CGRP upregulation in dorsal root ganglia and ileal mucosa during Clostridium difficile toxin A-induced enteritis

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Keates, Andrew C., Ignazio Castagliuolo, Bosheng Qiu, Sigfus Nikulasson, Ashok Sengupta, and Charalabos Pothoulakis. CGRP upregulation in dorsal root ganglia and ileal mucosa during Clostridium difficile toxin A-induced enteritis. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G196–G202, 1998.—We have previously reported that pretreatment of rats with capsaicin (an agent that ablates sensory neurons) or CP-96345 (a substance P receptor antagonist) dramatically inhibits fluid secretion and intestinal inflammation in ileal loops exposed to Clostridium difficile toxin A. The aim of this study was to determine whether calcitonin gene-related peptide (CGRP), a neuropeptide also found in sensory afferent neurons, participates in the enterotoxic effects of toxin A. Administration of toxin A was also found to increase CGRP content in dorsal root ganglia and ileal mucosa 60 min after toxin exposure. Furthermore, immunohistochemical studies demonstrated increased neuronal staining for CGRP 2 h after toxin A treatment. Pretreatment of rats with CGRP-(8—37), a specific CGRP antagonist, before instillation of toxin A into ileal loops significantly inhibited toxin-mediated fluid secretion (by 48%), mannitol permeability (by 83%), and histological damage. We conclude that CGRP, like substance P, contributes to the secretory and inflammatory effects of toxin A via increased production of this peptide from intestinal nerves, including primary sensory afferent neurons.

intestine; sensory nerves; neurotransmitters; calcitonin gene-related peptide

Over the last 10 years or so the concept of neurogenic (i.e., sensory neuropeptide-mediated) inflammation has received considerable support. Antidromic stimulation of sensory neurons has been shown to stimulate release of calcitonin gene-related peptide (CGRP) and substance P and to produce an intense inflammatory response in the skin of various animals (see Ref. 7 for review). Furthermore, depletion of neurotransmitters in sensory nerves with capsaicin abolishes the inflammatory response induced by antidromic stimulation (16). These findings are complemented by numerous studies that suggest that CGRP and substance P have proinflammatory properties and contribute to inflammatory changes associated with arthritis (13, 30).

CGRP I, or α-CGRP, is a 37-amino acid peptide that was originally detected as an alternative splice product of the calcitonin gene (2). It is now known that a closely related gene encodes CGRP II (β-CGRP), which possesses almost identical biological properties to CGRP-I and which in the rat differs by a single conservative amino acid substitution (1). In addition to its functions as a neurotransmitter/neuromodulator in the central nervous system, CGRP is also an important neurotransmitter in the enteric nervous system, where it has been localized to both primary sensory afferent nerves and intrinsic neurons (33).

Several experimental observations suggest that CGRP participates in immune and inflammatory responses (see Ref. 29 for review). First, CGRP is a vasodilator and potentiates vascular permeability and neutrophil recruitment induced by interleukin-1, platelet-activating factor, histamine, and substance P. Second, binding sites for CGRP have been detected on rat and mouse lymphocytes, rat macrophages, and canine mesenteric lymph nodes. Binding of CGRP to its receptor stimulates histamine release from mast cells and inhibits T lymphocyte proliferation and eosinophil chemotaxis. CGRP also inhibits antigen presentation and interferon-γ-induced H2O2 production by macrophages. Finally, antibodies to CGRP have been shown to ameliorate inflammation induced by arthritis and topical treatment with mustard oil in rats.

In contrast to the above findings, studies investigating the role played by CGRP in animal models of colitis suggest that sensory nerves function in an anti-inflammatory capacity. In the rabbit immune complex model of colitis, tissue CGRP content was found to be reduced by 80% 48 h after induction of inflammation (12). Decreases in colonic CGRP content have also been reported in rats treated with trinitrobenzenesulfonic acid and Formalin to induce inflammation (11, 24). Furthermore, in the immune complex- and trinitroben-
zenesulfonic acid-induced colitis, ablation of sensory neurons with capsaicin increases the severity of inflammation (27, 28).

Because primary sensory neurons and substance P participate in toxin A-mediated enteritis, we sought to determine whether CGRP also contributes to the secretory and inflammatory effects of C. difficile toxin A. To accomplish this we used the rat ileal loop model, in which toxin A produces a reproducible acute inflammatory response (25). We demonstrate that toxin A–induced fluid secretion, mucosal permeability, and inflammation in ileal loops are markedly attenuated by CGRP-(8—37), a specific CGRP antagonist. We also show that instillation of toxin A into ileal loops induces an early increase of CGRP content in lumbar dorsal root ganglia, which is followed by increased levels of CGRP in the ileal mucosa.

**MATERIALS AND METHODS**

Male Wistar rats weighing 150–200 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). All rats arrived at the animal facility at least 4 days before the experiment and were housed under standardized environmental conditions. Pentobarbital sodium was obtained from Abbott (Chicago, IL). [3H]mannitol (30 Ci/mmol) was obtained from New England Nuclear (Boston, MA). The rat CGRP antagonist CGRP-(8—37) was obtained from Peninsula Laboratories (Belmont, CA). CGRP-(8—37) was dissolved in phosphate-buffered saline (PBS) immediately before use and injected intravenously via a catheter. Toxin A was purified to homogeneity from brotch culture supernatants of C. difficile strain 10,463 as previously described (26). Enterotoxicity and cytotoxicity of toxin A were assessed as previously described (25). A dose of 5 µg of purified toxin A was used in all experiments, since previous studies showed this dose to stimulate fluid secretion, increase mannitol permeability, and cause an acute inflammatory infiltrate when injected into rat ileal loops (25). Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Laboratories, Rockford, IL).

Measurement of mannitol permeability and fluid secretion in rat ileal loops. Two days before the experiment, rats were anesthetized by intraperitoneal injection of pentobarbital sodium, and polyethylene catheters (1.27 mm in diameter; Clay Adams, Parsippany, NJ) were placed in the right jugular vein and subcutaneously exteriorized in the intrascapular region. After implantation of the catheters, animals were kept under continuous observation until the day of the experiment. Two days after surgery, fasted rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg ip), and a laparotomy was performed. Both renal pedicles were ligated, and 10 µCi of [3H]mannitol were injected into the inferior vena cava. Two 5-cm closed loops were then formed in the distal ileum, with at least 5 cm distance separating each loop. Five minutes before instillation of toxin A, 

A similar dose of CGRP-(8—37) was found to significantly inhibit neurogenic vasodilatation in rat paw skin after intraplantar injection of sodium nitroprusside (17). The same amount of PBS or CGRP-(8—37) was also administered 25, 55, and 85 min after injection of toxin A. Multiple doses of CGRP-(8—37) were given to minimize possible in vivo degradation of the antagonist over the course of the experiment. Animals were maintained under general anesthesia for the duration of the experiment with pentobarbital sodium (20 mg/kg ip) given ~20 min after the end of the operation. Animals were also placed on a heating pad to keep their body temperatures at 37–38°C. After 4 h, the animals were killed, the ileal loops were removed, and the weights and lengths were measured. Intestinal fluid secretion (weight-to-length ratio; mg/cm) and mucosal [3H]mannitol permeability (dissintegrations per minute (dpm) per centimeter loop) were determined as described previously (25).

Histological evaluation. Ileal tissues were fixed in Formalin, embedded in paraffin, and stained with hematoxylin and eosin for light microscopy. All sections were graded in a blinded fashion by a gastrointestinal pathologist (S. Nikulasson), taking into account the following features: 1) epithelial cell damage, 2) hemorrhagic congestion and edema of the mucosa, and 3) neutrophil margination and tissue infiltration, as described previously (25). A score of 0–3, denoting increasingly severe abnormality, was assigned to each parameter. The effect of CGRP-(8—37) on toxin A–induced histological damage was assessed after 4 h, since full-blown enteritis requires at least 2 h exposure to toxin A to develop (3). Measurement of CGRP content in ileal mucosa and dorsal root ganglia. Ileal loops were prepared as described above and exposed to 5 µg of toxin A. After 0, 30, 60, and 120 min, animals were killed and the loops were removed, opened, and washed in ice-cold Hank’s balanced salt solution (Sigma). The mucosa was then scraped from the underlying muscle layers and homogenized in 2.5 ml of ice-cold 0.2 M HCl for 20 s. The homogenate was centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was collected and stored at −80°C until use. To collect lumbar dorsal root ganglia, the spinal column was dissected bilaterally and, with the use of ultrafine forceps, the lumbar dorsal root ganglia (6–8 each animal) were removed from each side of the animal. In separate experiments thoracic dorsal root ganglia (T3–T4) were isolated in a similar manner. Dorsal root ganglia from each animal were pooled separately and processed as described above for intestinal mucosal scrapings.

CGRP levels in mucosal scrapings and lumbar dorsal root ganglia were determined with the use of a commercially available radioimmunoassay (RIA; Phoenix Pharmaceuticals, Mountain View, CA). Samples were reconstituted on C18 reverse-phase cartridge columns (Waters, Cambridge, MA). Briefly, columns were first washed with 5 ml methanol and then equilibrated with 0.1% (vol/vol) trifluoroacetic acid (TFA). Samples diluted 1:1 with 0.2% (vol/vol) TFA were slowly loaded onto the column, and the column was then washed with 10 ml of 0.1% (vol/vol) TFA. Adsorbed peptides were then eluted with 1.5 ml of 75% (vol/vol) acetonitrile and freeze-dried. Samples were reconstituted in 0.5 ml of RIA sample buffer, and the CGRP content was determined according to the manufacturer’s protocol. Results are expressed as picomoles CGRP per milligram protein.

Immunohistochemistry. Ileal loops were prepared as described above and exposed to toxin A for 0, 1, and 2 h. At the indicated time points, loops were removed, opened along their length, and washed in ice-cold PBS (Sigma). Samples were then fixed in Zamboni’s fixative for 18 h at 4°C. After fixation, tissues were washed exhaustively in ice-cold PBS containing 0.015% (wt/vol) sodium azide, oriented, frozen in optimal cutting temperature compound (Miles, Elkhart, IN), and cryosectioned onto gelatin-coated slides. Control and test sections were mounted onto each slide to ensure accurate comparison between samples. Slides were incubated with...
rabbit anti-rat CGRP antiserum (Peninsula Laboratories) at a dilution of 1:500 or nonimmune rabbit serum at the same dilution for 1 h at room temperature. After washing, primary antibodies were detected by the avidin-biotin peroxidase staining system, using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were then counterstained using hematoxylin and eosin.

Data analysis. Statistical analyses were performed using SigmaStat for Windows version 2.0 (Jandel Scientific Software, San Rafael, CA). Analysis of variance followed by protected t-test was used for intergroup comparisons, except for the histological grades, for which the nonparametric Kruskal-Wallis analysis of variance on ranks was used.

RESULTS

Effect of CGRP-(8—37) on toxin A-induced intestinal secretion and mucosal permeability. As previously reported (4, 25), exposure of rat ileal loops to 5 µg of highly purified C. difficile toxin A significantly increased intestinal fluid secretion (3.6-fold; P < 0.01) compared with loops from control animals (Fig. 1). Instillation of toxin A into ileal loops also caused a prominent elevation in mucosal [3H]mannitol permeability (35.5-fold, P < 0.01) compared with untreated loops (Fig. 2). Intravenous injection of the CGRP antagonist CGRP-(8—37) significantly inhibited intestinal fluid secretion in response to toxin A (by 48%, P < 0.01; Fig. 1). Administration of the same dose of CGRP-(8—37) also markedly reduced toxin A-mediated increases in mucosal [3H]mannitol permeability (by 83%, P < 0.01; Fig. 2). A similar dose of CGRP-(8—37) given in the absence of toxin A had no effect on basal intestinal fluid secretion or mucosal permeability (Figs. 1 and 2).

Effect of CGRP-(8—37) on toxin A-induced histological damage. Histological examination of ileal tissues from loops exposed to toxin A showed characteristic epithelial cell damage with disruption of villus architecture compared with buffer-treated control loops (Fig. 3). Pretreatment of animals with CGRP-(8—37) dramatically reduced histological damage induced by toxin A (Fig. 3). Quantitative histological scores for all three parameters studied (epithelial damage, mucosal congestion and edema, and neutrophil infiltration) were each significantly decreased in CGRP-(8—37)-treated animals compared with animals treated with toxin A alone (Fig. 4).

Effect of toxin A on CGRP content in intestinal mucosa and lumbar dorsal root ganglia. Because the results (Figs. 1–4) indicate that CGRP participates in the intestinal responses to toxin A, we next measured the CGRP content of the ileal mucosa from loops exposed to toxin A. Treatment of ileal loops with toxin A resulted in a time-dependent increase in the CGRP content of the mucosa as measured by specific radioimmunoassay (Fig. 5). Mucosal CGRP content was unaltered 30 min after ileal instillation of toxin A. One hour after toxin A administration mucosal CGRP content was increased approximately threefold compared with control (Fig. 5), and mucosal CGRP content remained elevated after 2 h.

Previous studies from our laboratory indicate that sensory afferent neurons play an important role in the mechanism of action of C. difficile toxin A (4, 25). Several publications have shown that sensory afferent neurons innervating the terminal ileum have their cell bodies in either lower thoracic or upper lumbar dorsal root ganglia (6, 34). Because we have recently shown that the substance P content in lumbar dorsal root ganglia is increased 30 min to 1 h after toxin A treatment (3), we next investigated whether administration of toxin A into ileal loops altered the CGRP content in these dorsal root ganglia. Our results show that the CGRP content in lumbar dorsal root ganglia was increased approximately twofold (P < 0.01) 1 h after toxin A administration compared with the content of this peptide in dorsal root ganglia from control animals (Fig. 6). However, 2 h after treatment of ileal loops with toxin A, the CGRP content of lumbar dorsal root ganglia had returned to control levels (Fig. 6). In contrast, the CGRP content of thoracic ganglia from the T9–T12 level (n = 5 animals) was not significantly altered 30 min (3,910 ± 324 pg/mg protein), 1 h
(4,786 ± 1,323 pg/mg protein), or 2 h (4,876 ± 1,140 pg/mg protein) after exposure to toxin A compared with CGRP levels in ganglia from control animals (5,101 ± 560 pg/mg protein).

Immunohistochemical studies. To determine the cell(s) of origin of increased CGRP production in ileal loops exposed to toxin A, immunohistochemical staining was performed using tissue sections from ileal loops exposed to toxin A (Fig. 7). CGRP immunoreactivity in control ileum was primarily associated with nerve terminals around myenteric and submucosal neurons and the villus plexus in the lamina propria. CGRP immunoreactivity was unaltered after 1 h exposure to toxin A. However, treatment of ileal loops for 2 h with toxin A markedly increased CGRP staining intensity compared with control. Elevated CGRP levels appeared to be associated with nerve fibers, particularly in the lamina propria.

DISCUSSION

The principal finding of this study is that instillation of purified C. difficile toxin A into rat ileal loops induces an early increase in the CGRP content of dorsal root ganglia and intestinal mucosa. Furthermore, CGRP-(8—37), a CGRP inhibitor, almost completely abolished

Fig. 3. Inhibition of toxin A-induced enteritis by CGRP-(8—37). Rat ileal loops were injected with 0.4 ml Tris buffer alone or with Tris buffer containing 5 µg purified C. difficile toxin A. Test animals were pretreated with either PBS or PBS containing CGRP-(8—37) (80 nmol/kg iv) 5 min before and 25, 55, and 85 min after toxin A administration. After 4 h, loops were removed, full-thickness samples were fixed in Formalin, and sections were stained with hematoxylin and eosin. A: buffer-treated ileal loop showing normal mucosa. B: ileal loop exposed to toxin A showing disruption of villus architecture, goblet cell discharge, and tissue necrosis. C: ileal loop pretreated with CGRP-(8—37) before challenge with toxin A, showing almost complete prevention of the intestinal effects of toxin A. Magnification, ×140.

Fig. 4. Inhibition of toxin A-mediated enteritis by CGRP-(8—37). Tissue sections from control and toxin-treated animals were prepared as described in Fig. 3 legend. Histological severity of enteritis was graded in each section by a score of 0–3, using the following parameters: 1) epithelial damage, 2) hemorrhagic congestion and mucosal edema, and 3) neutrophil margination and tissue infiltration. Results are means ± SE from 5 loops. *P < 0.05 compared with toxin A alone.

Fig. 5. Effect of toxin A (TxA) on CGRP content of ileal mucosa. Rat ileal loops were formed and injected with 0.4 ml Tris buffer containing 5 µg purified C. difficile toxin A. After 30, 60, and 120 min, loops were harvested, and mucosal CGRP content was determined as described in MATERIALS AND METHODS. Results are means ± SE from 5–6 loops. **P < 0.01 compared with control.
mucosal permeability to [3H]mannitol and substantially inhibited intestinal fluid secretion and histological damage induced by toxin A in rat ileum. These findings indicate that CGRP participates in the mechanism of action of C. difficile toxin A in the rat intestine.

Retrograde tracing studies have demonstrated that the primary afferent nerves innervating the distal ileum of the rat have cell bodies localized in the lower thoracic and upper lumbar dorsal root ganglia (6, 34). Increased levels of CGRP in lumbar dorsal root ganglia were observed 1 h after treatment with toxin A. Furthermore, in a similar experiment, increased levels of substance P in lumbar dorsal root ganglia were evident 30 min after toxin A administration (3). These findings suggest that activation of sensory neurons is an early event in toxin A-induced enteritis, since upregulation of CGRP and substance P in lumbar dorsal root ganglia precedes increases in intestinal fluid secretion, mucosal permeability, and histological damage mediated by toxin A (4). Interestingly, the CGRP content of thoracic ganglia (T9–T12) was unaltered when toxin A was applied to ileal loops. These data suggest either that toxin A only activates sensory neurons that have cell bodies in lumbar dorsal root ganglia or, alternatively, that toxin A may activate sensory neurons indirectly, possibly via activation of enteric nerves.

Increases in the CGRP and substance P content of lumbar dorsal root ganglia have also been reported in rat adjuvant-induced arthritis. For example, Donnerer et al. (9) reported a 30–40% increase in CGRP and substance P content of the lumbar dorsal root ganglia 5 days after induction of inflammation in rat hindpaws. Moreover, increases in CGRP levels in lumbar dorsal root ganglia were present as early as 12 h after injection of adjuvant, suggesting that activation of CGRP-containing sensory nerves is also an early event in this model. In a separate study Hanesch et al. (15) reported that the number of CGRP-positive cell bodies in the dorsal root ganglia increased significantly (30%) 2 days after adjuvant challenge.

In this study we show that increased levels of CGRP are associated with acute intestinal inflammation. In-

Fig. 6. Effect of toxin A (TxA) on CGRP content of lumbar dorsal root ganglia. Rat ileal loops were formed and injected with 0.4 ml Tris buffer containing 5 µg purified C. difficile toxin A. After 30, 60, and 120 min, lumbar dorsal root ganglia were harvested and CGRP content was determined as described in MATERIALS AND METHODS. Results are means ± SE from 5–10 loops. *P < 0.05 compared with control.

Fig. 7. CGRP immunohistochemistry in normal and toxin A-treated ileum. Rat ileal loops were formed and injected with 0.4 ml Tris buffer containing 5 µg purified C. difficile toxin A. After 60 and 120 min, loops were harvested and CGRP immunohistochemistry was performed as described in MATERIALS AND METHODS. In normal ileum CGRP immunoreactivity is mainly associated with neurons present in the lamina propria and the submucosa (Fig. 7A). No change in ileal CGRP immunoreactivity was observed after treatment with toxin A for 1 h (data not shown). Instillation of toxin A into ileal loops for 2 h markedly increased CGRP immunoreactivity compared with control. Increased staining is particularly apparent in lamina propria neurons (Fig. 7B). Control ileal sections processed in parallel but incubated with rabbit nonimmune serum showed no immunostaining (Fig. 7C). Magnification, ×200.
increased immunohistochemical staining of CGRP was apparent 2 h after exposure to toxin A in the intestinal mucosa and lamina propria. An important source of CGRP is capsaicin-sensitive sensory afferent neurons, which are distributed throughout the intestine. These nerves have their cell bodies in spinal dorsal root ganglia and regulate a number of immune and inflammatory intestinal functions (29). CGRP is also contained within intrinsic nerves and may be produced by intestinal immune cells (29). Although CGRP may be released from multiple sites in the intestine, our results in Fig. 7 and previous studies (4, 25) are consistent with release of this peptide from enteric neurons. Given the rapid time course (1–2 h) of CGRP upregulation in response to toxin A stimulation, it would seem unlikely that these changes are the result of de novo protein synthesis in neuronal cell bodies. A more likely explanation for the observed changes in CGRP staining is that toxin A increases axonal transport and secretion of CGRP. Although axonal transport of CGRP has been demonstrated in sensory afferent fibers innervating the gastrointestinal tract (36), the role of this process in acute intestinal inflammation is poorly understood. However, increased axonal transport of CGRP has been reported in the sciatic nerve after adjuvant-induced inflammation in rat hindpaw (10).

Although numerous studies implicate sensory neuropeptides in gastrointestinal pathophysiology, evidence for neurogenic inflammation in the gastrointestinal tract remains controversial. Levels of substance P have been reported to be increased in the jejunum of rats infected with Trichinella spiralis (35) and in the colon of patients with ulcerative colitis (22). In contrast, CGRP and substance P levels in the trinitrobenzenesulfonic acid and immune complex models of colitis are either unchanged or decreased (11, 12, 24, 27, 28). The discrepancy between these studies and the results of this investigation may be explained by the use of different models (bacterial toxin mediated vs. chemical/immune mediated), time courses of inflammation (acute vs. chronic), and animals (rats vs. rabbits).

The site of action of CGRP in the toxin A model of intestinal secretion and inflammation cannot be elucidated from our results. Previous anatomic studies suggest that primary sensory afferent neurons predominantly terminate in the myenteric and submucosal plexii and around submucosal blood vessels in the gastrointestinal tract (29). Indeed, localization of CGRP and substance P receptors to myenteric neurons has been demonstrated using quantitative receptor autoradiography and confocal microscopy (14, 23). Interestingly, Mantyh et al. (23) recently reported internalization of substance P receptors on enteric neurons after administration of toxin A to ileal loops. These data suggest that sensory neurons activated by toxin A may exert their effects, at least in part, by regulating the function of other nerves.

CGRP receptors may also be present on nonneuronal cells located in the lamina propria. The dose apposition of sensory afferent neurons and intestinal mast cells suggests that neuropeptides such as CGRP and substance P may directly interact with these cells (31, 32). In support of this hypothesis, we have shown that pretreatment of rats with the substance P receptor antagonist CP-96,345 can abolish release of rat mast cell protease II from ileal explants exposed to toxin A (25). Although CGRP receptors have not been identified on mucosal mast cells, this peptide can induce release of histamine from peritoneal mast cells (19). Another site of action of CGRP may be intestinal enterocytes. In a recent report, Cox and Tough (8) indicated that the human intestinal epithelial lines Col-29 and HCA-7 express functional CGRP receptors. Vascular cells regulating vasodilation and vascular permeability are also likely to interact with CGRP.

In this study we demonstrate for the first time increased production of CGRP in response to acute intestinal inflammation induced by C. difficile toxin A. We also show that luminal application of toxin A leads to an early activation of CGRP-containing sensory neurons in lumbar dorsal root ganglia. Furthermore, in vivo administration of a CGRP antagonist to rats significantly reduces the intestinal response to this toxin. These findings and previous studies suggest that primary sensory afferent nerves containing CGRP and substance P play an important role in the pathogenesis of toxin A-induced enteritis.

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