

Corticotropin-releasing hormone (CRH) requirement in *Clostridium difficile* toxin A-mediated intestinal inflammation

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Clostridium difficile, the causative agent of antibiotic-associated colitis, mediates inflammatory diarrhea by releasing toxin A, a potent 308-kDa enterotoxin. Toxin A-induced inflammatory diarrhea involves many steps, including mucosal release of substance P (SP) corticotropin-releasing hormone (CRH) and neutrophil transmigration. Here we demonstrate that, compared with wild type, mice genetically deficient in CRH (*Crh*^{-/-}) have dramatically reduced ileal fluid secretion, epithelial cell damage, and neutrophil transmigration 4 h after intraluminal toxin A administration. This response is associated with diminished mucosal activity of the neutrophil enzyme myeloperoxidase compared with that of wild-type mice. In wild-type mice, toxin A stimulates an increase in intestinal SP content compared with buffer administration. In contrast, toxin A administration in *Crh*^{-/-} mice fails to result in an increased SP content. Moreover, immunohistochemical experiments showed that CRH and SP are colocalized in some enteric nerves of wild-type mice, and this colocalization is more evident after toxin A administration. These results provide direct evidence for a major proinflammatory role for CRH in the pathophysiology of enterotoxin-mediated inflammatory diarrhea and indicate a SP-linked pathway.

Corticotropin-releasing hormone (CRH), a 41-aa peptide, is a major peptide hormone that modulates the activity of the hypothalamic–pituitary–adrenal axis during stress, including inflammation (1–3). CRH mediates its effects by binding to its G protein-coupled receptor subtypes, CRH1 and CRH2 (4–6). CRH plays a dual role in the pathophysiology of inflammation. CRH secreted from the hypothalamus has indirect antiinflammatory effects via stimulation of glucocorticoid release, but CRH is also expressed peripherally, and its expression is increased in leukocytes, nerve fibers, and other cells involved in inflammatory reactions (7–12). Moreover, *in vivo* and *in vitro* studies indicate that CRH is a potent proinflammatory peptide (7, 12–15).

A limited number of studies indicate that CRH and its receptors play a role in the pathophysiology of intestinal inflammation. Thus, Van Tol *et al.* (11) found increased CRH expression in the rat cecum in colitis induced by injection of peptidoglycan-polysaccharide polymers. Increased expression of CRH-immunoreactive cells was also evident in the colonic mucosa of patients with inflammatory bowel disease (16). CRH gene expression is increased in hypothalamic neurons during colitis after intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (17) and appears to play a protective role in the worsening of experimental colitis induced by stress (18). We have recently shown that expression of CRH as well as CRH1 and CRH2 was dramatically increased in mouse ileum shortly after intraluminal administration of *Clostridium difficile* toxin A (19). Moreover, experiments with CRH receptor antagonists indicate that CRH plays a proinflammatory role in toxin A-induced intestinal secretion and inflammation in mice and that CRH1 is important in the mediation of these responses (19).

C. difficile is the primary agent responsible for antibiotic-associated diarrhea and pseudomembranous colitis after antibiotic therapy (20). *C. difficile* causes diarrhea and colitis by releasing two high-molecular-mass protein exotoxins, toxin A and B, with potent cytotoxic and enterotoxic properties in animal and human intestine (21). Injection of toxin A into ileal or colonic loops of anesthetized animals triggers mucosal neutrophil infiltration and increases fluid secretion and mucosal permeability 1–4 h after toxin A administration (22–25). Although the *in vivo* mechanism(s) leading to acute enterocolitis are not completely understood, activation of sensory nerves (26, 27) and release of sensory neuropeptides, such as substance P (SP) (25, 28, 29), are pivotal in the mediation and amplification of the inflammatory signal in response to toxin A.

The development of CRH-deficient mice (30, 31) allows us to directly evaluate the contribution of CRH in *C. difficile* toxin A-induced ileal fluid secretion and intestinal inflammation. *Crh*^{-/-} mice have impaired hypothalamic–pituitary–adrenal axis activity as shown by their lower plasma corticosterone levels after physiological or psychological stimuli (30, 32), or carrageenin-induced acute inflammation (33). Because we have shown that endogenous corticosteroid controls the intestinal secretory and inflammatory effects of toxin A, we studied circulating corticosterone levels in *Crh*^{+/+} and *Crh*^{-/-} mice after toxin A administration. Because SP plays a major role in the development and progress of toxin A-induced intestinal secretion and inflammation (34), we studied whether CRH deficiency is associated with alterations in the intestinal levels of SP in response to toxin A and examined additionally whether SP and CRH colocalize in intestinal nerves.

Methods

Mouse Closed Ileal Loop Model. Male *Crh*^{+/+} and *Crh*^{-/-} mice, generated as described (30), and of 129 × C57BL/6 genetic background weighing 23–25 g were housed into individual cages under controlled conditions on a 12-h light–dark cycle. Mice were fasted 12–18 h before the experiments to avoid formation of stool but had free access to a 5% glucose solution to prevent hypoglycemia and hypothermia. Experiments were performed between 9:30 a.m. and 11:30 a.m. to minimize influence on the circadian rhythm. Mice were anesthetized with a mixture of ketamine (0.9 ml, 100 mg/ml) and xylazine (0.1 ml, 100 mg/ml) in 9 ml of saline at a dose of 0.15 ml/20 g of body weight. Before anesthesia and at the end of the experiment, blood samples were taken by retroorbital eye bleeding of conscious mice by using heparinized capillary tubes for plasma corticosterone measure-

Abbreviations: CRH, corticotropin-releasing hormone; MPO, myeloperoxidase; SP, substance P; TBS, Tris-buffered saline.

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ments. A laparotomy was then performed, and one 3- to 5-cm-long loop was formed at the terminal ileum as described (34). Loops were injected with either 0.15 ml of 50 mM Tris buffer (pH 7.4) containing 10 μ g of purified toxin A or buffer alone (control). The abdomen was then closed, and animals were placed on a heating pad at 37°C for the duration of the experiment. After 2 h, some animals ($n = 6$ per group) were killed by CO₂ inhalation, and loops were collected and washed in Hanks' Balanced Salt Solution containing 0.35 g/liter NaHCO₃ for SP measurements. At 4 h the remaining animals ($n = 6$ per group) were killed, and fluid secretion was estimated as the loop weight-to-length ratio as described (34, 35). Tissues were then washed in saline, cut into 5 \times 5-mm pieces, and snap-frozen for myeloperoxidase (MPO) measurements. Full-thickness loop sections were also fixed in formalin, paraffin-embedded, and stained with hematoxylin/eosin. Histologic severity of enteritis was graded under previously established toxin A-associated histologic parameters by a "blinded" gastrointestinal pathologist (M.O.) (28). Animal studies were approved by the Institutional Animal Care and Use Committee.

MPO Activity. MPO activity was determined by a modified method of Bradley *et al.* (36). After homogenization, loop samples were frozen and thawed three times and then sonicated (Heat Systems, Ultrasonics, Farmingdale, NY) in 1.5 ml of 50 mM phosphate buffer containing 0.5% of hexadecyl-trimethyl ammonium bromide. Samples were then centrifuged (10,000 \times g for 15 min at 4°C), and supernatants were further diluted into the same phosphate buffer containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 5.10⁻⁴% of hydrogen peroxide. MPO activity was measured spectrophotometrically (Lambda 20, UV/VIS spectrophotometer, Perkin-Elmer) at 450 nm by using human MPO (0.1 unit/100 μ l; Sigma) as a standard. Protein concentration was determined by using the Bio-Rad Detergent Compatible Protein Assay, and MPO activity is expressed as MPO units/g of protein.

Plasma Corticosterone. Blood samples from both mice genotypes were centrifuged at 2,000 \times g (10 min at 4°C), plasma was collected, and aliquots were stored at -80°C. Corticosterone levels were measured by an RIA kit (ICN) as described (19), and results are expressed in μ g/dl.

SP Content. Ileal loops were cut longitudinally and washed in ice-cold Hanks' balanced salt solution. Samples were then homogenized in 1.5 ml of 0.1 M ice-cold HCl for 10 sec and then centrifuged at 10,000 \times g (15 min at 4°C). The supernatants were collected and absorbed on C18 cartridge columns (Waters) as described (34). SP was measured in the eluates by an immunoassay (EIA, Peninsula Laboratories). Protein concentration was measured by the BCA protein assay (Pierce), and results are expressed in pmol/mg of protein.

SP Immunohistochemistry. Toxin A or buffer was injected into ileal loops of wild-type and *Crh*^{-/-} mice ($n = 3$ mice per group), and after 2 h mice were killed, and freshly frozen sections were prepared. Ileal sections were embedded in OCT compound (Tissue-Tek, Redding, CA) for 10 min and then cut at 6- μ m thickness. The sections were then fixed in cold acetone (80%) and allowed to air-dry before being washed in Tris-buffered saline (TBS) (pH 7.5) and blocked of endogenous peroxidase with 0.6% H₂O₂ in methanol for 20 min at room temperature. Slides were washed in TBS and incubated at room temperature, serially, in avidin and biotin (20 min each) to block endogenous biotin and avidin. Slides were then washed in TBS and incubated at room temperature for 1 h in 1% BSA and goat (for anti-SP) serum. The sections were blotted and incubated overnight at 4°C with a polyclonal antibody directed against SP (Santa Cruz

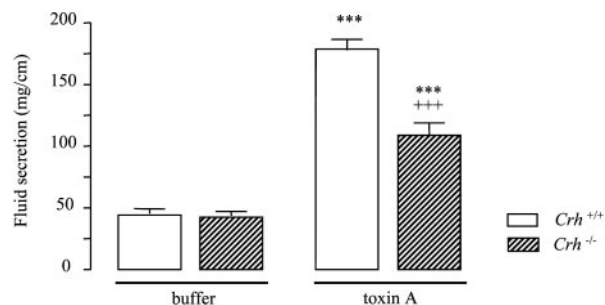


Fig. 1. Effect of toxin A in ileal fluid secretion of *Crh*^{+/+} and *Crh*^{-/-} mice. Ileal loops of *Crh*^{+/+} and *Crh*^{-/-} mice were exposed for 4 h to buffer or buffer-containing toxin A (10 μ g). Bars represent mean \pm SEM changes in fluid secretion ($n = 6$ per group). ***, $P < 0.001$ vs. respective buffer-treated groups; +++, $P < 0.001$ vs. toxin A-treated *Crh*^{+/+} mice.

Biotechnology) at a dilution of 1:100 or PBS for control slides. After washing in TBS, slides were washed after incubation, incubated in biotinylated IgG (1 h at room temperature), washed again, and incubated in peroxidase conjugated with streptavidin (1 h, room temperature). After washing, color was developed with 3,3'-diaminobenzidine and enhanced with copper sulfate. The slides were then counterstained with methyl green and mounted, and images were viewed under light microscopy (Eclipse E800, Nikon) by using a plan Apo 40 \times /0.95 objective, imported via a SPOT Insight camera (Diagnostic Instruments, Sterling Heights, MI), and stored digitally by using SPOT software (Diagnostic Instruments).

Immunohistochemistry for SP, CRH, and Neurofilaments. Frozen intestinal sections were prepared as described above, placed on Fisher superfrost slides, and fixed in 80% acetone at room temperature for 60 sec. Slides were air-dried and stored overnight at -20°C in a sealed container in Drierite (Drierite, Xenia, OH). Before labeling, sections were rehydrated in TBS, blocked for endogenous avidin and biotin, and washed in TBS. Sections were incubated with a rat anti-SP antibody (Novus Biologicals, Littleton, CO) (1:100) linked to a Texas red-labeled rabbit anti-rat IgG, a rabbit anti-CRH antibody (Advanced Targeting Systems, San Diego) (1:750) linked to a biotinylated goat anti-rabbit IgG then avidin conjugated to FITC, or with a rabbit anti-neurofilament antibody (provided by Georges Perides, Tufts University School of Medicine, Boston) (1:100) linked to a biotinylated goat anti-rabbit IgG and avidin-conjugated cy-5. All antibodies were incubated overnight at 4°C. All second- and third-stage antibodies were incubated at room temperature for 60 min. After final washing, slides were coverslipped with VECTASHIELD (Vector Laboratories), sealed with nail polish,

Table 1. Reduced histological severity of ileitis in *Crh*^{-/-} mice

Treatment	Histology		
	Epithelial damage	Congestion and edema	Neutrophil infiltration
<i>Crh</i> ^{+/+} + buffer	0.22 \pm 0.17	0.39 \pm 0.11	0.11 \pm 0.07
<i>Crh</i> ^{+/+} + toxin A	1.23 \pm 0.23**	1.44 \pm 0.24**	1.64 \pm 0.28***
<i>Crh</i> ^{-/-} + buffer	0	0.71 \pm 0.29	0.42 \pm 0.29
<i>Crh</i> ^{-/-} + toxin A	0.33 \pm 0.21	0.58 \pm 0.26 [†]	0.41 \pm 0.19 [‡]

Histological severity was graded by a score of 0–3, taking into account epithelial cell damage, congestion, and edema of the mucosa and mucosal neutrophil infiltration in ileal loops of *Crh*^{+/+} and *Crh*^{-/-} mice treated with buffer or toxin A for 4 h. Data are presented as mean \pm SEM. ** and ***, $P < 0.01$ and 0.001, respectively, vs. buffer-treated *Crh*^{+/+} mice. [†] and [‡], $P < 0.05$ and 0.001, respectively, vs. toxin A-treated *Crh*^{+/+} mice.

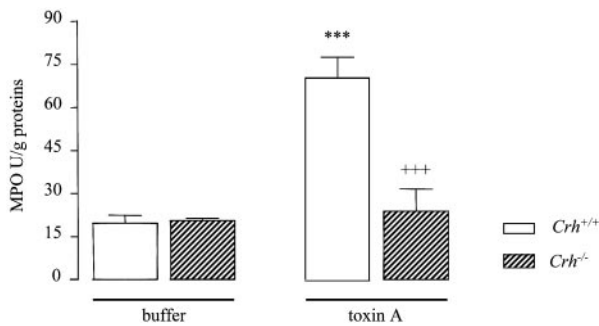


Fig. 2. Effect of toxin A in ileal MPO levels in *Crh*^{+/+} and *Crh*^{-/-} mice. Four hours after injection of buffer or toxin A, ileal loops were homogenized, and MPO activity was determined by colorimetric assay as described in *Methods*. Values are mean \pm SEM ($n = 6$ per group). ***, $P < 0.001$ vs. respective buffer-treated *Crh*^{+/+} mice; +++, $P < 0.001$ vs. toxin A-treated *Crh*^{+/+} mice.

and single- and triple-immunolabeling viewed on a confocal microscope (MRC 1024, Bio-Rad). To verify the specificity of the immunolabeling, some sections were incubated without the primary antibodies, before addition of the second- and third-stage antibodies.

Statistical Analyses. Results were expressed as mean \pm SEM, and data were analyzed by using the statistics software program PRISM (GraphPad, San Diego). An ANOVA was performed, and, when appropriate, a Tukey post hoc test was used for intergroup comparisons. $P < 0.05$ was considered significant.

Results

CRH-Deficient Mice Have Reduced Ileal Secretion, Histologic Damage, and Inflammation in Response to Toxin A. Our previous results indicated that CRH receptor blockade is associated with reduced fluid secretion and intestinal inflammation after intraluminal toxin A administration (19). To directly assess the role of CRH in the toxin A model of intestinal inflammation, we injected ileal loops of anesthetized *Crh*^{+/+} and *Crh*^{-/-} mice with purified toxin A, and after 4 h we measured ileal fluid secretion and neutrophil infiltration. Our results show that basal intestinal fluid secretion was similar in *Crh*^{+/+} and *Crh*^{-/-} mice (Fig. 1*A*). As expected, ileal toxin A administration in *Crh*^{+/+} animals stimulates a 4-fold increase of fluid secretion expressed as a weight/length ratio (Fig. 1*A*). In contrast, *Crh*^{-/-} mice have significantly diminished (2-fold) fluid secretion in response to toxin A compared with

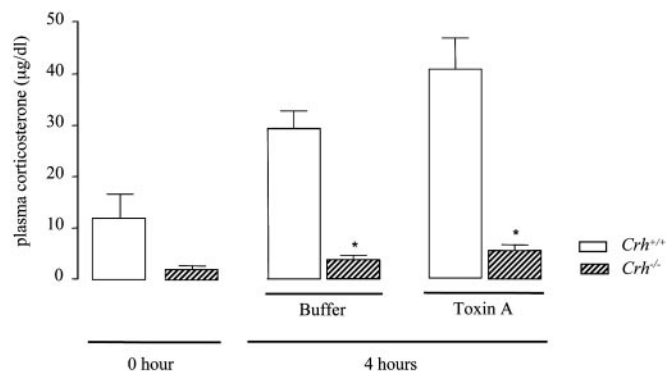


Fig. 3. Effect of toxin A in blood corticosterone levels in *Crh*^{+/+} and *Crh*^{-/-} mice. Before anesthesia and at the end of the experiment, blood was collected by retroorbital phlebotomy. After centrifugation, 5 μ l of plasma was used to measure corticosterone with an RIA kit. Bars represent mean \pm SEM of plasma corticosterone ($n = 6$ per group). *, $P < 0.05$ vs. buffer and toxin A-treated *Crh*^{+/+} mice, respectively (4 h).

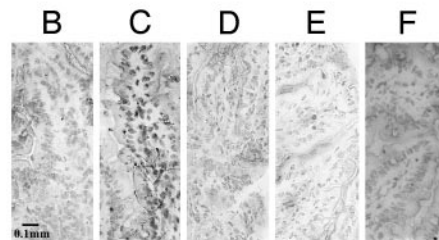
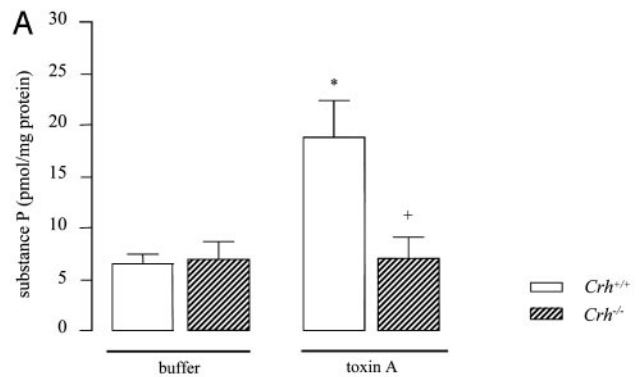


Fig. 4. Effect of toxin A in ileal SP content in *Crh*^{+/+} and *Crh*^{-/-} mice. Mice ileal loops were injected with either toxin A (10 μ g) or buffer (control). (*A*) Animals were killed after 2 h; full-thickness ileal samples were assayed for SP content by using an enzyme-immunosorbent assay, and SP-immunoreactive cells were assayed for SP content by immunohistochemistry with a polyclonal antibody directed against SP (see *Methods* for details). Values are mean \pm SEM ($n = 6$ per group). *, $P < 0.05$ vs. buffer-treated *Crh*^{+/+} mice; +, $P < 0.05$ vs. toxin A-treated *Crh*^{+/+} mice. *B* and *C* illustrate buffer- or toxin A-treated ileal sections, respectively, of *Crh*^{+/+} mice; *D* and *E* represent buffer- or toxin A-treated ileal sections, respectively, of *Crh*^{-/-} mice; and *F* represents buffer-exposed tissues incubated with PBS instead of primary antibody. Arrows indicate positive cells. Very little SP staining is present in the control (buffer-exposed) mouse ileum of both genotypes (*B* and *D*). However, in *Crh*^{+/+} mice, after toxin A injection, a substantial increase in the expression of SP immunoreactive cells is noted (*C*), whereas very little increase is observed in tissues from toxin A-exposed *Crh*^{-/-} mice (*E*). Immunohistochemical results are representative of three separate experiments per experimental condition. (Magnification, $\times 40$.)

wild-type mice (Fig. 1*A*). Histologic evaluation of ileal tissues reveals normal mucosal architecture of buffer-treated *Crh*^{-/-} mice compared with buffer-exposed *Crh*^{+/+} mice, whereas toxin A exposure in *Crh*^{+/+} mice results in increased epithelial cell damage, congestion and edema of the mucosa, and neutrophil transmigration (Table 1). In contrast, *Crh*^{-/-} mice demonstrate significantly reduced epithelial damage, congestion and edema of the mucosa, and neutrophil infiltration in response to toxin A (Table 1). Moreover, wild-type animals exposed to toxin A show a 3.5-fold increase in ileal MPO activity (Fig. 2). Interestingly, tissue MPO levels after toxin A administration are almost normalized in *Crh*^{-/-} mice (Fig. 2). Taken together, these results clearly demonstrate the importance of CRH in the pathophysiology of *C. difficile* toxin A-mediated intestinal secretion and inflammation.

Corticosterone Responses Before and After Toxin A Exposure in *Crh*^{+/+} and *Crh*^{-/-} Mice. Because previous studies indicated that endogenous corticosteroids play a protective role in the development of toxin A-induced enteritis (37), we measured blood corticosterone levels after buffer and toxin A administration into ileal loops in *Crh*^{+/+} and *Crh*^{-/-} mice. Before induction of anesthesia and surgery, plasma levels of corticosteroids were \approx 6-fold lower in CRH-deficient mice compared with *Crh*^{+/+} mice (Fig. 3).

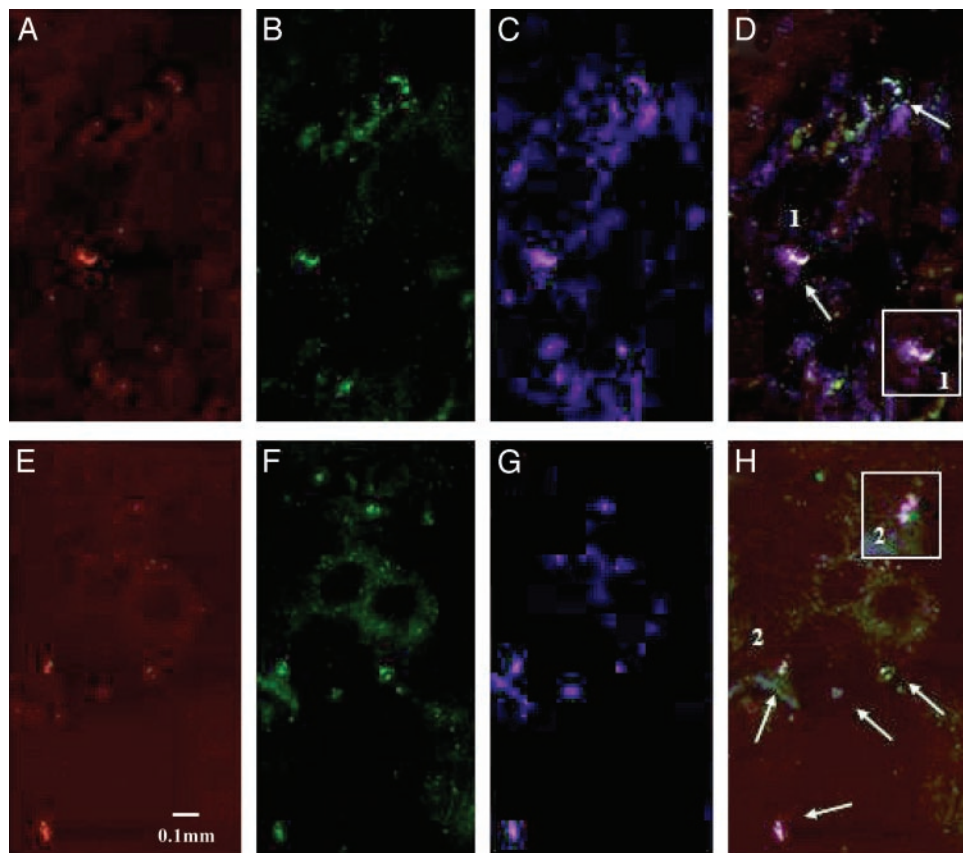


Fig. 5. CRH–SP colocalization in intestinal nerves. Mouse ileal loops were injected with either toxin A (10 μ g) or buffer (control). Animals were killed after 2 h, and full-thickness ileal samples were processed for identification of CRH- (green), SP- (red), and neurofilament-immunoreactive (blue) cells by using polyclonal antibodies as described in *Methods*. Treatment groups are displayed in rows: *A–D*, buffer-treated *Crh*^{+/+} mice; *E–H*, toxin A-treated *Crh*^{+/+} mice. Triple labeling (*D* and *H*) is illustrated by arrows. Single immunolabeling shows that all treatment groups have SP and CRH immunoreactivity that is significantly increased in toxin A-treated *Crh*^{+/+} mice (*E* and *F*). Moreover, the merged images demonstrate that intestinal nerves colocalize both SP and CRH in *Crh*^{+/+} mice at a significantly higher level after toxin A exposure compared with buffer-treated animals (*D* and *H*). Results are representative of three separate experiments per experimental condition. (Magnification, $\times 100$.)

Consistent with our prior studies in wild-type mice (19), plasma corticosterone levels in buffer-exposed loops are higher compared with preanesthesia levels (Fig. 3), probably because of the stress of the operation. In *Crh*^{-/-} mice, corticosterone levels are substantially lower after buffer or toxin A administration compared with *Crh*^{+/+} mice, in line with previous studies suggesting their unresponsiveness to stress (30).

SP Content in Response to Toxin A Is CRH-Dependent. As discussed above, several prior studies indicate that SP and its neurokinin 1 receptor are major proinflammatory mediators in the pathogenesis of toxin A-mediated enterocolitis (reviewed in refs. 38 and 39). Thus, we next determined whether CRH deficiency is linked to altered SP response during toxin A-induced inflammation. In both wild-type and *Crh*^{-/-} mice, basal tissue levels of SP obtained from buffer-exposed loops are similar (Fig. 4*A*). Moreover, only a few cells are stained positive for SP in tissue sections obtained from buffer-exposed ileal loops of both *Crh*^{+/+} and *Crh*^{-/-} mice (Fig. 4*B*). In line with prior observations (25, 40), toxin A administration in *Crh*^{+/+} mice results in increased SP content (Fig. 4*A*) and immunostaining (Fig. 4*B*). In contrast, SP content and immunostaining is not increased in toxin A-exposed *Crh*^{-/-} mice compared with wild-type mice (Fig. 4*A* and *B*). These results suggest that in the mouse, CRH-mediated ileal secretion and inflammation may involve SP.

SP and CRH Colocalize on Intestinal Nerves. Because our results suggested a CRH–SP-related pathway (Fig. 4), we next used an

immunofluorescence approach to examine whether CRH and SP are present in separate neurons and whether they colocalize on the same intestinal nerves. Single immunolabeling experiments identified SP and CRH immunoreactive cells in both buffer- and toxin A-exposed ileum (Fig. 5*A* and *E* for SP and *B* and *F* for CRH). Moreover, there are more SP and CRH immunoreactive cells in toxin A-exposed ileal loops (Fig. 5*B* and *F*) compared with controls (Fig. 5*A* and *B*). Using three different antibodies, we demonstrate colocalization of SP and CRH in neurofilament-positive cells in buffer-exposed sections of wild-type mice (Fig. 5*D*) that is more evident in toxin A-exposed sections (Fig. 5*H*). No CRH was detectable by immunohistochemistry in *Crh*^{-/-} mice (data not shown).

Discussion

Our present results provide direct evidence for the importance of CRH in ileal fluid secretion, histologic damage, and mucosal neutrophil transmigration caused by *C. difficile* toxin A. These results support our previous findings with CRH antagonists, indicating a proinflammatory role of peripheral CRH in *C. difficile* toxin A-induced enteritis (19). Interestingly, the increase in SP content that occurs after toxin A treatment in wild-type mice is not observed in *Crh*^{-/-} mice. To our knowledge, this is the first report indicating that a CRH–SP-related pathway is involved in the pathogenesis of inflammatory diarrhea.

We found that, compared with wild-type mice, *Crh*^{-/-} mice have significantly lower fluid secretion and diminished neutro-

phil transmigration and activation after toxin A administration. This result is clearly indicated by the diminished number of neutrophils infiltrating the ileal mucosa (Table 1), and their degree of activation is demonstrated by lower MPO activity (Fig. 2). This finding is consistent with previous data demonstrating a reduction of leukocytic infiltration after turpentine administration in *Crh*^{-/-} mice (41). The pathogenesis of *C. difficile* toxin A-mediated ileal fluid secretion involves a neutrophil-dependent release of proinflammatory cytokines from the intestinal mucosa (24). Moreover, CRH expression is increased in the ileal mucosa after toxin A exposure, whereas CRH antagonism inhibits neutrophil activation and intestinal cytokine release in toxin A-exposed mouse ileal loops (19). Similarly, immunoneutralization of peripheral CRH inhibits carrageenin-induced granuloma in rats (7), whereas CRH receptor antagonism reduces neutrophil infiltration in the same model of inflammation (33, 42). Based on these findings, we conclude that the reduced inflammatory responses of *Crh*^{-/-} mice during enterotoxin-mediated enteritis are likely due to the absence of CRH expressed in the intestinal mucosa.

An interesting question relates to the contribution of corticosteroids in the reduced enterotoxic responses after toxin A exposure in *Crh*^{-/-} mice. Our findings (Fig. 3) indicate that the diminished intestinal secretion and inflammation observed in *Crh*^{-/-} mice are not related to their glucocorticoid levels. *Crh*^{-/-} mice have adrenocortical atrophy and, thus, reduced normal and stress-induced corticosterone levels (43, 44). We have previously shown that carrageenin-induced acute inflammation does not induce corticosterone levels to the same degree in wild-type and *Crh*^{-/-} mice (33). Furthermore, we and others have shown a delayed corticosterone rise but finally similarly increased levels of corticosterone in wild-type and *Crh*^{-/-} mice in response to other inflammatory stimuli such as lipopolysaccharide, turpentine, and 2C11 administration (33, 41, 45).

Several pieces of evidence indicate that release of SP and its subsequent interaction with enteric nerves and immune and inflammatory cells of the intestinal lamina propria represent an important amplification system in the pathophysiology of *C. difficile* toxin A-induced inflammation (38). Mice deficient in neurokinin 1 receptor have dramatically reduced intestinal responses to toxin A, underscoring the importance of SP and its neurokinin 1 receptor in toxin A-associated diarrhea (34). Here we demonstrate that in *Crh*^{-/-} mice the rise in SP content after toxin A exposure does not occur (Fig. 4). We also found that SP colocalizes with CRH in enteric nerves, and that expression of both is increased after toxin A exposure (Fig. 5). Skofitsch *et al.* (46) showed that SP is colocalized with CRH in sensory ganglia and capsaicin-sensitive sensory neurons. A previous study also suggested the possibility of SP-CRH communication at the periphery. Jessop *et al.* (3) presented the hypothesis that release of SP and CRH from neuronal terminals in response to inflammatory stimuli as well as stimulation of CRH release from macrophages by SP may represent an important proinflammatory pathway in a rodent model of rheumatoid arthritis. However, to our knowledge, a functional relationship between SP and CRH at inflammatory peripheral sites has not been investigated. Our current findings (Fig. 5), together with results from previous studies (6, 11, 16, 19), suggest that CRH secreted from neurons of the intestinal mucosa in response to administration of toxin A may stimulate the release of SP and possibly other peptides from enteric nerves and sensory neurons that initiate and perpetuate a cascade of neuroimmunointeractions associated with the acute intestinal inflammatory response. More studies are needed to identify the mechanism of interaction(s) between these two important proinflammatory neuropeptides.

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