Cortagine, a CRF₁ agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways

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Submitted 25 February 2009; accepted in final form 28 April 2009

Larauche M, Gourcerol G, Wang L, Pambukchian K, Brunnhuber S, Adelson DW, Rivier J, Million M, Taché Y. Cortagine, a CRF₁ agonist, induces stresslike alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. Am J Physiol Gastrointest Liver Physiol 297: G215–G227, 2009. First published April 30, 2009; doi:10.1152/ajpgi.00072.2009.—Corticotropin-releasing factor (CRF) 1 receptor (CRF₁) activation in the brain is a core pathway orchestrating the stress response. Anatomical data also support the existence of CRF signaling components within the colon. We investigated the colonic response to intraperitoneal (ip) injection of cortagine, a newly developed selective CRF₁ peptide agonist. Colonic motor function and visceral motor response (VMR) were monitored by using a modified miniaturized pressure transducer catheter in adult conscious male Sprague-Dawley rats and C57Bl/6 mice. Colonic permeability was monitored by the Evans blue method and myenteric neurons activation by Fos immunohistochemistry. Compared with vehicle, cortagine (10 μg/kg ip) significantly decreased the distal colonic transit time by 45% without affecting gastric transit, increased distal and transverse colonic contractility by 35.6 and 66.2%, respectively, and induced a 7.1-fold increase in defecation and watery diarrhea in 50% of rats during the first hour postinjection whereas intracerebroventricular (icv) cortagine (3 μg/rat) had lesser effects. Intraperitoneal (ip) cortagine also increased colonic permeability, activated proximal and distal colonic myenteric neurons, and induced visceral hypersensitivity to a second set of phasic colorectal distention (CRD). The CRF antagonist astressin (10 μg/kg ip) abolished ip cortagine-induced hyperalgesia whereas injected icv it had no effect. In mice, cortagine (30 μg/kg ip) stimulated defecation by 7.8-fold, induced 60% incidence of diarrhea, and increased VMR to CRD. Stresslike colonic alterations induced by ip cortagine in rats and mice through restricted activation of peripheral CRF₁ receptors support a role for peripheral CRF₁ signaling as the local arm of the colonic response to stress.

CRF₁ agonist; colonic motility; visceral pain; rat; mice; colonic permeability; astressin; CP-154,526

CORTICOTROPIN RELEASED FACTOR (CRF) acting on CRF₁ receptors in the brain is well released to play an important role in stress-related neuroendocrine, behavioral, autonomic, immunological, and visceral responses (3). In particular, convergent studies showed that central injection of CRF in rats results in colonic motor stimulation and hypersensitivity to colorectal distention (CRD), thereby mimicking the effects of stress and features of symptoms in patients with irritable bowel syndrome (IBS) (56). In addition, the blunting of centrally injected CRF or stress-induced colonic response by peripheral injection of CRF₁ receptor antagonists that cross the blood-brain barrier or, in some studies, direct administration of CRF antagonists into the cerebrospinal fluid in rodents implicated this brain pathway in functional colonic alterations and hypersensitivity occurring during acute or repeated stress (51, 56).

More recently, peripheral injection of CRF and related endogenous peptides, urocortin 1 (Ucn 1), Ucn 2, and Ucn 3 (20) have been demonstrated to modulate cardiovascular and gastrointestinal function in experimental animals (4, 16, 45, 54). Mammalian ligands of the CRF family exert their biological actions by binding to either both CRF₁ and CRF₂ receptors (CRF and Ucn 1) or selectively to CRF₂ receptors (Ucn 2 and Ucn 3) (20). Peripheral injection of CRF or Ucn 1 potently activates colonic myenteric neurons, increases colonic propulsive contractile activity and permeability, and induces diarrhea in rodents (57). By contrast, peripheral injection of Ucn 2 or the amphibian CRF-related peptide sauvagine, which has high affinity for CRF₂ receptors (48), induces a CRF₂-mediated inhibition of basal gastric emptying, suppresses stimulated colonic motor function, and prevents the hypersensitivity to repeated CRD in rats or mice (36, 41, 43, 44). Direct peripheral stimulatory actions of CRF on motility and permeability have also been demonstrated by using rat colonic preparations or human colonic biopsies (4, 26, 31, 32, 63). Moreover, the expression of CRF ligands and cognate receptors in the immune, neuronal, and endocrine cells of rodent and human colon (6, 26, 63, 65) provides anatomical support to the emerging view that peripheral CRF/Ucn 1/CRF₁ signaling system may be a component of the efferent arm of the colonic activation induced by stress. However, so far, the assessment of the role of CRF₁ receptors in stress-related colonic responses has relied on pharmacological manipulations using peripheral injection of astressin or astressin-B, which are peptide antagonists blocking both CRF₁ and CRF₂ receptors (17), nonpeptide-selective CRF₁ antagonists crossing the blood-brain barrier (CP-154,526, NBI-35965) (25, 40); and CRF₁ receptor-deficient mice, rendering the identification of brain vs. peripheral CRF₁ sites of action difficult (5, 14, 28, 31, 42, 61, 64). Moreover, it is still unknown whether selective activation of peripheral CRF₁ receptors recapitulates all the colonic alterations induced by stress.

Recently, two peptide CRF agonists, cortagine and stresssin₁-A, have been developed that display higher selectivity to CRF₁ than CRF₂ receptors (10, 17, 49, 60), providing novel tools to restrict to CRF₁ the activation of CRF receptors. Study

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so far showed an increase in colonic myenteric activity as monitored by Fos expression along with an increase in defecation and induction of diarrhea in response to the intraperitoneal (ip) injection of stressin1-A at one dose in rats (49, 65). The objectives of the present study were twofold. First, we characterized the selectivity of cortagine to peripheral activation of CRF1 receptors under our conditions of administration by assessing its influence on gastric emptying known to be inhibited by CRF2 receptor activation in rodents (36, 42). Then we established in rats whether selectively targeting CRF1 receptor using ip injection of cortagine at doses having no effect on gastric transit induces stresslike alterations of colonic function (increase in enteric motility activity, permeability, and occurrence of diarrhea) as well as hypersensitization to CRD. In addition, although CRF peptides have limited access to the brain when injected peripherally (24, 38), we ascertained the peripheral hyperalgesic action of ip cortagine by comparing the effects of intracerebroventricular (icv) and ip injections of the CRF antagonist astressin (34). The other objective was to extend these observations in mice to develop a pharmacological murine model of stress representing key colonic features of diarrhea-predominant IBS patients (D-IBS) (increased propulsive colonic motor function, diarrhea, and visceral hyperalgesia) (39) by using ip injection of cortagine and a novel noninvasive method to monitor visceromotor response (VMR) to CRD in rodents.

MATERIAL AND METHODS

Animals

Adult male Sprague-Dawley rats (200–250 g) and C57Bl/6 mice (6–8 wk old) (Harlan Laboratory, San Diego, CA) were kept under controlled conditions of illumination (12:12-h light-dark cycle starting at 6 AM), temperature (21–23°C), and humidity (30–35%). Animals were housed in groups (2–4 per cage) except otherwise stated and maintained on a standard rodent food diet (Purina chow) and water ad libitum except otherwise indicated. Animals were allowed to acclimate to the animal facility for 1 wk after their arrival. Experiments were initiated between 9 AM and 10 AM and ended no later than 2 PM to avoid circadian influence on parameters under study. All protocols were approved by the Research Committee of the Veterans Affairs (VA) Greater Los Angeles Healthcare System (99127-07).

Compounds and Treatments

Cortagine ([Glu21, Ala40][sauvagine1–12]×rat CRF14–30)×[sauvagine30–40]), astressin, and astressin-B (Peptide Biology Laboratories, Salk Institute, La Jolla, CA) stored in powder form at −10°C were weighed and dissolved in sterile water immediately before use as previously described (49). CP-154,526 (Pfizer, Groton, CT) was dissolved in dimethyl sulfoxide (DMSO)-cremaphor EL-saline (5: 5:90 vol/vol/vol), and Evans Blue (molecular weight 960.83) in 0.01 M phosphate-buffered saline. Except as otherwise mentioned, ip injections were performed in 0.2 ml in rats and 0.1 ml in mice.

Rats were equipped with a chronic icv cannula as previously described (35). The guide cannula (22 gauge, Plastic One Products, Roanoke, VA) was implanted into the right lateral brain ventricle in animals anesthetized with an ip injection of a mixture of ketamine hydrochloride (75 mg/kg; Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5 mg/kg; Rompun, Mobay, Shawnee, KS) by using the following coordinates (mm from bregma: anteroposterior, −0.8; lateral, −1.5; dorsoventral, −4.0). The guide cannula was maintained in place by dental cement anchored by four stainless steel jewelry screws fixed to the skull. During the 5–7 days following surgery, animals were housed individually on direct bedding and handled for 5 min daily and habituated to the manipulation of the cannula. The icv injection was performed in lightly hand-restrained rats as previously described (35). A 28-gauge injection cannula, 1 mm longer than the guide cannula, was then connected to a 50-μl Hamilton syringe by a PE-50 catheter (Intramedic Polyethylene Tubing, Clay Adams, Sparks, MD) filled with distilled water. An air bubble (5 μl) was drawn at the distal end of the PE-50 catheter to separate the injected solution from the water and for visual inspection of the injection (5 μl except otherwise stated). Injection was performed over 30 s. At the end of the experiments, the correct location of the cannula into the lateral ventricle was verified by injecting 10 μl of dye (0.1% toluidine blue). Visualization of dye on the wall of the lateral ventricle indicates correctness of the icv injections.

Assessment of Colonic and Gastric Motor Function

Defecation and diarrhea. Fecal pellet output (FPO) and incidence of diarrhea (defined as the percentage of rats displaying at least one watery stool during the observation period) were monitored every 15 min for 1 h after the following treatments: 1) ip injection of vehicle or cortagine at 3, 6, 10, and 30 μg/kg in different groups of naïve conscious rats or at 10, 30, and 100 μg/kg in different groups of naïve conscious mice; 2) icv injection of either vehicle or cortagine (10 μg/kg) in two groups of chronically icv cannulated rats; 3) ip injection of vehicle or cortagine (10 μg/kg) in chronically icv cannulated rats, then 4 days later in the same animals an icv injection of either vehicle or cortagine (10 μg/kg, i.e., 3 μg/rat); 4) subcutaneous pretreatment (0.3 ml) with the CRF1-selective antagonist CP-154,526 (20 mg/kg) or its vehicle (pH adjusted to CP-154,526) 30 min before the ip injection of vehicle or cortagine (10 μg/kg) in naïve conscious rats. In all experiments, the icv cannulated rats were single housed and handled daily for 5 days before the first administration of cortagine or vehicle (ip or icv). The ip doses of cortagine were on the basis of previous dose-response studies of ip CRF-induced defecation or shortening of colonic transit in rats and mice (31, 36). The route and dose of CP-154,526 administration were based on previous studies showing maximal efficacy to antagonize the colonic motor response to ip CRF (10 μg/kg) or acute stress (31, 42).

Colonic motility. Spontaneous colonic contractions were recorded for 1 h before and after the ip injection of vehicle or cortagine (10 μg/kg) in conscious rats maintained in Bollman cage. Transverse and distal colonic contractions were monitored as change in intraluminal colonic pressure (ICP) by using a recently developed noninvasive approach in rodents (15). Rats were accustomed to Bollman cages for 2 h/day for 3 consecutive days. The following day, rats were briefly anesthetized with isoflurane (3% in O2), and two miniaturized pressure transducer catheters (SPR-524 Mikro-Tip catheter; Millar Instruments) lubricated with medical grade lubricant were introduced into the colon such that the middle of the pressure sensors (3.5 F) were placed at 4 and 8 cm past the anus. The pressure transducer catheters were then secured to the tail with tape and connected to preamplifiers (model 600; Millar Instruments, Houston, TX). The signal was then amplified using a transducer amplifier in differential mode (TBM4, World Precision Instruments, Boca Raton, FL), acquired via an analog-to-digital interface Micro1401 (Cambridge Electronic Design, Cambridge, UK) at 100 samples/s and recorded using Spike 2 version 5 data acquisition software. At the start of each experiment, the system was calibrated using known pressures at 0, 20, 40, and 60 mmHg to convert voltage output to intraluminal pressure. The fast changes in ICP resulting from abdominal muscle contractions and breathing artifacts were excluded by smoothing the original trace with a time constant of 2 s. The phasic component of ICP was extracted by 10.220.33.6 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from
Time course of motility index change was expressed for every minute as means pAUC (pAUCm) by using the formula pAUCm = average (pAUCm_2 min) or as mean pAUC over 30-min periods pre- and postinjection.

**Distal colonic transit and gastric emptying.** Conscious rats fasted for ~16 h with water ad libitum were given access to preweighed Purina chow for a 2-h period, then, under brief isoflurane (3% in O2) anesthesia (2–3 min), a single 5-mm colored plastic bead was inserted into the distal colon (3 cm past the anus) with a lubricated plastic rod, and then vehicle or cortagine (10 μg/kg) was injected ip and rats were placed in individual cages without water and food. The time required for expulsion of the bead (in minutes) was monitored over a 4-h period; thereafter animals were euthanized and the 4-h gastric emptying of the ingested meal was assessed as previously described (15, 42). In separate experiments, conscious ~16-h fasted rats were injected ip with vehicle or cortagine (10 μg/kg) followed 10 min later by an orogastric administration of 1.5 ml of methylcellulose (1.5%)–phenol red (0.5%) viscous suspension. The gastric emptying of nonnutrient meal was monitored 20-min later as previously described (37).

**Fos Immunolabeling in Colonic Myenteric Neurons**

Conscious rats were injected ip with vehicle or cortagine (10 μg/kg) and euthanized 1-h later with an overdose of pentobarbital sodium (100 mg/kg ip, Nembutal, Abbott Laboratories, Chicago, IL). Segments of proximal and distal colon were processed for Fos immunolabeling in whole-mount longitudinal muscle myenteric preparations as in our previous studies (65). In each animal, the number of cells with Fos-immunoreactive (IR) nuclei was counted in 25 myenteric ganglia randomly selected in each examined field (0.25 cm²). The mean number of Fos-positive cells/ganglion in 25 ganglia from each animal was used to calculate the group mean.

**Assessment of Colonic Permeability**

Colonic permeability (CP) was assessed by the Evans blue permeation method (27). Previous studies showed that Evans blue instilled into the rat colon lumen can be detected in the blood through transport over the mucosa in a time- and dose-dependent manner, reflecting epithelial permeability (27). In 18-h fasted rats anesthetized with urethane (25%, 1.5 g/kg ip), the jugular vein was catheterized and a laparotomy was performed. The proximal colon was ligated at the junction with the cecum, and fecal contents were evacuated by using a Bollman cage (after 3 consecutive days of training) and recorded by using a miniaturized pressure catheter equipped with an unrestricted balloon during the recording period. The same signal was processed by using off-line extract side scripts to specifically extract signals from abdominal muscle contractions (middle trace) and colonic contractions (top trace). By using these respective parameters, colonic contractions (long lasting events > 2 s) indicated with asterisks are totally excluded from the abdominal muscle contraction signal and only the fastest event (<2 s) abdominal contractions (indicated with black arrows) are present in the final trace to be analyzed. Similarly, abdominal muscle contractions are excluded from the colonic contraction signal when assessing only colonic contractions.

**CRD procedure.** Rats and mice were trained to the experimental conditions (Bollman cage in rats and restrainer in mice) for 3 h/day once (mice) or for 3 days (rats) before the experiment. The day after the end of training, mice and rats were briefly anesthetized with isoflurane (3% in O2) and the modified miniaturized pressure transducer catheter equipped with a custom-made polyethylene plastic balloon tied below the pressure sensor was inserted into the colorectum up to 0.5 and 1 cm past the anal verge of mouse and rat, respectively. The catheter was secured to the tail with tape, and mice were placed in mouse restrainers or rats in Bollman cages and left to rest for 30 min before the CRD procedure. The CRD protocol consisted of graded phasic distentions to constant pressures including in rats, 2 CRD at 60 mmHg (to unfold the balloon) immediately followed by CRD at 10, 20, 40, and 60 mmHg (20-s duration; 4-min interstimulus interval) and in mice CRD at 15, 30, 45, and 60 mmHg (3 times each, 10-s duration, 4-min interstimulus interval). Similar CRD paradigms have been used previously to assess visceral pain-related responses in rats and mice (8, 23, 28).

**CRD experimental protocols.** In conscious rats and mice, the VMR to a first set of CRD was obtained and taken as baseline response. After a 1-h rest period, animals were injected ip with vehicle or cortagine (10 μg/kg in rats and 30 μg/kg in mice) and the VMR to a second set of CRD was recorded 15 min after the injection. Cortagine dose in rats and mice was selected on the basis of the maximal effective dose influencing colonic secretory motor function in the above studies. In separate experiments, groups of rats equipped with icv cannulae were injected with vehicle (3 μl icv) and the VMR to a first CRD was obtained 5 min later (baseline response). After a 1-h rest period, vehicle or astressin (3 μg/rat, 7 μl) or ip (10 μg/kg), and then cortagine (10 μg/kg) was injected ip 5 min after icv or 15 min after ip injection. The second CRD was performed 15 min later. The CRF antagonist-to-CRF1 agonist ratio selected was shown previously to antagonize icv CRF-induced colonic motor response (34).

**CRD signal acquisition.** The modified miniaturized pressure transducer was connected to a preamplifier (model 600; Millar Instruments, Houston, TX) and the balloon to an electronic barostat (Distender Series II, G&J Electronics, Toronto, ON, Canada). The barostat controlled for balloon pressure variation and minimized

**Assessment of Visceral Pain**

Visceral pain in rodents is traditionally assessed by monitoring the pseudoaffective reflexes i.e., cardiovascular changes and VMR induced by CRD (46). Classically, the VMR is monitored by recording the abdominal contractions occurring in response to CRD through electromyographic signals captured by electrodes chronically implanted into the abdominal musculature (8, 23, 28). In the present study, we measured the VMR to CRD using a noninvasive method based on a slightly modified manometric method of ICP recording previously reported in mice (2). We used a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX), equipped with a custom-made polyethylene plastic balloon (1 cm width × 2 cm length for mice, 2 cm width × 5 cm length for rats) tied below the pressure sensor. While in our study, ICP monitoring was used to record both visceral sensitivity and colonic motility signals (see Colonic motility above), the parameters used for the two measurements allowed us to discriminate the abdominal muscle from the colonic smooth muscle contractions. Figure 1 shows a raw ICP trace (bottom trace) obtained from a conscious rat placed in a Bollman cage (after 3 consecutive days of training) and recorded by using a miniaturized pressure catheter equipped with an unrestricted balloon during the recording period. The same signal was processed by using off-line extract side scripts to specifically extract signals from abdominal muscle contractions (middle trace) and colonic contractions (top trace). By using these respective parameters, colonic contractions (long lasting events > 2 s) indicated with asterisks are totally excluded from the abdominal muscle contraction signal and only the fastest event (<2 s) abdominal contractions (indicated with black arrows) are present in the final trace to be analyzed. Similarly, abdominal muscle contractions are excluded from the colonic contraction signal when assessing only colonic contractions.

CRD procedure. Rats and mice were trained to the experimental conditions (Bollman cage in rats and restrainer in mice) for 3 h/day once (mice) or for 3 days (rats) before the experiment. The day after the end of training, mice and rats were briefly anesthetized with isoflurane (3% in O2) and the modified miniaturized pressure transducer catheter equipped with a custom-made polyethylene plastic balloon tied below the pressure sensor was inserted into the colorectum up to 0.5 and 1 cm past the anal verge of mouse and rat, respectively. The catheter was secured to the tail with tape, and mice were placed in mouse restrainers or rats in Bollman cages and left to rest for 30 min before the CRD procedure. The CRD protocol consisted of graded phasic distentions to constant pressures including in rats, 2 CRD at 60 mmHg (to unfold the balloon) immediately followed by CRD at 10, 20, 40, and 60 mmHg (20-s duration; 4-min interstimulus interval) and in mice CRD at 15, 30, 45, and 60 mmHg (3 times each, 10-s duration, 4-min interstimulus interval). Similar CRD paradigms have been used previously to assess visceral pain-related responses in rats and mice (8, 23, 28).

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**CRD signal acquisition.** The modified miniaturized pressure transducer was connected to a preamplifier (model 600; Millar Instruments, Houston, TX) and the balloon to an electronic barostat (Distender Series II, G&J Electronics, Toronto, ON, Canada). The barostat controlled for balloon pressure variation and minimized
any interference of colonic motor activity changes during balloon inflation. The signal was acquired by use of the CED Micro1401/SPIKE2 program (Cambridge Electronic Design). The phasic component of the ICP signal was extracted from the original signal recorded by applying the DC Remove process in Spike 2 (CED, Cambridge Electronic Design) component with a time constant of 1 s to exclude the slower, tonic changes in ICP resulting from colonic smooth muscle activity and by applying the root mean square amplitude process with a time constant of 1 s to the resulting trace. ICP activity was recorded for 10 s (mice) or 20 s (rats) before, during, and after termination of CRD. The VMR was defined as the increase in AUC of ICP during CRD over means of pre- and postdistension periods (10 s in mice or 20 s in rats each) and quantified by using the “modulus” process in Spike 2. To examine the pressure-response relationship and adjust for interindividual variations of the signal (46), ICP amplitudes were normalized for each rat or mouse to the highest pressure (60 mmHg) in the first set of CRD. This value served as 100% response (control) in the baseline period of data collection before ip cortagine or vehicle. The VMR to the first set of CRD before treatment represents baseline VMR. The VMR to the second CRD with or without treatment are shown either as % from their normalized control values (% control) or mean change from the baseline response (ΔVMR in % control) at different pressures of distention as validated in our previous studies (28).

**Histological Evaluation**

Mice injected ip with cortagine (30 μg/kg) or vehicle were euthanized by isoflurane anesthesia followed by cervical dislocation 1 h after injection. The proximal and distal colonic segments were fixed in 4% paraformaldehyde overnight before paraffin embedding. Sections (5 μm) were stained with hemalun-eosin. Microscopic examination for the presence of lesions or cell infiltration in the colonic tissue was performed in a blind manner.

**Data Analysis**

The results are expressed as means ± SE, and P values <0.05 were considered statistically significant. For gastric emptying, colonic transit, and Fos immunolabeling, comparisons between two groups were performed by unpaired Student’s t-test. Defecation time course, cumulative FPO, CP time course, and the effects of cortagine or vehicle on VMR to CRD within one group of animals (comparison of the baseline vs. vehicle or cortagine values at each distention pressure) were all analyzed by a one-way ANOVA followed by Newman-Keuls post-test comparisons. The VMR response to the second CRD expressed as mean change from baseline of cortagine- vs. that of vehicle-treated rats or mice was analyzed by two-way ANOVA and Bonferroni posttest.

**RESULTS**

**Cortagine Injected Peripherally Activates Colonic Propulsive Motor Function and Myenteric Neurons Without Modifying Gastric Emptying in Conscious Rats**

Defecation and diarrhea. Cortagine injected ip at 3, 6, and 10 μg/kg induced a dose-related increase in pellet output per hour compared with vehicle (0.17 ± 0.17, 5.0 ± 1.8, and 7.0 ± 2.0 vs. 0.6 ± 0.4 pellets/h; P < 0.05) and diarrhea in 0, 20, and 50% of naive rats, respectively, whereas a higher dose (30 μg/kg) did not result in higher colonic responses compared with the 10 μg/kg (Fig. 2A). Time-course study revealed that the onset of defecation occurred after 15 min and was maintained throughout the 60-min period (Fig. 2B). Subcutaneous injection of CRF1 receptor antagonist CP-154,526 (20 mg/kg) reduced the pellet output by 84.8% and abolished the diarrhea response induced by the maximal effective dose of ip cortagine (Fig. 2C) and did not influence basal output (data
not shown). When cortagine (10 μg/kg) was injected ip in singly housed icv cannulated rats, the defecation was similar to that in naive group-housed rats (9.0 ± 1.8, n = 6 vs. 0.0 ± 0.0, n = 2 for vehicle; P < 0.05) and diarrhea occurred in 50% of the animals. The same animals injected 4 days later icv with cortagine (10 μg/kg) resulted in low defecation (1.0 ± 1.0 n = 6 vs. vehicle: 0.0 ± 0.0, n = 2) and diarrhea (16.6%) at 1-h postinjection. In two separate additional experiments, a single icv injection of cortagine (3 μg/rat) in otherwise naive icv-equipped rats resulted in similar low incidence of diarrhea or defecation response (1.3 ± 0.9 vs. 0.0 ± 0.0; n = 12).

**Gastric and distal colonic transit.** Simultaneous measurement of gastric and distal colonic transit in conscious fasted-refed rats showed that cortagine (10 μg/kg ip) decreased significantly the colonic transit time to 90 ± 25 min compared with 165 ± 21 min in vehicle-treated rats (Fig. 3A). By contrast, in the same animals, gastric emptying of a solid meal ingested for 2 h before the ip cortagine injection was not modified compared with ip vehicle as monitored 4-h postinjection either as wet or dry weight of gastric content (Fig. 3B). Likewise, under conditions of short gastric transit (20 min) of a viscous nonnutrient solution, cortagine (10 μg/kg ip) did not modify gastric emptying (44.0 ± 9.7%, n = 6) compared with vehicle (52.4 ± 8.0%, n = 6; P > 0.05).

**Colonic motility.** Cortagine (10 μg/kg ip) enhanced the mean motility index in both distal and transverse colons throughout the first and second 30-min periods postinjection compared with ip vehicle in conscious nonfasted rats (Figs. 4, C and D). The time-course response shows that the increase in mean...
The preinjection motility index was not different between the two groups and between pre- and postinjection of ip vehicle although there was a trend to a decrease particularly in the distal colon (Fig. 4C).

**Fos expression in colonic myenteric neurons.** Cortagine (10 μg/kg ip) induced a significant increase in the number of motility index in the distal colon started almost immediately following the injection, lasted for ~40 min, and returned thereafter to values similar to the vehicle-injected group (Fig. 4A). In the transverse colon, there was a sustained increase in motility index throughout the 60 min postinjection with a peak response occurring 15 min after cortagine injection (Fig. 4B).

**Fig. 4.** Intraperitoneal cortagine increases the colonic motility index in conscious nonfasted rats maintained in Bollman cages. Time course of the motility index changes expressed as area under the curve (AUC) of the phasic component of the intracolonic pressure trace (pAUC) per min (A and B) or mean pAUC per 30 min (C and D) after the ip injection of cortagine (10 μg/kg) or vehicle in the distal (A and C) and transverse (B and D) colons. C and D: each bar represents mean ± SE of mean motility index (pAUC/30 min) in number of rats indicated at the bottom of bars; *P < 0.05 vs. vehicle postinjection, +P < 0.05 vs. cortagine preinjection.
Fos-positive cells/ganglion at 60 min after the injection in the colon proximal (7.8 ± 0.6) and distal (9.2 ± 0.6), whereas in vehicle-pretreated rats there were only rare Fos-IR cells per ganglion (Fig. 5).

Cortagine Injected Intraperitoneally Increases Colonic Permeability in Anesthetized Rats

Cortagine (3 or 10 μg/kg ip) dose dependently increased the permeation of Evans blue through the colonic mucosa into the blood with a peak increase response occurring within 15 min (223.0 ± 43.6 and 312.5 ± 36.6% from vehicle, respectively, \( P < 0.05 \)), followed by a linear time-related return to vehicle group values at 60 min in urethane-anesthetized rats (Fig. 6A). Astressin-B (100 μg/kg) injected intravenously 5 min before cortagine (10 μg/kg ip) abolished the increase in CP at all time points compared with iv vehicle-plus-cortagine-treated group (Fig. 6B).

Cortagine Injected Intraperitoneally Increases the Visceromotor Response to Colorectal Distention in Conscious Rats

Cortagine (10 μg/kg ip) increased significantly the VMR to the second set of CRD at 40 and 60 mmHg compared with the first set of CRD (185.7 ± 49.4 and 226.3 ± 51.0 vs. 56.11 ± 6.8 and 100.0 ± 0.0 AUC/min in % control, respectively) whereas there was no significant changes after injection of ip vehicle group (Fig. 7, A and B). The ΔVMR at 40 and 60 mmHg after cortagine vs. vehicle over baseline was 111.3 ± 33.9 and 107.8 ± 36.5 vs. 1.6 ± 11.3 and −3.1 ± 15.1.

![Fig. 5. Intraperitoneal cortagine induces Fos expression in proximal and distal colon myenteric ganglia in conscious rats. Photomicrographs of ganglionic Fos-immunoreactive (IR) cells (arrow) in whole-mount preparations of proximal (A and B) and distal (C and D) colon at 1 h after the ip injection of vehicle (B and D) and cortagine (A and C). Scale bar, 100 μm. E: mean ± SE of number of Fos-IR cells/myenteric ganglion. *P < 0.05 vs. respective control groups.](http://ajpgi.physiology.org/DownloadedFrom/)
Intraperitoneal Cortagine-Induced Defecation, Diarrhea, and Hyperalgesia to CRD in Conscious Mice

Cortagine (30 μg/kg) injected ip increased significantly the pellet output per hour compared with vehicle (9.4 ± 1.4 vs. 1.2 ± 0.7; \( P < 0.05 \)) and induced diarrhea in 60% of mice (\( P < 0.05 \)) within 30 min (Fig. 8A), whereas a higher dose (100 μg/kg) did not further enhance the colonic propulsive responses and the lowest dose, 10 μg/kg had no significant effect (Fig. 8A). Histological evaluation of the proximal and distal colons of mice treated with ip cortagine (30 μg/kg) did not reveal signs of inflammation or structural changes compared with vehicle-treated animals (data not shown).

In conscious mice, cortagine at 30 μg/kg injected ip increased the VMR to the second set of CRD at the pressures of 30, 45, and 60 mmHg compared with the first set of CRD (70.9 ± 9.2, 111.0 ± 11.5, and 131.0 ± 8.6 vs. 44.1 ± 6.2, 79.5 ± 5.0, and 100.0 ± 0.0 AUC/min in % control, respectively, \( P < 0.05, n = 7–18 \)) as well compared with vehicle (ΔVMR after cortagine vs. vehicle over baseline: 26.9 ± 11.1, 31.7 ± 12.1, and 31.0 ± 8.3 vs. −10.1 ± 10.6, −8.5 ± 12.3, and −13.8 ± 8.9 AUC/min in % control, respectively, \( P < 0.05, n = 7–18 \)) (Fig. 8, C and D).

DISCUSSION

The present study shows that cortagine under our conditions of administration (10 μg/kg ip) induced a peripheral CRF1-restricted activation of CRF receptors resulting in stimulation of colonic propulsive motor function linked with myenteric activation, alterations of mucosal barrier function, as well as visceral hyperalgesia in rats. In addition, we developed a novel murine model that features colonic manifestations of IBS-D as shown by the defecation, diarrhea, and increased visceral hypersensitivity to CRD without histological evidence of inflammation induced by ip injection of cortagine in naive mice.

Cortagine has been characterized to display high affinity to CRF1 receptor (average IC\(_{50}\) = 2.6 nM), limited binding to CRF2 (average IC\(_{50}\) = 540 nM) and no affinity to the CRF binding protein (average IC\(_{50}\) > 1,000 nM) in in vitro binding assays (60). However, a recent study indicates that this chimeric peptide may somewhat be less selective (\( K_I \) for CRF3 = 102 nM) than has been initially reported (49). In addition, in vivo, the selectivity of cortagine to activate CRF1 receptors has only been established upon injection into the mouse brain in doses ranging from 0.1 to 0.5 μg (10, 60). Earlier experiments using selective CRF receptor antagonists have revealed differential biological actions in the gut induced by the activation of CRF1 and CRF2 receptors. In particular, mixed CRF1/CRF2 agonists such as CRF, Ucn 1, and sauvagine injected ip at 10 μg/kg were shown to elicit both a CRF1-mediated stimulation of colonic function and a CRF2-mediated inhibition of gastric emptying of solid meal as monitored simultaneously in rats or mice (36, 42, 54). Therefore, to ascertain cortagine selectivity to CRF1 receptors under our conditions of administration, we assessed in conscious rats its influence on upper and lower gut transit simultaneously. Cortagine injected ip at 10 μg/kg shortened the distal colonic transit time by 45% while not altering the gastric emptying of a solid meal 4 h after peptide injection.
The lack of cortagine effect on gastric emptying cannot be related to the vanishing of its biological action because of the 4-h period between peptide injection and gastric emptying determination that is required to assess simultaneously gastric and distal colonic transit. Indeed, when gastric emptying of a nonnutrient solution was determined 30 min after the ip injection of cortagine, there was also no change. In contrast, cortagine injected at a similar dose induced a near-maximal increase in transverse colonic motility and defecation within 30 min after ip injection. Moreover, the CRF₁ receptor antagonist CP-154,526 (53) prevented ip cortagine-induced defecation by 85% and abolished the diarrhea consistent with our previous observations using the same antagonist against ip CRF (31, 52). This, combined with existing evidence that activation of peripheral CRF₂ receptors has no effect on basal and inhibits stress-stimulated distal colonic propulsive motor function (26, 36, 43), support the selectivity of CRF₁ receptor-mediated action of the peptide under our in vivo conditions. Whether the
remaining component reflects activation of CRF1 receptor variants on myenteric neurons (21) not recognized by the antagonist remains to be established.

The colonic motor response to peripheral injection of CRF peptides has been little studied in mice compared with rats (36, 43). We previously reported a CRF1-mediated stimulation of distal colonic transit in fasted-refed mice in response to ip CRF characterized by the use of selective CRF1 antagonists, whereas selective CRF2 receptors activation by ip injection of Ucn 2 had no effect on basal colonic motor function and potently inhibited novel environmental stress-related defecation in fed mice (36, 43). In the present study, 30 μg/kg of cortagine injected ip in naive mice was the maximal effective dose resulting in elevated defecation and diarrhea in 60% of the animals within 1 h postinjection. The dose of 100 μg/kg showed a trend to be less effective, which may be indicative at such a high dose of additional inhibitory signaling through CRF2 receptors (49, 60), which are known to dampen CRF1 stimulatory effect on colonic motor function in mice (43). Also, of note is the threefold difference in cortagine dose at which the maximal defecation and diarrhea responses occurred in mice compared with rats which had a significant dose-related increase in defecation at doses ranging from 6 to 10 μg/kg with a plateau response at higher dose. Cortagine binding affinity has been determined in human and rat but not in mouse CRF1-transfected cells (49, 60). Because CRF1 receptors differ by few amino acids between species (48), this may reflect a lower affinity of cortagine on mice vs. rat CRF1 receptors and/or a lesser expression of CRF1 receptors in murine than rat colon that will need to be assessed under similar conditions. We previously found that CRF1 receptors mRNA levels are much lower in distal than proximal colon in mice (43). Nevertheless, taken together these data establish that cortagine injected ip at 10–30 μg/kg elicits a highly CRF1-restricted activation of CRF1 receptors in both rats and mice.

Convergent sets of evidence derived from our data and earlier work indicate that the activation of propulsive colonic motor function by ip cortagine most likely reflects a peripheral action on colonic myenteric nervous system. It has been shown that CRF1 receptors are expressed at the gene and protein levels on myenteric neurons and fibers of rat proximal and distal colon (6, 26, 65). Previous in vitro studies in rat colonic preparations also demonstrated that CRF or Ucn 1 evokes a CRF1 receptor-mediated and tetrodotoxin-sensitive excitatory action on contractile activity consistent with a direct action on myenteric neurons (26, 31, 32). In the present study ip cortagine at 10 μg/kg induced Fos expression in myenteric neurons in both the proximal and distal colons, indicative of neuronal activation, along with a rapid onset increase in both proximal and distal colonic motility monitored in conscious rats using a novel noninvasive method (15). In addition, we found that in chronically icv implanted rats and subsequently single housed, a similar dose of cortagine was more efficient to stimulate defecation when injected ip than icv in the same animals at a 4-day interval. The low efficacy of central cortagine was reproduced upon a single icv injection supporting a peripheral site of cortagine action after ip injection. The low potency of cortagine injected icv to induce defecation was unexpected on the basis of previous reports of colonic motor response to CRF injected icv at similar or lower doses in rats (30, 34, 64). The characterization of behavioral responses to cortagine injected icv in mice indicates similar and also some differential actions compared with CRF; in particular an antidepressant-like effect in the forced swim test not found with icv injection of CRF (60). Therefore additional examinations are needed to delineate whether there is distinct influence of cortagine and CRF on gut function upon injection into the brain. The induction of diarrhea within 1 h of ip injection in both rats and mice can be the consequence of hypermotility, as observed in both proximal and distal colon and/or hypersecretion (from the ileal and/or colonic mucosa). Whether cortagine also alters intestinal epithelial secretion remains to be determined; however, indirect evidence supports such a contention. We showed that stres-
sin1-A (10 μg/kg), another selective CRF1 agonist (49), injected ip acutely in rats, selectively activates rat ileal submucosal enteric neurons (65), key components of the gut secretory function (13). Collectively these data demonstrate that CRF receptors activation limited to CRF1 subtype induced by peripheral injection of cortagine is sufficient to produce a potent and rapid in onset stimulation of colonic secretory-motor function and myenteric neurons in rodents.

Our findings also established in both rats and mice that cortagine injected ip at the maximal effective dose selectively influencing colonic motor function induces visceral hypersensitivity to graded phasic CRD. This was observed within the first 30 min of peptide injection with pressures of 40 and 60 mmHg in rats and 30, 45, and 60 mmHg in mice, in animals naive of surgical intervention for chronic implantation of electrodes into the abdominal wall, a procedure most commonly used to assess VMR in rodents (8). This is the first demonstration that the peripheral activation of CRF1 receptors induces visceral hyperalgesia in rodents. Since CRF injected icv was previously reported to induce a CRF1-mediated visceral hypersensitivity to CRD in rats (18, 19), one may argue that peripheral injection of cortagine could have induced its effects by penetrating the central nervous system or by acting on circumventricular organs such as the area postrema or the median eminence. Convergent evidence, however, does not support such a contention. First, pharmacokinetic studies have established that CRF peptides are not effectively transported from the blood to the brain (24, 38). Cortagine is a chimeric peptide of similar molecular weight and structure to other CRF-related peptides and is therefore unlikely to cross the blood-brain barrier. Second, CRF1 receptors have not been detected in rodent area postrema at either the mRNA or protein level (7, 62). Lastly, if peripheral cortagine was acting at medullary sites outside of the blood-brain barrier, direct delivery of the CRF antagonist astressin at these sites should be equally or more potent than if administered peripherally. However, astressin injected at 10 μg/kg icv did not significantly antagonize ip cortagine-induced visceral hypersensitivity, whereas such a dose administered ip resulted in a complete blockade of cortagine action. Interestingly, we previously reported that peripheral injection of Ucn 2, a selective endogenous CRF2 agonist, or sauvagine, a CRF1/CRF2 agonist with high affinity to CRF2 receptor (48) at similar dose range (10–20 μg/kg), prevented repeated tonic CRD-induced visceral hyperalgesia in rats through a peripheral site of action (41, 44). Therefore the present findings unmask a dual and opposite peripheral effect of CRF1 and CRF2 receptor activation in the visceral response to CRD. Combined together, these data may imply that peripheral injection of CRF1 receptor antagonists known to cross the blood-brain barrier or CRF1 knockout mice reported to prevent visceral hyperalgesia induced in different models (18, 40, 51, 61) could involve both peripheral and central blockade of CRF1 signaling involved in visceral hyperalgesia.

It is thought that alterations of the colonic barrier integrity may play a role in the development of visceral hypersensitivity to distension in rodents (1). We found that ip cortagine at 10 μg/kg increased CP monitored by the increased permeation of Evans blue from the colonic mucosa to the blood in rats, a method validated in other studies (27). Cortagine action was rapid in onset, with a maximal peak increase of 312% over ip vehicle values occurring at 15 min, and blocked by the peripheral injection of CRF receptor antagonist. Of interest, the dose-response study showed that ip cortagine at 3 μg/kg induced a significant 223% increase in permeability whereas such a dose did not result in significant alterations of defecation or induction of diarrhea, which occurred at 6 and 10 μg/kg. These data may be indicative of higher responsiveness and/or effectiveness of the colonic mucosa vs. myenteric-muscle layers to CRF1 receptor activation.

Lastly, in the present study we showed that ip cortagine alone at a dose influencing the VMR to CRD did not produce obvious colonic damage or infiltration of inflammatory cells as assessed by hemalun-eosin staining in mice. Hence, the peripheral injection of cortagine, which induces watery diarrhea, increased permeability and visceral hyperalgesia to phasic CRD, mimicked the main features of symptoms observed in D-IBS patients (9, 66), and thereby provides novel and easy-to-use rat and murine models. The potential relevance of the present model is also supported by clinical studies showing that systemic injection of the preferential CRF1 agonist ovine CRF (48) lowers pain thresholds to colonic distension in healthy humans (29, 47) and human CRF increases colonic motility index and more so in IBS patients (12). In in vitro human biopsies, CRF increases mucosal macromolecular permeability via CRF receptors (63). In addition, systemic administration of the nonselective and peripherally restricted CRF receptor antagonists α-helical CRF8-41 and astressin reduces visceral hyperalgesia in D-IBS patients subjected to colonic electrical stimulation (50, 59) and acute or chronic stress-induced defecation and VMR in rats (15, 28, 31, 64). By contrast, the first phase IIa clinical trial with the selective CRF1 antagonist BMS-562086 in 39 D-IBS patients showed no improvement in regional colonic transit or bowel function (55). Since the CRF1 antagonist NBI-34041 has shown efficacy in attenuating elevated stress response in a phase I clinical trial (22), additional studies are required to determine whether the first phase IIa clinical trial negative results reflect differential efficacy of CRF1 antagonists or a lack of translational application of stress-related mechanisms to the pathophysiology of IBS. In addition, in human tissues, there is evidence of alternative splicing of CRF1 receptors leading to 11 isoforms and dimerization of the receptors along with differential regulation under pathophysiological conditions (21). This creates additional regulatory elements in the CRF1 signaling pathways that have been shown to have biological relevance (21, 33, 67). The expression and regulation of alternative splicing of CRF1 receptors in the colon, their biological actions, and interaction with CRF1 antagonists are unknown and may be additional components to take into account at the light of this clinical trial.

In summary, cortagine injected peripherally at a dose of 10 μg/kg in rats induces CRF1 receptor-restricted actions to the colon, resulting in a rapid increase in proximal and distal motility, permeability, shortening of distal colonic transit, defecation, watery diarrhea, and visceral hypersensitivity to CRD in fed rats. Likewise in mice ip cortagine at 30 μg/kg reproduces colonic landmark manifestations of IBS-D, namely increased bowel motor function, diarrhea, and visceral hyperalgesia, without producing macroscopic evidence of colonic inflammation. The lesser potency of cortagine injected icv to stimulate propulsive colonic motor function and lack of block-
ade of visceral hyperalgesia by astressin injected iv on CRF₁ receptors present in the colon. (6, 26, 65). On the basis of these data and recent experimental and clinical evidence (11), we speculate that enhanced peripheral activation of CRF₁ in addition to central activation bears clinical relevance as part of the peripheral efferent components responsible for altering the colonic secretory-motor and visceral responses to stress. Cortagaine injected ip may represent a novel, reliable, dose-related easy-to-achieve experimental rodent model to further investigate underlying mechanisms of stress-induced alterations of colonic function.

ACKNOWLEDGMENTS

The authors thank Jing Fang Zhao, Honghui Liang, and Chrysanshy Ha for helpful technical support.

GRANTS

The research was supported by a VA Career Scientist Award (Y. Tache´), National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 DK-57238 and DK-33061 (Y. Tache´), P50 DK-64539 (Emeran Mayer), DK AM 41301 (Animal Model Core, Y. Tache´), DK PO1-26741 (J. Rivier), DK-068155 (M. Million), T32 DK07180-32 (M. Larache´), and the French Society of Gastroenterology (SNFGE; G. Gourcerol).

REFERENCES


