and immunoreactive uroguanylin are bioactive and are mature guanylin and uroguanylin, respectively. Peak 2 in Fig. 4 was not detected by the bioassay, which means that it is biologically inactive. According to a previous report (25), peak 2 is most likely to be proguanylin. Uroguanylin may act as an endocrine mediator for the kidney and is thought to be secreted to both the luminal and basolateral sides of the intestine (7). The difference in molecular forms observed in the perfusate may reflect differences in the secretion behavior of uroguanylin and guanylin. Further study is required to clarify the basolateral secretion of uroguanylin.

The basal secretion rates of immunoreactive guanylin and immunoreactive uroguanylin were almost 2 and 1 pmol/min, respectively. Immunoreactive guanylin seemed to contain almost the same amounts of mature guanylin and proguanylin, and thus almost the same amounts of mature guanylin and uroguanylin should be secreted into the perfusate. Guanylin and uroguanylin showed different distributions of expression in the longitudinal axis of the gastrointestinal tract (31), but the present study demonstrated that almost equal amounts of mature peptides were secreted from the total small intestine. Guanylin and uroguanylin showed bioactivities at a concentration in
the 10⁻⁹ M range with T84 cells (15), and the basal secretion rate of both peptides (1 pmol/min) could be enough to cause chloride secretion from the small intestine.

The main target of this study was to clarify the secretions of guanylin and uroguanylin into the lumen of the small intestine and the reaction of the guanylin family to salt loading. It must be emphasized that guanylin and uroguanylin and their receptor GCC are predominantly expressed in the gastrointestinal tract and that the peptides act on the luminal surface of the intestine (11, 18, 20, 25, 26, 28–31). The gastrointestinal tract has not received much attention as a regulator of water and electrolytes. The identification of the guanylin family may shed light on the role of the gastrointestinal tract in water and electrolyte regulation. In addition, an endocrine link seems to exist between the intestine and kidney via uroguanylin (7).

In the present study, stable and large basal secretions of guanylin and uroguanylin from rat small intestine were observed. Moreover, it was clearly demonstrated that salt loading to the lumens of the small intestine causes a dose-dependent increase of guanylin and a lesser but significant increase of uroguanylin. Interestingly, guanylin reacts, although not significantly, with isomolar solution; moreover, the reaction of uroguanylin seems to reach a plateau at the isomolar solution. Because the osmotic pressures of the contents (e.g., foods, saliva, and gastric juice) of the small intestine are relatively low, isomotic pressure could be enough to induce a change of guanylin and uroguanylin secretions from the small intestine. Guanylin also reacts with hyperosmolar solution containing mannitol. This observation suggested that guanylin secretion is partially driven by the osmotic pressure of the contents of the small intestine. The increases of guanylin and uroguanylin in response to salt or hyperosmolar loading could be expected to induce increased chloride secretion to the lumen of the intestine and simultaneously increase the flow of water and sodium to the lumen to maintain the ion balance. This would primarily contribute to maintaining local osmotic homeostasis in the small intestine because induced water secretion could dilute hyperosmolar content in the small intestine. On the other hand, guanylin reacted more to salt loading than to mannitol loading and uroguanylin reacted with only salt loading (Fig. 5). This observation strongly suggested that salt itself, besides osmotic pressure, causes a specific reaction in the secretion of guanylin family members. Increased guanylin secretion could depress sodium absorption and increase chloride secretion through the intestinal tract (28). Uroguanylin may also act as a natriuretic factor in the kidney. This observation raises the possibility that the guanylin family acts as regulatory peptides of sodium and water homeostasis in the intestine.

Address for reprint requests and other correspondence: T. Kita, First Dept. of Internal Medicine, Miyazaki Medical College, 5200 Kihara Kiyotake, Miyazaki 889-1692, Japan (E-mail: t-kita@po.sphere.ne.jp).

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