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Gastrointestinal pain: Unraveling a novel endogenous pathway through uroguanylin/guanylate cyclase-C/cGMP activation

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ABSTRACT

The natural hormone uroguanylin regulates intestinal fluid homeostasis and bowel function through activation of guanylate cyclase-C (GC-C), resulting in increased intracellular cyclic guanosine-3′,5′-monophosphate (cGMP). We report the effects of uroguanylin-mediated activation of the GC-C/cGMP pathway in vitro on extracellular cGMP transport and in vivo in rat models of inflammation- and stress-induced visceral hypersensitivity. In vitro exposure of intestinal Caco-2 cells to uroguanylin stimulated bidirectional, active extracellular transport of cGMP into luminal and basolateral spaces. cGMP transport was significantly and concentration dependently decreased by probenecid, an inhibitor of cGMP efflux pumps. In ex vivo Ussing chamber assays, uroguanylin stimulated cGMP secretion from the basolateral side of rat colonic epithelium into the submucosal space. In a rat model of trinitrobenzene sulfonic acid (TNBS)-induced visceral hypersensitivity, orally administered uroguanylin increased colonic thresholds required to elicit abdominal contractions in response to colorectal distension (CRD). Oral administration of cGMP mimicked the antihyperalgesic effects of uroguanylin, significantly decreasing TNBS- and restraint stress–induced visceromotor response to graded CRD in rats. The antihyperalgesic effects of cGMP were not associated with increased colonic spasmolytic activity, but were linked to significantly decreased firing rates of TNBS-sensitized colonic afferents in rats in response to mechanical stimuli. In conclusion, these data suggest that the continuous activation of the GC-C/cGMP pathway along the intestinal tract by the endogenous hormones guanylin and uroguanylin results in significant reduction of gastrointestinal pain. Extracellular cGMP produced on activation of GC-C is the primary mediator in this process via modulation of sensory afferent activity.

1. Introduction

The endogenous hormones guanylin and uroguanylin are cyclic guanosine-3′,5′-monophosphate (cGMP)–regulating signaling peptides involved in intestinal fluid homeostasis and bowel function of the gastrointestinal tract [17,23,25]. Their effects occur through activation of guanylate cyclase-C (GC-C), a type I transmembrane receptor expressed predominantly on the apical surface of intestinal epithelial cells [9,29,47]. Receptor activation by selective GC-C agonists such as uroguanylin results in increased intracellular concentrations of the second messenger cGMP [17,39], which mediates its biological effects. Recently, it was shown that the synthetic peptide linaclotide, closely related to the endogenous hormones guanylin and uroguanylin, also increased intracellular concentrations of cGMP, resulting in augmented intestinal fluid secretion and accelerated transit in rodents [7,8]. In addition, linaclotide reduced colonic hypersensitivity in several rodent models of postinflammatory and stress-induced visceral hypersensitivity, in a GC-C–dependent manner, a mechanism not previously linked to the GC-C/cGMP pathway [16]. Moreover, clinical studies have also documented the benefit of GC-C agonists in improving pain in conditions like irritable bowel syndrome (IBS) with constipation (IBS-C). IBS-C patients treated with linaclotide, a drug approved by the FDA for the treatment of patients with IBS-C and chronic

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idiopathic constipation, showed statistically significant and clinically meaningful improvements in abdominal pain compared with placebo-treated patients, as well as an improvement in bowel habits [13,22,42]. It has been hypothesized that the analgesic effects of linaclotide may also be mediated by extracellular cGMP.

An increase in intracellular cGMP is known to modulate a variety of cellular processes, and its well-characterized intracellular effects are primarily mediated through interaction with 3 groups of target proteins: cGMP-dependent protein kinases, cyclic nucleotide–gated ion channels, and cGMP-regulated phosphodiesterases [46]. In contrast, the effects of cGMP after transport out of intestinal epithelial cells after activation of GC-C remain unknown.

It is known that cGMP can be actively transported out of cells through membrane-bound transport proteins [44]. In the central nervous system (CNS), activation of glutamate receptors have been shown to increase intracellular cGMP and to induce cGMP release in cultured cerebellar neurons or in cerebellar slices [30,33,51]. Several studies that investigated the effects of extracellular cGMP on neuronal cells showed direct inhibitory effects of cGMP on CNS neurons resulting in reduced excitability and inhibition of neurotransmitter release, suggesting a role for cGMP in processes modulating neuronal activity [12,30,40].

In this study, we investigated whether uroguanylin, through activation of the GC-C/cGMP pathway, elicits analgesic effects in models of gastrointestinal pain and whether these analgesic effects could be mimicked by exogenous cGMP, thus suggesting a novel role for the GC-C/cGMP pathway in modulating gastrointestinal sensory signaling.

2. Methods

2.1. Animals

Male and female Sprague-Dawley and Wistar rats, respectively, were obtained from Elevage Janvier S.A. (Le Genest St. Isle, France) to measure visceromotor (abdominal contractions) responses in rats to colorectal distension (CRD) in postinflammatory TNBS-induced visceral hypersensitivity and after partial restraint stress. For colonic afferent responses to colonic distension after TNBS-induced colitis, female Sprague-Dawley rats were obtained from Hilltop Lab Animals (Scottsdale, PA). All animal studies were approved by institutional committees based on approved guidelines: Animal Care and Use Committee of the Institut National de la Recherche Agronomique, University of Pittsburgh Institutional Animal Care and Use Committee in accordance with the standards set by the Animal Welfare Act and the National Institutes of Health (Guide for the Care and Use of Laboratory Animals), the Committee for Research and Ethical Issue of the IASP (1983) and the European Guidelines 2010/63/UE, and the Ironwood Pharmaceuticals Institutional Animal Care and Use Committee.

2.2. Reagents

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Probenecid, *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide, 8-bromo-cGMP, and cGMP were obtained from Sigma (St. Louis, MO) and acetylcholine (Ach) from MP Biomedicals (Santa Ana, CA). Human uroguanylin-A (uroguanylin) was obtained from American Peptide (Sunnyvale, CA).

2.3. Caco-2 cell differentiation

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mmol/L glutamine in a humidified atmosphere containing 5% CO_2 in air at 37°C for 21 days on 24-well collagen-coated insert plates (BD Biosciences, Bedford, MA). Caco-2 cell monolayer integrity was confirmed by measurement of transepithelial electrical resistance using an epithelial tissue volt-ohm meter (SYS-WVOM with STX2 electrode; World Precision Instruments Inc., Sarasota, FL), and monolayers with resistance values of >250 Ωcm^2 were considered completely confluent and used in the transport experiments. Furthermore, apical-to-basolateral permeability of mannitol was measured to monitor the integrity of tight junctions and was compared with the permeability of propranolol, a highly monolayer-permeable drug.

2.4. Measurement of intracellular and extracellular concentrations of cGMP

Confluent (day 21) Caco-2 cell monolayers were washed twice with prewarmed transport buffer (Hanks' balanced salt solution supplemented with 15 mmol/L glucose and 10 mmol/L *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0). Transport buffer containing either vehicle (1% dimethyl sulfoxide) or probenecid (0.5, 2 mmol/L) in vehicle was then added to both the apical and basolateral chambers and incubated for 15 minutes at 37°C. After incubation for 15 minutes, the transport buffer was aspirated, and the apical (volume: 0.3 mL) and basolateral chambers (volume: 1 mL) of triplicate wells were treated with vehicle, uroguanylin (1 μmol/L), or uroguanylin (1 μmol/L) plus 0.5 and 2 mmol/L of probenecid and incubated at 37°C for 60 minutes. After the 60-minute incubation period, 0.2-mL aliquots from both the apical and basolateral chambers were collected, and the concentration of cGMP in each chamber was determined by liquid chromatography coupled to mass spectrometry (LC/MS/MS) analysis. To determine the concentration of intracellular cGMP, the Caco-2 cells were lysed in 0.1 mL of 0.1 M hydrochloric acid at room temperature for 15 minutes, followed by centrifugation (3200 rpm, 15 minutes) to pellet cell debris. The supernatants (90 μL) were transferred to 96-well plates and neutralized (pH 7.0) with 90 μL of 1 mol/L ammonium acetate, and analyzed by LC/MS/MS. A cGMP standard curve (10 nmol/L–10 μmol/L) was prepared in transport buffer (pH 7.0) or 0.1 mol/L hydrochloric acid, neutralized with an equal volume of 1 mol/L ammonium acetate. The data were processed using LCquan 2.5.6 software (Thermo-Fisher Scientific, Pittsburgh, PA). Analyte peak areas were used to generate a $1/x^2$-weighted linear calibration curve, which was used to extrapolate the cGMP concentration in each sample.

2.5. Expression of GC-C, guanylin and uroguanylin, and cGMP transporters multidrug resistance proteins 4 and 5, and organic anion transporter 2 in Caco-2 cells and in rat tissue panel

Rat tissue total RNA and RNA from Caco-2 cells was extracted using Trizol (Life Technologies, Grand Island, NY) and further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were synthesized using a high-capacity cDNA reverse-transcription kit (Life Technologies). cDNA 500 ng was used for TaqMan quantitative real-time polymerase chain reaction gene expression analysis. Human and rat TaqMan cDNA probes specific for GC-C, guanylin, uroguanylin, multidrug-resistance proteins (MRP) 4 and 5, organic anion transporter (OAT) 2, and glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene, control) were obtained from Life Technologies. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression [31].

2.6. Preparation of rat peritoneal mast cells

Mast cells from Sprague-Dawley rats were obtained by peritoneal lavage of adult animals with 30 to 35 mL of Tyrode's buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L NaH_2PO_4,

1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 10 mmol/L HEPES, pH 7.2) containing 0.1% BSA (fatty acid–free bovine serum albumin). Purification of peritoneal mast cells was performed according to the method previously described by Lee et al. [27]. Briefly, peritoneal cells ($6–10 \times 10^7$ cells) were suspended in 2 mL of Tyrode's buffer layered on 4 mL of 22.5% (wt/vol) Histo-Denz density gradient medium (Sigma, St. Louis, MO), centrifuged at ambient temperature for 15 minutes at 400*g*. Mast cells sedimented at the bottom of the tube and were washed and suspended in 2 mL of Tyrode's buffer. After this protocol, mast cells represented 70% to 80% of the nucleated cells.

2.7. Mast cell degranulation assay

The release of β-hexosaminidase that parallels the release of histamine was measured as an index of degranulation [36]. Degranulation of rat peritoneal cells (2.5×10^4 cells/well in 96-well plates) was induced by stimulating these cells either with substance P (30 μmol/L), A23187 (1 μmol/L) or compound 48/80 (10 μg/mL) in the presence of either cGMP (1, 5, 10 mmol/L) or the cGMP degradation product 5'GMP (10 mmol/L) (triplicate wells). Incubations were conducted in Tyrode's buffer containing 0.1% BSA under gentle agitation for 45 minutes at 37°C.

Culture supernatants were collected and transferred to a 96-well plate. 100 μL of Triton X-100 solution (0.5%) was added to the cells to quantify the enzyme activity remaining in the cells. 50 μL of either supernatant or Triton X-100 cell extracts were transferred to a new 96-well plate and 100 μL of Tyrode's buffer was added to each well, followed by 50 μL substrate solution (1.3 mg/mL *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.04 mol/L sodium citrate, pH 4.5). The plate was incubated at 37°C for 120 minutes under gentle agitation. The reaction was stopped by adding 20 μL of 1 mol/L glycine adjusted to pH 10.0 with NaOH, and the absorbance at OD_{405nm} (Optical density) of each well was measured with a microplate reader. Mast cell degranulation (as a percentage) was calculated by the following formula: % degranulation = OD supernatant/(OD supernatant + OD pellet) × 100.

2.8. Acetylcholine and electric field stimulation of colonic tissues

Rat (Sprague-Dawley, male) colon tissue was divided into 2-cm segments and transferred to a Schuler Organ Bath. Colon segments were suspended in tissue bath vessels containing Krebs solution (117.9 mmol/L NaCl, 4.7 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.3 mmol/L NaH_2PO_4, 1.2 mmol/L $MgSO_4(H_2O)$, 2.5 mmol/L $CaCl_2$, 11.1 mmol/L D-glucose) continuously aerated with 95% O_2, 5% CO_2 and maintained at a temperature of 37°C. Tissue resting tension was 0.5 g for electrical field stimulation (EFS) studies and 1 g for ACh concentration-response curve studies. For EFS studies, colonic tissues were stimulated at a fixed frequency (8 Hz, 0.5-ms duration, 10 V, 3 trains of 10-second width, with a 2-minute delay between trains). Two control stimulations were performed, and tissues were allowed to recover for 10 minutes. After the recovery period, tissues were treated with vehicle (n = 10), uroguanylin (3 μmol/L) (n = 6), cGMP (1 mmol/L) (n = 12), or 8-bromo-cGMP (100 μmol/L) (n = 5) at a volume of 100 μL, and EFS at the fixed frequency was repeated. Contraction amplitude is expressed as the percentage of baseline amplitude of the second set of 8-Hz control stimulations. The amplitude of stimulations before treatment is considered 100% amplitude. Before measuring the effects of cGMP on ACh-stimulated contractions, an ACh concentration-response curve (n = 8, 10 nmol/L–100 μmol/L) was performed. After a washing step and tissue stabilization period, colon tissue segments were incubated with either vehicle (n = 6) or cGMP (10–100 μmol/L) (n = 6), and the ACh concentration-response curve was repeated. The maximum contraction during the pretreatment concentration response curve is considered the maximum (100%) response. Treatment with test article was initiated 5 minutes before EFS or pharmacological stimulation. Intracolonic instillation of TNBS, under isoflurane anesthesia, in male Sprague-Dawley rats was performed as described in the following.

2.9. Inflammation (TNBS)–induced colonic hypersensitivity

TNBS colitis in male Sprague-Dawley rats was induced as previously described [14]. Briefly, under anesthesia with ketamine (80 mg/kg, i.p.) and acepromazine (12 mg/kg, i.p.), rats received either TNBS (50 mg/kg) or vehicle (1% methylcellulose, 0.9% saline) administered in the proximal colon (1 cm from the cecum). Seven days after TNBS administration, the number of abdominal contractions was measured in response to CRD by progressively inflating a balloon (5 cm in length) inserted in the anus from 0 to 75 mm Hg, each step of inflation lasting 30 seconds. Colonic thresholds were determined by the pressure (mm Hg) required to elicit abdominal contractions. To evaluate the ability of uroguanylin and cGMP to modulate the hypersensitivity response, rats (n = 8 per group) were orally administered either uroguanylin (3, 10, 30, 100, 300 μg/kg), cGMP (0.3, 3, 30 mg/kg), morphine (1 mg/kg), or vehicle 30 minutes before CRD.

2.10. Myeloperoxidase activity in colon tissue

Colon tissue (100 mg wet weight, n = 8) was suspended in 1 mL of buffer (0.5% hexadecyltrimethylammonium bromide in 10 mmol/L phosphate buffer, pH 6.0) and homogenized using Qiagen Tissue Lyser II. Homogenates were freeze-thawed 3 times to disrupt the cells and were cleared by centrifugation at 13,000*g* at 4°C. Myeloperoxidase (MPO) activity in the supernatants was assayed by measuring the change in absorbance (460 nm) after 15 minutes resulting from the decomposition of H_2O_2 in the presence of *O*-dianisidine hydrochloride. The reactions (96-well format) were carried out with 90 μL of 10 mM phosphate buffer, containing 0.2 mg/mL of *O*-dianisidine hydrochloride and 0.0006% of H_2O_2, and started by the addition of 10 μL of tissue supernatant.

2.11. Acute partial restraint stress–induced colonic hypersensitivity

Partial restraint stress is a relatively mild, nonulcerogenic model of restraint stress [54]. Under general anesthesia induced by intraperitoneal administration of 0.6 mg/kg acepromazine (Calmivet; Vetoquinol, Lure, France) and 120 mg/kg ketamine (Imalgene 1000; Rhone Merieux, Lyon, France), 3 pairs of nichrome electrodes were each implanted in the striated muscles of the abdomen of female Wistar rats. The electrodes were exteriorized on the back of the neck and protected by a glass tube attached to the skin. Electromyography recordings were initiated 5 days after surgery. The electrical activity of the abdominal striated muscle was recorded with an electromyograph (Mini VIII; Alvar, Paris, France) using a short time constant (0.03 seconds) to remove low-frequency signals (<3 Hz) and a paper speed of 3.6 cm/min.

Briefly, rats were lightly anesthetized with ethyl ether, and their upper forelimbs and thoracic trunk were wrapped in a confining harness of paper tape to restrict but not prevent body movements and placed in their home cages for 2 hours. Control sham-stress animals were anesthetized but not wrapped in the confining harness. Colorectal distension was performed using a balloon inflated from 0 to 60 mm Hg using increments of 15 mm Hg for 5 minutes each. Rats were accustomed to polypropylene tunnel devices (diameter: 7 cm; length: 20 cm) during 5 days before the start of CRD procedures to minimize recording artifacts caused by movement of the animals. The balloon used for distension

(4 cm in length) was inserted in the rectum at 1 cm from the anus and fixed at the base of the tail. The balloon, connected to a barostat, was inflated progressively in increments of 5 minutes of 15 mm Hg each, ranging from 0 mm Hg to 60 mm Hg. Nonstressed rats received either vehicle (2% dimethyl sulfoxide/15% β-cyclodextrin in water) or cGMP (3 mg/kg) by gavage (n = 10 per group). For the stress protocol, rats received CRD directly before and 15 minutes after 2 hours of restraint stress. Treatment groups of rats (n = 8–12 per group) were administered either vehicle or cGMP (3 mg/kg) by gavage 1.25 hours after the start of restraint stress treatment, and CRD was performed 15 minutes after completion of the 2 hours of restraint stress.

2.12. Rat colonic mucosa transepithelial current and uroguanylin-induced basolateral cGMP secretion

Proximal colons (n = 9) were collected from male Sprague-Dawley rats, and the seromuscular layers were removed by blunt dissection. Colonic tissue was mounted on slides with the 0.5-cm^2 aperture. Three milliliters of Krebs bicarbonate Ringer solution containing (in mM) 115 NaCl, 15 $NaHCO_3$, 2.4 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 0.4 KH_2PO_4 at pH 7.4 was added to the apical and basolateral chambers of the Ussing chamber. The apical and basolateral chambers also contained mannitol (10 mM) and glucose (10 mM), respectively. The temperature in both chambers was maintained constant at 37°C, and Krebs bicarbonate Ringer solution was oxygenated throughout the experiment. Measurements of transepithelial short-circuit current (I_{sc}) were performed using an automatic voltage clamp (Model VCC MC8; Physiologic Instruments, San Diego, CA). At 0 minutes, 5 μL of vehicle (water) was added to the apical chamber, followed by uroguanylin (1 μM) at 60 minutes. Aliquots of 100 μL were collected from the serosal chamber every 15 minutes for analysis of secreted cGMP. Extracellular cGMP was measured using the cGMP Biotrak (GE Healthcare, Little Chalfont, UK) (Enzyme Immunoassay) System, and the rate of cGMP secretion per surface area of tissue R [fmol/(min × cm^2)] was calculated using the equation: R = dC × V/A (where dC is the rate of change in cGMP concentration, V is the volume of the Ussing chamber [3 mL], A is the area of the slider aperture [0.5 cm^2]).

2.13. Colitis-induced colonic afferent sensitization

To induce colitis, TNBS (30 mg per rat in vehicle [50% ethanol/water]) was instilled with the rats under anesthesia (4% isoflurane) via a transanal approach using a PE-90 catheter whose tip was placed approximately 6 cm proximal to the anus. As an added precaution, Surgilube (Fougera, Melville, NY) was applied to the perineum to minimize any potential contaminant irritation due to anal leakage. This model of colitis is characterized by local areas of acute inflammation peaking at 4 to 7 days, followed by a chronic, mononuclear inflammatory cell infiltrate that persists up to 6 weeks until it resolves without spontaneous relapse. For the measurement of colonic afferent responses, female Sprague-Dawley rats were divided into 2 groups. In the first group (n = 10), the responses of colonic afferents to colonic distension were measured in control animals before and after intraduodenal administration of cGMP (3 mg/kg in vehicle [2% dimethyl sulfoxide/15% β-cyclodextrin in distilled water]). In the second group (n = 11), the afferent responses were tested in animals 8 to 10 days after TNBS-induced colitis, before and 20 to 30 minutes after intraduodenal administration of cGMP (3 mg/kg).

2.14. In vivo physiological instrumentation for afferent recording studies

In vivo physiological instrumentation was performed with the rats under urethane anesthesia (1.2 g/kg, s.c.). A latex balloon (~10 mm wide and 30 mm long) was inserted through the anus so that the tip of the balloon was 6 cm proximal to the anal verge. The balloon was connected to a pressure transducer (World Precision Instruments, Sarasota, FL) and a syringe pump (Harvard Apparatus, Holliston, MA) via 3-way stopcocks for filling and continuous measurement of intracolonic pressure. A Transbridge transducer amplifier was used to amplify the signal from the pressure transducer, which was processed using a PowerLab 8s unit data acquisition system (AD Instruments, Mountain View, CA) connected to an Apple G5 computer [37,38].

2.15. Recording of nerve activity and identification of afferent endings

The right pelvic nerve was isolated at the major pelvic ganglion, dissected free from surrounding tissue, and cut at a maximal distance from the ganglion. The cut end, still contiguous with the colon, was positioned on a small platform and covered with mineral oil. Fine bundles were dissected and placed on 1 arm of a silver electrode, while a second arm was grounded. Impulses were amplified (Grass QP511; Grass, West Warwick, RI) and acquired with the PowerLab software as above and counted by a rate meter in 1-second intervals. The rate meter threshold was set to count potentials of desired amplitude. A bundle that had 1, or at most 2, easily distinguishable active units was used. The afferent firing rate was calculated as the average number of impulses per second over a 20-second period. Resting activity represented maximal activity recorded 1 minute before each intervention. Changes in afferent activity in response to interventions were expressed as the percentage of change from the resting firing rate. Only afferents that clearly responded to inflation of the intracolonic balloon were studied.

2.16. Mechanical testing of afferents

After identification of the sensory ending, the mechanical sensitivity of the afferent was tested by CRD with saline infusion to the maximal pressure of 60 mm Hg. Afterward, the balloon was immediately emptied and returned to a baseline pressure of 2 to 5 mm Hg. CRDs were repeated 2 to 3 times at 10- to 15-minute intervals to ensure the stability of the responses. Afferent firing during CRD was averaged over 10-mm Hg increments of intracolonic pressure.

2.17. Statistical analysis

The determination of statistical differences between groups was determined by 1-way analysis of variance, and differences between mean values were determined by Bonferroni correction post hoc for multiple *t* tests. Statistical analysis of the number of abdominal contractions obtained during 5-minute periods was performed by analysis of variance using Student *t* test for paired or unpaired values, as appropriate. A paired *t* test was used to compare the responses of the same group of afferents before and after cGMP administration. Unpaired *t* tests were performed to determine statistically significant differences in cGMP assays. All data are expressed as the mean ± SEM. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Uroguanylin reduces inflammation (TNBS)-induced colonic hypersensitivity

The pharmacological effects of the synthetic GC-C peptide agonist linaclotide in significantly reducing colonic hyperalgesia in models of inflammation- and stress-induced visceral

hypersensitivity [16] prompted us to investigate whether the endogenous hormone uroguanylin elicits similar effects on colonic hyperalgesia in a rat TNBS inflammation model. Oral administration of uroguanylin at doses of 30, 100, and 300 μg/kg before CRD resulted in dose-dependent, significantly ($P < 0.01$, 100 and 300 μg/kg) increased colonic thresholds required to elicit abdominal contractions compared with TNBS vehicle–treated animals (Fig. 1). In addition, the colonic thresholds at the 100 and 300-μg/kg dose levels were not significantly different from those of naive animals (Fig. 1). Increased colonic thresholds were also observed after orally administered uroguanylin at doses of 3 and 10 μg/kg, but were not statistically significant (data not shown). Animals treated with a subcutaneous dose of morphine (1 mg/kg), were used as positive controls, as previously described [14] (Fig. 1). MPO activity as a marker of tissue inflammation was significantly ($P < 0.05$) increased only in the proximal colon (site of TNBS instillation), but not in the noninflamed distal colon ($P = 0.03$ between vehicle groups) (Fig. 2). Orally administered uroguanylin at all dose levels elicited no significant effects on MPO activity either in the noninflamed distal colon or under inflammatory conditions in the proximal colon when measured 3 hours after dosing (Fig. 2).

3.2. Expression of GC-C, guanylin, and uroguanylin in rat small and large intestine

GC-C is predominantly expressed in the intestine, and activation of GC-C accounts for the primary source of cGMP in intestinal epithelial cells [9,21,29,39,47]. In the rat intestinal tract, high levels of GC-C mRNA expression were detected in all regions, especially in the jejunum, cecum, and ascending and transverse colon (Supplemental Fig. 1A). Similarly, expression of guanylin and uroguanylin was detected in all regions of the rat intestine (Supplemental Figs. 1B and 1C). In TNBS-treated rats, GC-C expression was decreased in the proximal colon (site of TNBS administration) compared with the distal colon. Uroguanylin at all dose levels had no effect on GC-C expression in either region of the colon (data not shown). We further investigated GC-C expression in a broad panel of nonintestinal tissues including dorsal root ganglia neurons in naive rats. No detectable levels of GC-C, guanylin, and uroguanylin expression were found in most nonintestinal tissues, including dorsal root ganglion neurons (Supplemental Figs. 1A–C).

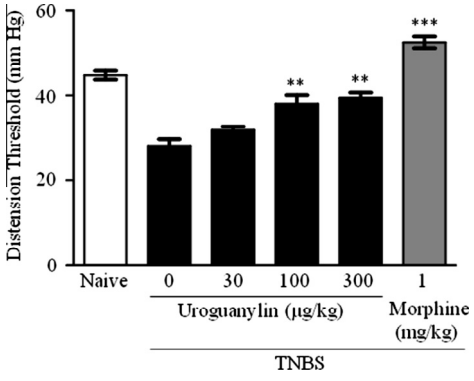

Fig. 1. Effects of uroguanylin on inflammation-induced colonic hypersensitivity in rats. Inflammatory colitis in rats (n = 8 per group) was induced by intracolonic instillation of trinitrobenzene sulfonic acid (TNBS) (50 mg/kg), and colonic thresholds required to elicit abdominal contractions were determined in response to graded colorectal distension 7 days after TNBS treatment. Uroguanylin (30, 100, 300 μg/kg) and vehicle were orally administered 30 minutes before colorectal distension. Data are expressed as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated animals (1-way analysis of variance, followed by Bonferroni correction post hoc for multiple *t* tests).

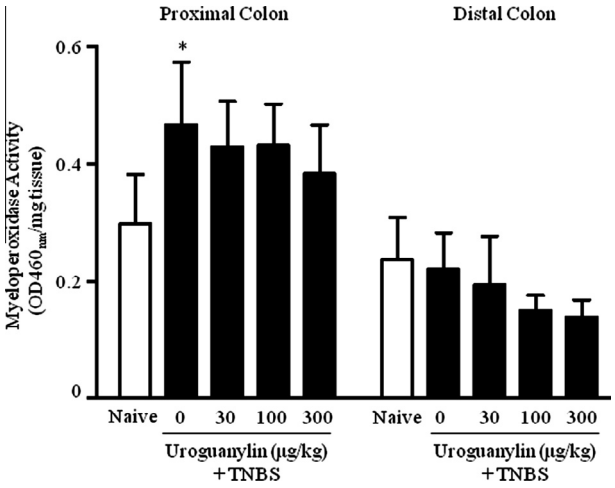

Fig. 2. Effects of uroguanylin on myeloperoxidase (MPO) activity in the inflamed rat proximal colon and in the noninflamed distal colon. Colonic tissues were homogenized, and MPO activity in cleared supernatants was measured after 15 minutes by the change in absorbance (460 nm) resulting from the decomposition of H_2O_2 in the presence of 0.2 mg/mL *O*-dianisidine hydrochloride. MPO activity is expressed in units per milligram of colon tissue. All data are expressed as the mean ± SEM (n = 8). $^{*}P = 0.03$ versus vehicle-treated group, distal colon.

3.3. Effects of uroguanylin on intracellular accumulation of cGMP and on apical and basolateral transport of cGMP from Caco-2 cells

Ligand binding of GC-C agonists stimulates the intrinsic guanylate cyclase activity of the receptor, resulting in the conversion of guanosine-5′-triphosphate to cGMP and increased intracellular concentrations of this signaling molecule [21]. As shown in Fig. 3A, exposure of human colorectal adenocarcinoma Caco-2 cells, a commonly used model system for intestinal epithelium [45] that express GC-C (data not shown) [52], to the endogenous hormone uroguanylin increased intracellular concentrations of cGMP. Incubation of Caco-2 cells with uroguanylin in the presence of probenecid, a pharmacological inhibitor of the cGMP transporters MRP4 and MRP5 and the solute carrier (SLC) family member OAT2, did not affect intracellular cGMP concentrations (Fig. 3A).

We then investigated whether increased intracellular cGMP concentrations in Caco-2 cells after uroguanylin activation of GC-C are associated with extracellular cGMP transport into the apical and basolateral compartments. As shown in Fig. 3B and C, exposure of Caco-2 cells to uroguanylin resulted in the concomitant transport of cGMP into the apical and basolateral compartments, respectively, with apical cGMP concentrations 7- to 8-fold increased over basolateral concentrations. Both apical and basolateral export of cGMP was significantly inhibited in the presence of probenecid in a concentration-dependent manner (Fig. 3B and C), indicative of active cGMP transport out of Caco-2 cells. To further confirm the involvement of active cGMP transport processes, unstimulated Caco-2 cells were incubated with either 200 nM or 1 μM cGMP added to the apical compartment, in the presence or absence of probenecid. Under those conditions, no measurable amounts of cGMP were found in the basolateral compartment (data not shown).

3.4. Expression of MRP4, MRP5, and OAT2 transporters in Caco-2 cells and in the rat small and large intestine

Previous studies investigating MRP4, MRP5, and OAT2 expression in Caco-2 cells and in different regions of the human intestine found either low or nondetectable levels of these transporters [20,32,41,48,53]. We confirm these findings, but show that their

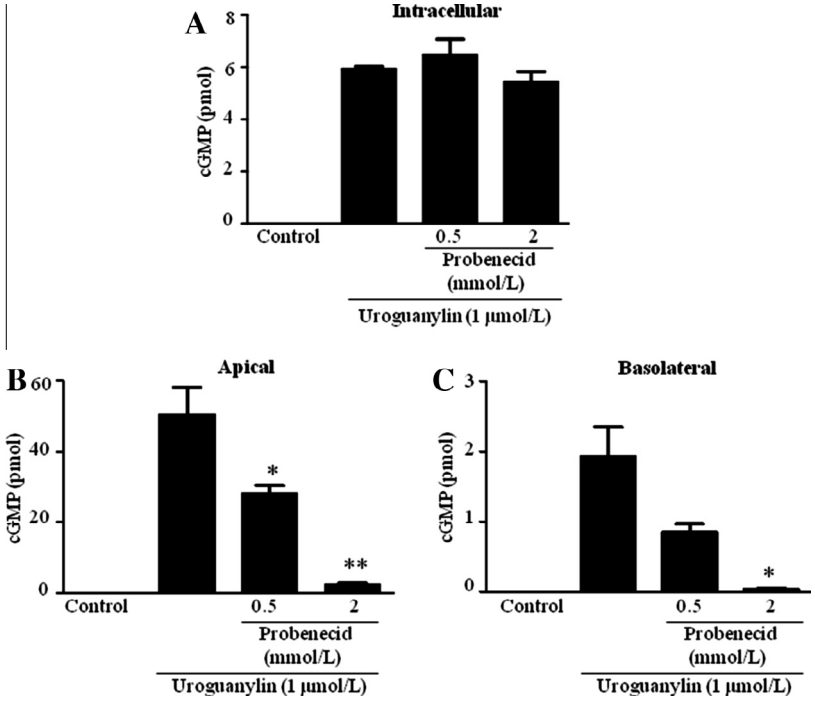

Fig. 3. Uroguanylin activation of guanylate cyclase-C on Caco-2 cells: effects on intracellular cyclic guanosine-3′,5′-monophosphate (cGMP) production and on apical and basolateral cGMP transport. Confluent Caco-2 cell monolayers (day 21), confirmed by transepithelial electric resistance measurement, were preincubated with probenecid (0.5 and 2 mmol/L) for 15 minutes, followed by incubation either with vehicle, uroguanylin (1 µmol/L), or uroguanylin (1 µmol/L) plus probenecid (0.5 and 2 mmol/L) for 60 minutes (n = 3). Intracellular cGMP concentrations (A) and cGMP concentrations in the apical (B) and basolateral compartments (C) were determined by liquid chromatography coupled to mass spectrometry (LC/MS/MS) analysis, derived from a cGMP standard curve. The amount of cGMP indicates the total amount of cGMP recovered from each compartment. The limit of quantitation in this assay is 0.05 pmol/mL. Data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus uroguanylin-treated cells.

relative expression levels in Caco-2 cells exhibit a distinct rank order: MRP4 > MRP5 > OAT2 (Supplemental Fig. 2A, insert). To explore whether similar mechanisms of cGMP transport could occur in vivo in the rat intestinal tract, we measured the expression of MRP4, MRP5, and OAT2 in segments of the rat intestine ranging from the duodenum to the rectum. In general, we found only low levels of MRP4 expression (Supplemental Fig. 2A), whereas MRP5 expression levels were markedly elevated in the colon compared with MRP4 (Supplemental Fig. 2B). Similarly, expression of MRP4 and MRP5 was found in mucosal tissue extruded from each region of the intestine (data not shown). OAT2 expression was generally below detectable threshold levels (data not shown). The rank order of cGMP transporter expression in the rat intestine (MRP5 > MRP4 > OAT2) was different from that of Caco-2 cells. In TNBS-treated rats, MRP4 and MRP5 expression levels were neither altered in the inflamed proximal colon nor in the noninflamed distal colon. Similarly, uroguanylin at all dose levels had no effect on MRP4 and MRP5 expression in either region of the colon (data not shown).

3.5. cGMP reduces inflammation (TNBS)-induced colonic hypersensitivity

We then asked whether the effects of uroguanylin in the TNBS-induced model of colonic hypersensitivity to distension could be mimicked by the primary downstream effector of uroguanylin, cGMP. Oral administration of cGMP at doses of 0.3, 3, and 30 mg/kg 30 minutes before CRD significantly ($P < 0.01$, 3 mg/kg; $P < 0.001$, 30 mg/kg) and dose dependently increased colonic thresholds in this model compared with vehicle-treated animals (Fig. 4). In addition, the colonic threshold after an oral dose of 30 mg/kg cGMP was not significantly different from that of naive animals (Fig. 4). Animals treated with a subcutaneous dose of morphine (1 mg/kg) were used as positive controls, as previously described [14] (Fig. 4).

3.6. cGMP reduces acute restraint stress–induced colonic hypersensitivity

We then assessed whether cGMP also elicits analgesic effects in a mechanistically different model of colonic hypersensitivity, using a nonulcerogenic mild restraint stress (wrap restraint) model. Oral

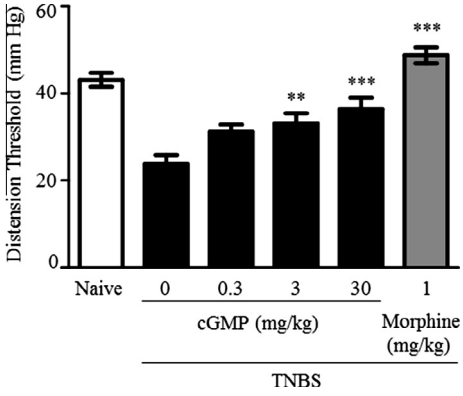

Fig. 4. Effects of cyclic guanosine-3′,5′-monophosphate (cGMP) on inflammation-induced colonic hypersensitivity in rats. Inflammatory colitis in rats (n = 7–8 per group) was induced by intracolonic instillation of trinitrobenzene sulfonic acid (TNBS) (50 mg/kg), and colonic thresholds required to elicit abdominal contractions were determined in response to graded colorectal distension 7 days after TNBS treatment. cGMP (0.3, 3, 30 mg/kg) and vehicle were orally administered 30 minutes before colorectal distension. Data are expressed as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated animals (1-way analysis of variance, followed by Bonferroni correction post hoc for multiple *t* tests).

administration of cGMP (3 mg/kg) to male Wistar rats, a strain with high stress responsiveness, had no significant effect on colorectal sensitivity to distension under basal conditions (sham stress) when compared with vehicle-treated animals (data not shown). After 2 hours of restraint stress, the number of abdominal contractions in vehicle-treated rats was significantly ($P < 0.05$) increased at all distension pressures tested compared with sham-stressed animals (Fig. 5). Orally administered cGMP (3 mg/kg) significantly ($P < 0.05$) reduced stress-induced visceral hypersensitivity at all distension pressures tested, without affecting colonic volumes (Fig. 5 and data not shown).

3.7. cGMP decreases colonic afferent firing rates in response to colonic distension in rats

Because visceral afferent sensitization is thought to play a key role in IBS symptoms of abdominal pain and discomfort, we assessed the analgesic effects of cGMP in a model of TNBS-induced colonic afferent sensitization, using single-unit colonic afferent recordings. Before colonic irritation, incremental colonic distension led to incremental increases in colonic afferent firing. cGMP, administered intraduodenally at a dose of 3 mg/kg, had no significant effect on afferent firing rates during colonic distension in control animals at intracolonic pressures of 10 to 50 mm Hg, but significantly decreased afferent firing rates (50%) at 60 mm Hg (noxious range) ($P < 0.05$). In the 40- to 50-mm Hg distension range (suprathreshold, subnoxious), a nonsignificant downward trend in afferent firing was observed in the cGMP-treated animals (data not shown). In animals with subacute colitis, 8 to 10 days after intrarectal instillation of TNBS, incremental CRD led to significant incremental increases in colonic afferent firing rates. cGMP, intraduodenally administered at a dose of 3 mg/kg, significantly ($P < 0.05$) decreased colonic afferent firing rates in response to colonic distension at distension pressures ranging from 30 to 60 mm Hg (pressure range of C-fiber afferents) compared with their responses to distension before cGMP administration (Fig. 6).

3.8. Uroguanylin stimulates cGMP secretion from the basolateral surface of rat colonic mucosa

Because exogenous cGMP significantly decreased colonic afferent firing rates in response to colonic distension, we investigated whether uroguanylin, through activation of the GC-C/cGMP pathway could stimulate the secretion of cGMP into the rat colon submucosa, using an ex vivo Ussing chamber assay. Ion transport and epithelial barrier function were monitored by measuring I_{sc} and transepithelial electrical resistance. Uroguanylin stimulation (1 μM) elicited a robust I_{sc} across rat colonic epithelium (Fig. 7A), which reached a maximum within 10 minutes after stimulation and remained constant until the end of the study. The secretion of cGMP into the chamber exposed to the basolateral side of the colonic epithelium was measured before and after the addition of uroguanylin. In this assay, the basal rate of cGMP secretion was 5.46 fmol/min × cm^2. Exposure of rat colonic mucosa to uroguanylin resulted in an approximately 6-fold increased cGMP secretion from the basolateral side of the epithelium, with a calculated rate of cGMP secretion of 32.34 fmol/min × cm^2 (Fig. 7B). The transepithelial electrical resistance of the colonic mucosa remained high during the course of the study, indicating that the diffusion barrier for cGMP between apical and basolateral sides remained intact throughout the study (data not shown).

3.9. cGMP has no effect on rat peritoneal mast cell degranulation

Intestinal mucosal mast cells are located in close proximity to afferent fibers in the mucosa and have been implicated in the pathophysiology of IBS [1,2]. We used rat peritoneal mast cells to further investigate whether the pharmacological effects of cGMP, observed in vivo in a rat model of postinflammatory visceral hyperalgesia, are linked to a mechanism associated with the inhibition of mast cell degranulation. Measurement of β-hexoaminidase release, a marker of mast cell degranulation, after incubation of peritoneal mast cells with substance P, the calcium ionophore A23187, or compound 48/80 confirmed the ability of these agents to potently stimulate their degranulation (Supplemental Figs. 3A–C). When rat peritoneal mast cells were stimulated with these agents in the presence of increasing concentrations of cGMP (1–10 mmol/L), no effects on mast cell degranulation were observed (Supplemental Figs. 3A–C). Furthermore, cGMP alone did not modulate degranulation of vehicle-treated mast cells. Similarly, the cGMP degradation product 5′-GMP (10 mmol/L) had no effect on either substance P–, A-23187-, or compound 48/80–stimulated mast cell degranulation, nor did 5′-GMP modulate degranulation of vehicle-treated mast cells (data not shown).

3.10. cGMP has no effect on EFS and ACh-induced contractions of rat colonic tissue

Next, we investigated whether the antihyperalgesic effects of cGMP in vivo in rat models of after inflammatory and stress-induced

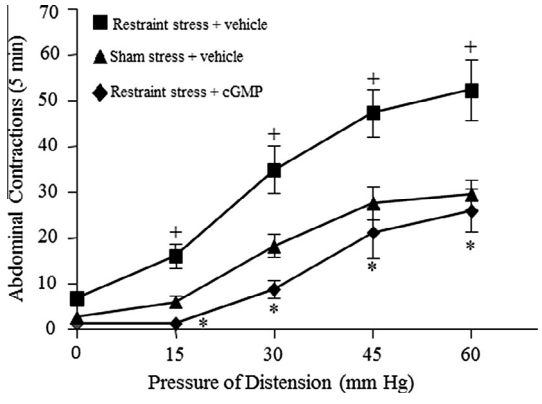

Fig. 5. Effects of cyclic guanosine-3′,5′-monophosphate (cGMP) on stress-induced colonic hypersensitivity in rats. The animals (n = 9–10 per group) were subjected for 2 hours to partial restraint stress and received either orally dosed vehicle (n = 12) or cGMP (3 mg/kg) (n = 8) 1.25 hours after the initiation of restraint stress. Sham-stressed rats were treated orally with vehicle (n = 12). Abdominal contractions in response to graded colorectal distension were determined 15 minutes after completion of 2 hours of restraint stress treatment. Data are expressed as the mean ± SEM. *$P < 0.05$ versus vehicle-treated animals, +$P < 0.05$ versus sham stress-treated animals.

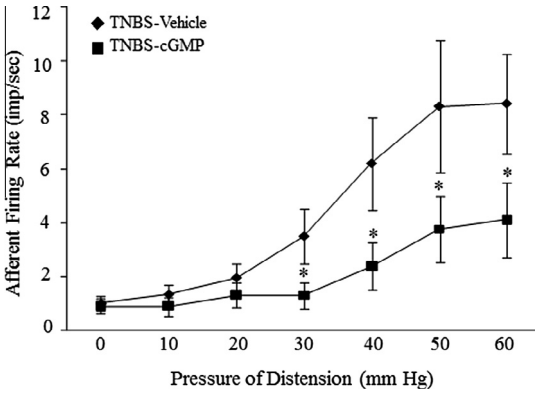

Fig. 6. Effects of cyclic guanosine-3′,5′-monophosphate (cGMP) on colonic afferent firing rates in trinitrobenzene sulfonic acid (TNBS)–sensitized rats. Colonic single-unit afferent responses to graded colorectal distension in rats 10 days after intracolonic instillation of TNBS (30 mg per rat), treated intraduodenally either with vehicle (n = 11) or cGMP (3 mg/kg) (n = 11). Data are expressed as the mean ± SEM. *$P < 0.05$ versus vehicle-treated animals.

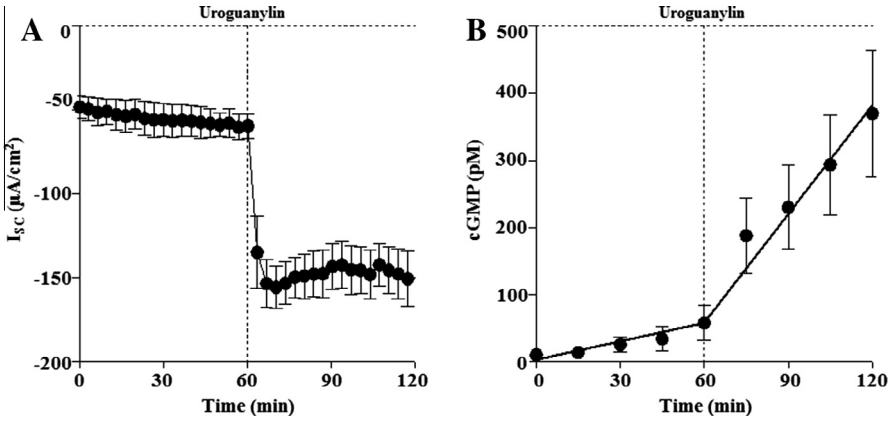

Fig. 7. Effects of uroguanylin on transepithelial short-circuit current and cyclic guanosine-3′,5′-monophosphate (cGMP) secretion from the basolateral side of rat colonic mucosa. Vehicle and uroguanylin (1 μM) were added to the apical chamber of the Ussing chamber at time 0 and 60 minutes, respectively. Treatment with uroguanylin elicited a robust transepithelial short-circuit current (A) and increased secretion of cGMP from the basolateral side of the rat colonic mucosa (B). Data (n = 9) are presented as mean ± SEM.

visceral pain are mechanistically associated with altered colonic smooth muscle contractility. In vitro, neither cGMP (1 mmol/L) nor uroguanylin (3 μmol/L) attenuated EFS-induced contractions of rat colonic tissue (Fig. 8A); in contrast, membrane-permeable 8-bromo-cGMP (100 μmol/L) significantly ($P < 0.01$) reduced EFS-induced contractions of colonic tissue (Fig. 8A). The EC_{50} for ACh-induced colonic contractions in the presence of 100 μmol/L cGMP (550 nmol/L) were similar to the EC_{50} for vehicle (340 nmol/L), indicating no functionally relevant difference (Fig. 8B). We then assessed the potential effects of uroguanylin on smooth muscle contractility in inflamed rat colonic segments, 7 days after intracolonic instillation of TNBS. Similar to colonic tissue in naive rats, uroguanylin (3 μmol/L) did not attenuate EFS-induced contractions in inflamed colonic tissue (Supplemental Fig. 4). 8-Bromo-cGMP (100 μmol/L) significantly reduced EFS-induced contractions ($P < 0.05$); however, this effect was markedly less pronounced than the significant decrease ($P < 0.001$) in EFS-induced contractions observed in colonic tissue from naive rats (Supplemental Fig. 4).

4. Discussion

We provide evidence that activation of intestinal GC-C by uroguanylin reduces colonic hypersensitivity and, importantly, that these analgesic effects of uroguanylin are mimicked by cGMP, the primary GC-C downstream effector in several models of colonic hypersensitivity. These data reveal a novel, previously unrecognized mechanism linking the GC-C/cGMP pathway to the modulation of gastrointestinal pain (Fig. 9).

The function of uroguanylin as an intestinal secretagogue via activation of the GC-C/cGMP pathway in intestinal epithelial cells has been firmly established [15,23]. Thus, the GC-C/cGMP pathway is considered the principal regulator of intestinal fluid homeostasis. Additionally, GC-C signaling plays important roles in the restoration of mucosal barrier function in intestinal disorders and homeostatic control of the intestinal crypt-villus axis [18,28]. Moreover, recent findings that linaclotide, a selective GC-C agonist peptide acting locally within the gastrointestinal tract, decreased colonic hypersensitivity in models of inflammatory and stress-induced visceral hypersensitivity, has for the first time implicated GC-C in regulation of visceral pain [16]. Furthermore, in 2 phase 3 clinical trials, linaclotide significantly improved abdominal pain in patients with IBS-C (13,42). These findings prompted us to further investigate whether the modulation of colonic hypersensitivity after uroguanylin activation of GC-C in the gastrointestinal tract may have evolved as a function not previously linked to the GC-C/cGMP pathway and whether these effects of uroguanylin are mechanistically linked to the primary GC-C downstream effector, cGMP.

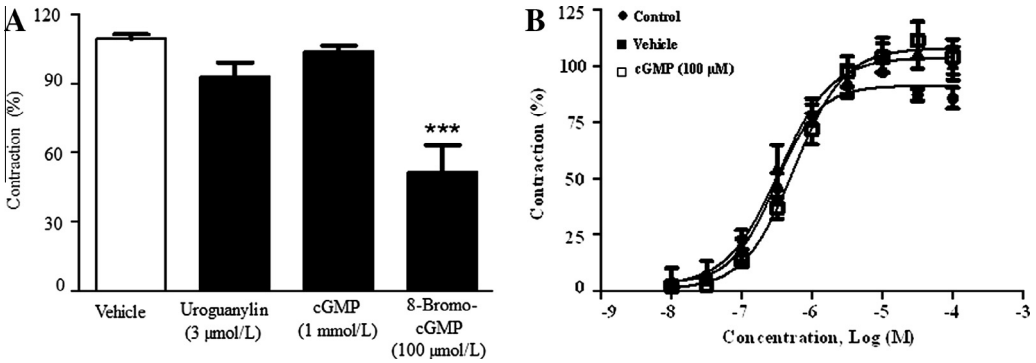

Fig. 8. Effects of cyclic guanosine-3′,5′-monophosphate (cGMP) on electric field stimulation (EFS)– and acetylcholine-induced contractions of rat colonic tissues. Rat colonic tissue segments (2 cm) were suspended in tissue bath vessels containing Krebs solution and continuously aerated with 95% O_2, 5% CO_2 at 37°C. (A) Colonic tissue contractions were induced by electric field EFS at a fixed frequency (8 Hz, 0.5 ms, 10 V, 3 trains of 10-second width, with a 2-minute delay between trains). After a 10-minute recovery after 2 control stimulations, colonic tissues were incubated with vehicle (n = 10), cGMP (1 mmol/L) (n = 12), uroguanylin (3 μmol/L) (n = 6), or 8-bromo-cGMP (100 μmol/L) (n = 5) for 5 minutes before EFS at the fixed frequency. Contraction amplitude is expressed as the percentage of baseline amplitude of the second set of 8-Hz control stimulations. (B) After stabilization after a control acetylcholine (ACh) concentration-response curve (10 nmol/L–100 μmol/L) (n = 8), colonic tissues were incubated for 5 minutes either with vehicle (n = 6) or cGMP (100 μmol/L) (n = 6) before repeating the ACh concentration-response curve. Maximum contraction during the pretreatment concentration response curve is considered the maximum (100%) response. Data are expressed as the mean ± SEM. ***$P < 0.001$.

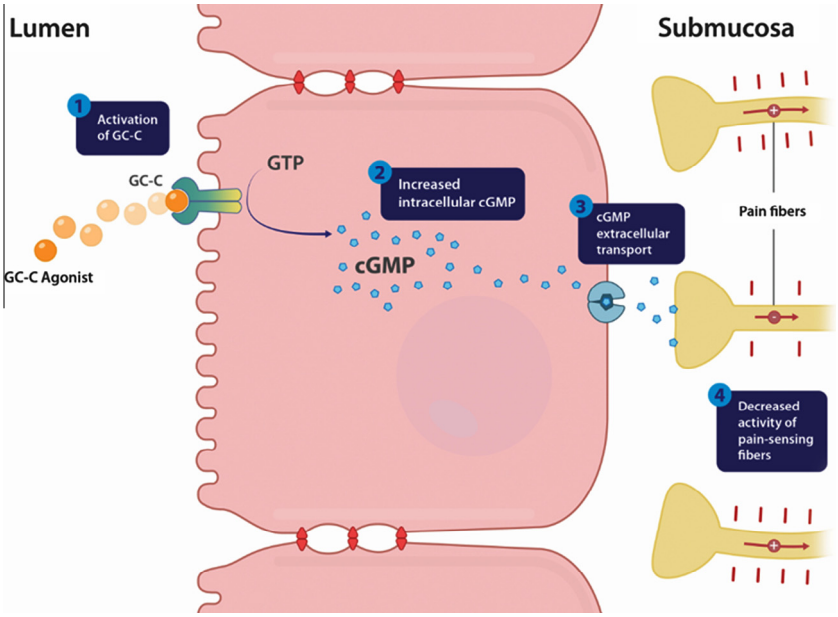

Fig. 9. Proposed mechanism of action of guanylate cyclase-C (GC-C) agonists, through activation of the GC-C/cyclic guanosine-3′,5′-monophosphate (cGMP) pathway, as gastrointestinal analgesics. 1. GC-C agonists bind and activate GC-C at the apical surface of intestinal epithelial cells, the location of GC-C expression. 2. Activation of GC-C results in hydrolysis of guanosine triphosphate (GTP) and production of cGMP inside the epithelial cells. 3. Intracellular cGMP is actively transported out of epithelial cells by efflux pumps into the submucosa. 4. Extracellular cGMP is proposed to inhibit colonic nociceptors.

First, we examined the ability of uroguanylin to stimulate extracellular transport of cGMP. Exposure of human Caco-2 cells to uroguanylin led to increased cGMP concentrations in the apical and basolateral compartments, with higher cGMP concentrations found on the apical side. Because cell membranes are virtually impermeable to cyclic nucleotides such as cGMP, requiring energy-dependent transport, we measured the expression of known cGMP transporters in Caco-2 cells and in a panel of rat intestinal tissues. Consistent with previous reports, we found that expression of MRP4 in Caco-2 cells, a transporter localized predominantly in the apical membrane, is higher than that of MRP5, which is localized predominantly in the basolateral membrane. Together, these findings most likely account for the observed differences in apical and basolateral cGMP concentrations. Incubation of Caco-2 cells with uroguanylin in the presence of probenecid inhibited cGMP export in a concentration-dependent fashion, confirming the involvement of active transport mechanisms in this process. In contrast to Caco-2 cells, we found greater expression of MRP5 compared with MRP4 in all regions of the rat small and large bowel, suggesting increased cGMP transport in vivo into the submucosal space. The expression of MRP4 and MRP5 was generally greater in the colon compared with the small intestine, a pattern similar to that observed in the human intestine [55]. When we measured intracellular cGMP concentrations in Caco-2 cells after exposure to uroguanylin in the presence of probenecid, we detected no significant elevation of cGMP in these cells, as previously reported [53]. The activity of cGMP-binding phosphodiesterases is considered the major cGMP elimination pathway controlling cellular cGMP levels. However, MRP4 and MRP5 have been characterized as overflow pumps, supporting the decrease in intracellular cGMP levels under conditions in which cGMP synthesis is strongly induced and, importantly, providing extracellular cyclic nucleotides for possible paracrine actions [43,55].

The synthetic GC-C agonist linaclotide is an orally administered, minimally absorbed peptide eliciting its effects locally in the gastrointestinal tract, and is closely related to the cGMP-regulating hormone uroguanylin [7,8]. Linaclotide potently decreases colonic hypersensitivity in several models of visceral pain in a GC-C-dependent manner, leading us to investigate the effects of an endogenous GC-C agonist, uroguanylin, on modulating gastrointestinal pain [16]. In a model of inflammation-induced colonic hypersensitivity, orally administered uroguanylin decreased colonic hyperalgesia, as evidenced by increased colonic thresholds required to elicit abdominal muscle contractions during CRD, suggesting that the modulation of colonic sensation may have evolved as a previously unrecognized effect of GC-C agonism in intestinal epithelial cells. Although the role of cGMP and its mechanism in the regulation of intestinal fluid homeostasis is firmly established, no role of non-neuronal cGMP in modulating gastrointestinal pain has been previously reported. Thus, we investigated whether the analgesic effects of uroguanylin could be mimicked by the GC-C downstream effector cGMP. In several mechanistically different models of gastrointestinal pain, orally administered cGMP significantly decreased visceral hypersensitivity. Together, these results have uncovered a novel role of an endogenous GC-C agonist, uroguanylin, in the peripheral modulation of gastrointestinal sensation, effects mimicked in vivo by cGMP and providing a strong rationale for targeting the GC-C/cGMP pathway for treatment of functional gastrointestinal disorders characterized by abdominal pain, such as IBS-C.

Although the pathophysiology of IBS is not completely understood, immunological, psychological, genetic, microbial, visceral nociceptive, motility, and mast cell factors may all be involved [1,2,19,24]. When we studied the effects of uroguanylin and cGMP on colonic contractility in naive and TNBS-treated rats in response to ACh or EFS as a potential mechanism mediating inhibition of colonic visceral pain, we did not observe any effect on the GC-C/cGMP pathway, indicating that this pathway neither affects colonic contractility nor has spasmolytic activity.

Several lines of evidence from both animal models and clinical studies appear to support a mechanistic role of colonic mast cells in the pathogenesis of visceral hypersensitivity and generation of abdominal discomfort and pain associated with IBS [1,2,26,34,35]. However, their role in this condition remains controversial [5,50]. When we investigated the pharmacological effects of cGMP on modulating the release of mast cell–specific mediators from rat

peritoneal mast cells stimulated with substance P, the calcium ionophore A23187, or compound 40/80, we found that cGMP had no effect on mast cell degranulation, consistent with previously reported data that the cyclic nucleotides cGMP and cyclic adenosine monophosphate did not affect the release of histamine from activated rat mast cells [49]. These findings suggest that the cGMP-mediated decrease of colonic hypersensitivity observed in our animal models of gastrointestinal pain is not directly associated with modulating the release of mast cell mediators.

Visceral sensory afferents modulate their firing properties in response to the local environment in both normal and pathological conditions. During disease states such as chronic visceral pain, chronic changes in afferent pathways (sensitization) lead to persistently enhanced perceptions of noxious stimuli responses to normally non-noxious stimuli. Because second-order neurons in the CNS have been shown to receive convergent input from different visceral organs, it has been suggested that this mechanism underlies viscerosomatic and viscerovisceral (cross-organ) referral and sensitization [37,38]. Such sensitization pathway changes are considered a central process in the development of chronic visceral pain, and such changes can be measured in primary colonic afferent nerve terminals (peripheral sensitization). The development of altered mechano- and chemosensitive properties of primary colonic afferents is likely a critical step in the transmission of painful stimuli to the CNS. Thus, primary colonic afferents are potential targets for visceral pain because they directly link the gastrointestinal tract to the CNS. Several ion channels and receptors expressed on terminals of visceral primary afferent neurons are currently under investigation as potential targets for visceral pain therapy [3,4,6]. Because cGMP elicits potent analgesic effects in several models of gastrointestinal pain, we hypothesize that cGMP may act on intestinal afferent fibers while traveling along the small intestine, the projections of which converge on second-order neurons that also receive input from colonic afferents, thus affecting their function. This is consistent with our findings that cGMP concentrations needed to potentially modulate afferent nerve firing are present in the extracellular mucosal space of the duodenum, jejunum, and ileum (Supplemental Table 1). In addition, we investigated whether in a TNBS model of experimental colitis, cGMP affects colonic afferent sensitization and found that cGMP significantly decreased the firing rates of sensitized pelvic afferent neurons in response to mechanical stimuli. Moreover, we recently showed, using an in vitro preparation, that both cGMP and uroguanylin decrease the mechanosensitivity of high-threshold colonic afferent from both splanchnic and pelvic nerves [10,11], as well as the responses of control and sensitized muscular and muscular-mucosal afferents to stretch (Feng et al, accepted for publication). In addition, using ex vivo Ussing chamber assays, uroguanylin stimulates cGMP secretion in the colonic submucosa. These results uncover a previously undescribed function of cGMP in modulating gastrointestinal pain and further provide a strong rationale for targeting the GC-C/cGMP pathway for the treatment of functional gastrointestinal disorders characterized by symptoms of abdominal pain, such as IBS-C.

4.1. Conclusions

These studies have demonstrated that uroguanylin activation of the GC-C/cGMP pathway increases extracellular transport of cGMP and significantly decreases colonic hypersensitivity. The analgesic effects of uroguanylin are mimicked in vivo by its primary downstream effector cGMP, suggesting that cGMP is the primary mediator in this process and that regulation of visceral sensory signaling has evolved as a physiological function not previously linked to the GC-C/cGMP pathway. Moreover, the translational concept of therapeutically targeting the GC-C/cGMP pathway for the treatment of gastrointestinal pain has now been validated in the clinic. Phase 2b and 3 trials conducted in IBS-C patients have confirmed that linaclotide improves the defining symptoms of this disease—abdominal pain and discomfort in addition to constipation symptoms [13,22,42].

Conflict of interest statement

The authors declare no conflicts of interest pertaining to this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.05.044.

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