Substance P receptor expression in intestinal epithelium in Clostridium difficile toxin A enteritis in rats

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Pothoulakis, Charalabos, Ignazio Castagliuolo, S. E. Leeman, Chi-Chung Wang, Hanzong Li, Beth J. Hoffman, and Eva Meze. Substance P receptor expression in intestinal epithelium in Clostridium difficile toxin A enteritis in rats. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G68–G75, 1998.—We previously reported that the inflammatory effects of Clostridium difficile toxin A on rat intestine can be significantly inhibited with a specific neurokinin-1 receptor (NK-1R) antagonist. In this study we investigated the localization and expression of NK-1R mRNA and protein in rat intestine by in situ hybridization, Northern blot analysis, and immunohistochemistry, respectively, after exposure to toxin A. Northern blot analysis showed increased mucosal levels of NK-1R mRNA starting 30 min after toxin A administration. In situ hybridization showed that toxin A increased NK-1R mRNA expression in intestinal epithelial cells after 30, 120, and 180 min. In rats pretreated with the NK-1R antagonist CP-96345 the increase in NK-1R mRNA levels after exposure to toxin A was inhibited, indicating that NK-1R upregulation is Substance P (SP) dependent. One hour after exposure to toxin A many of the intestinal epithelial cells showed staining for NK-1R compared with controls. Specific [125I]-SP binding to purified epithelial cell membranes obtained from ileum exposed to toxin A for 15 min was increased twofold over control and persisted for 4 h. This report provides evidence that NK-1R expression is increased in the intestinal epithelium shortly after exposure to toxin A and may be important in toxin A-induced inflammation.

DURING THE LAST FEW YEARS a great deal of research has been conducted on the pathways by which neuropeptides control intestinal epithelial cell function and inflammation. One of the peptides involved in intestinal inflammation is Substance P (SP), an 11-amino acid peptide member of the tachykinin family (9, 10). Binding sites for SP are elevated in the colon of patients with Crohn’s disease and ulcerative colitis (20, 23), and pretreatment of animals with neurokinin-1 receptor (NK-1R) antagonists inhibits colonic inflammation in several animal models of colitis (24, 39). Many studies also showed that application of SP to animal intestine causes Cl− secretion from intestinal epithelial cells (10, 17, 27, 33, 40). Studies from our laboratory and others documented that SP is a major mediator in inflammatory diarrhea caused by Clostridium difficile toxin A in an animal model of intestinal secretion and inflammation (30). Pretreatment of rats with either capsaicin, a drug that targets primary sensory neurons, or specific nonpeptide NK-1R antagonists dramatically inhibited ileal fluid secretion, mucosal permeability, and inflammatory infiltrate mediated by C. difficile toxin A (6, 22, 31), the primary cause of antibiotic-associated diarrhea and colitis in animals and humans (29). Recent interesting results showed increased NK-1R expression in C. difficile-induced pseudomembranous colitis (21). Furthermore, Castagliuolo et al. (4) showed that injection of toxin A into rat ileum causes a rapid increase in SP content in lumbar dorsal root ganglia and ileal mucosal scrapings 30 to 60 min after toxin A administration, suggesting an early involvement of SP in toxin A enteritis.

SP mediates its effects by binding to its specific NK-1R (12, 41), and many in vivo and in vitro studies show that the small and large intestine can respond to stimulation by SP. Brown et al. (2) showed that SP produces sodium and bicarbonate secretion in porcine jejunal mucosa sheets through actions on enteric neurons and intestinal epithelial cells. In another study, Keast et al. (13) provided presumptive evidence for the presence of SP receptors on mucosal cholinergic and noncholinergic nerves as well as on intestinal epithelial cells. Rangachari et al. (33) also concluded that SP stimulates canine colonic transport directly by acting on colonocytes and indirectly via stimulation of enteric neurons. Recently, Cooke et al. (11), using in situ hybridization and RT-PCR techniques, showed the presence of NK-1Rs on isolated colonocytes from guinea pigs.

The NK-1R, cloned by Yokota et al. (41) and Hershey and Krause (12), has 407 amino acids and belongs to the family of G protein-coupled receptors. Many studies indicate that one gene and one mRNA encode the NK-1R (12, 25, 41). Although NK-1R mRNA (11, 15) and SP binding sites (15) have been reported in the epithelial cells of the colon, direct evidence for the localization of the NK-1R and its mRNA on the epithelial cells of the small intestine and its modulation during inflammatory and secretory processes has been lacking. Studies presented here show time-dependent increases in the expression of the rat intestinal NK-1R mRNA and receptor protein after intraluminal administration of C. difficile toxin A. We also report that prior in vivo administration of the specific NK-1R antagonist CP-96345 inhibits the increase of the abundance of mRNA encoding for the NK-1R after toxin A.
MATERIALS AND METHODS

Materials

Toxin A was purified to homogeneity from broth culture supernatants of C. difficile strain 10,463 as previously described (14, 31, 32). Enterotoxicity and cytotoxicity of toxin A were determined in rat ileal loops and human lung (IMR-90) fibroblasts, respectively (31, 32). Because previous studies showed that 5 µg of purified toxin A induce fluid secretion, increase mannitol permeability, and cause an acute inflammatory infiltrate in rat ileal loops in vivo (4, 6, 31, 32), the same dose was used in all experiments. The NK-1R antagonist CP-96345 was generously provided by Pfizer Diagnostics. CP-96345 was dissolved in 0.9% saline immediately before use and injected intraperitoneally as described previously (31). Pentobarbital sodium was purchased from Abbott (Chicago, IL). Protein concentrations were determined by the bicinchoninic acid protein assay reagent (Pierce Laboratories, Rockford, IL).

Methods

Preparation of ileal loops. Fasted male Wistar rats (200-250 g; Charles River Breeding Laboratories, Wilmington, MA) were anesthetized by an intraperitoneal injection of pentobarbital sodium (35 mg/kg). Laparotomy was then performed, and two closed 5-cm-long ileal loops for each animal were formed and injected with either toxin A (5 µg) in 0.4 ml 50 mM Tris buffer or buffer alone as previously described (4, 6, 32). The abdomen was then closed, and body temperature was maintained at 37°C by a heating pad. At the indicated time points animals were killed by a bolus of intraperitoneal pentobarbital (120 mg/kg) and the ileal loops were removed and harvested for the different types of experiments as will be described. For immunohistochemical studies full-thickness sections of loops were fixed in Formalin at several time points after toxin A administration, paraffin embedded, and stored at room temperature until use. This study was approved by the Beth Israel Deaconess Medical Center and Boston University Medical Center Hospital Institutional Animal Care and Use Committee.

Northern analysis of NK-1R mRNA. Ileal mucosa from toxin A- or buffer-injected loops obtained from different exposure times as previously described (n = 6/group) was scraped off with a glass slide and stored at −70°C. Tissue was homogenized in 4 M guanidium isothiocyanate (Stratagene, La Jolla, CA) and centrifuged at 1,000 g for 10 min at room temperature. The supernatant was layered onto an equal volume of 5.7 M cesium chloride, and this mixture was centrifuged at 35,000 rpm in an SW40Ti rotor (Beckman Instruments, Fullerton, CA) for 20 h. The pellet containing purified RNA was resuspended in diethyl pyrocarbonate-treated water, precipitated with ethanol, and stored at −70°C. Total RNA (20 µg) was denatured by heating at 75°C in a sample buffer containing ethidium bromide, 2.2 M formaldehyde, and 50% formamide, and fractionated on a 1.2% formaldehyde-agarose gel in 1× MOPS buffer. Optimal resolution of the RNA was achieved by the use of thin surface tension gels. Equal lane loading was visualized under ultraviolet light and further examined by the 32P-labeled structural gene cDNA probe. RNA was electrophoresed onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). A 588-bp Xho I and Xba I NK-1R cDNA fragment was released and labeled with 32P by random primer extension (Amersham, Arlington Heights, IL) to a specific activity of 2 × 109 counts·min−1·µg−1. The labeled cDNA probe was used to hybridize overnight to the NK-1R mRNA on nitrocellulose filter in a buffer containing 6× saline-sodium citrate (SSC), 0.5% SDS, 10 mM EDTA, 1× Denhardt's solution, and 100 µg/ml of salmon sperm DNA at 65°C. The filter was washed four times in low-stringency buffer (2× SSC and 0.1% SDS) at room temperature. The NK-1R mRNA was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak, Rochester, NY). The band intensity of NK-1R versus cyclophilin was achieved by densitometric scanning (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software, and the mean average ratios of NK-1R versus cyclophilin were calculated. Data were analyzed using the SIGMA-STAT program (Jandel Scientific Software, San Rafael, CA). ANOVA with protected t-test was used for intergroup comparisons.

In situ hybridization of NK-1R mRNA. Rats were anesthetized, and ileal loops were then prepared and injected with toxin A or buffer as previously described. At the indicated time points tissues were removed and quickly frozen on powdered ice. Longitudinal sections (12 mm thick) were cut in a Reichert Fricot 2500 cryostat and mounted on silanized slides, and in situ hybridization histochemistry was performed as described previously (1). Briefly, the sections were fixed in 4% formaldehyde in 1× PBS and dehydrated in ethanol and chloroform. Hybridization buffer containing the probe was put on the sections (1 million dpm per 50 µl hybridization buffer). The slides were incubated overnight at 55°C in a humidified chamber and washed the next day. The slides were exposed to X-ray film (Hyperfilm MP; Amersham) and subsequently dipped in Kodak NTB3 emulsion for 2–8 wk, developed, and stained with toluidine blue (1%). The results were examined with a Leitz Dialux 20 microscope. Several ileal sections from six rats per each time point were used for these experiments.

Antisense and sense riboprobes were generated from a rat SP receptor cDNA (12), generously provided by Dr. James E. Krause (Washington University), as a template. The probes were generated using RNA polymerase enzymes and [35S]UTP. The template was a 1,224-base insert in a pBS vector. The vector was cut using Kpn I (to generate antisense probe) and S1I (to generate sense probe), and T7 and T3 polymers were used, respectively. The T3 (sense) and T7 (antisense) polymerase sites were incorporated into the primer annealing labeling.

NK-1R antibodies. An antisera generated against a peptide representing the last 15 amino acids of the rat NK-1R carboxy terminus (12) was prepared by Immuno-Dynamics (La Jolla, CA) according to the maleim-dibenzoyl-N-hydroxysuccinimide coupling method described by Kitigawa and Aikawa (16) and characterized by ELISA. Immunoprecipitation experiments using the method described by MacDonald et al. (17) for immunoprecipitation of the human SP receptor showed that this antibody immunoprecipitated photoaffinity-labeled NK-1Rs expressed in CHO cells transfected with the rat NK-1R (18) (data not shown).

Immunohistochemistry of NK-1R: confocal fluorescence microscopy. Immunohistochemical detection of the NK-1R protein was performed in frozen rat ileal sections. Tissues were fixed for 30 min in 4% paraformaldehyde in PBS (pH 7.4), washed three times at 4°C in PBS, and then cryoprotected overnight in PBS containing 30% sucrose before they were embedded in OCT (Miles, Elkhart, IN). Five-micrometer sections were cut and mounted on Superfrost plus slides (Fisher Scientific) and fixed for 3 min in 4% paraformaldehyde. Sections were washed in Tris-buffered saline (TBS, pH 7.5) and, after incubation (1 h at room temperature) with blocking solution (TBS containing 50 mM ammonium chloride, 1% normal donkey serum, and 3% BSA) they were incubated (1 h at room temperature) with 1:200 dilution of
the rat NK-1R antiserum described previously. The NK-1R antiserum (1:200 dilution) was also preincubated (1 h at 4°C) with an excess (10 µM) of the carboxy-terminal 15-amino acid peptide of the SP receptor that was used to generate the SP receptor antiserum. This mixture was then bound to Pansorbin cells (Calbiochem, La Jolla, CA) following the manufacturer's protocol. Unbound antibody was then released and used as a negative control in the experiments. Sections were washed in TBS and incubated (1 h at room temperature) with 1:100 dilution of FITC-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Sections were washed in TBS containing 0.2% Triton X-100 (Sigma, St. Louis, MO) and then mounted in anti-bleach solution (10% PBS, 90% glycerol, containing 1 mg/ml n-propyl gallate) and examined and photographed using a laser confocal microscope (Zeiss, Thornwood, NY). Sections from six rats per each time point were examined in these experiments.

Binding Experiments

Purification of intestinal epithelial cells. Intestinal epithelial cells were purified from ileal loops at different time points after toxin A or buffer injection using a modification of the method described by Panja et al. (26, 27). Loops were dissected, cut in small fragments (2 × 2 mm), washed in HBSS, and incubated for 5 min at room temperature in HBSS containing 1 mM dithiothreitol (Sigma) to remove adherent mucus. Ileal fragments were incubated (30 min at 37°C) in RPMI complete medium containing dispase (3 mg/ml) and DNase (0.1 mg/ml; Boehringer Mannheim, Indianapolis, IN) with gentle shaking every 5 min. Supernatants were then collected and centrifuged (600 g for 5 min at 4°C), and epithelial cells were purified by centrifugation through a discontinuous density gradient made with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Percoll was diluted to a final 30% and 60% concentration using RPMI complete medium, and the cells were resuspended in the 30% solution. The gradient was prepared by gently sequentially overlaying 3 ml of 60% Percoll, 6 ml of 30% Percoll, and finally 2 ml of RPMI medium alone. The mixture was then centrifuged (600 g for 20 min at 4°C), and epithelial cells migrating at the interface between 30% and 0% Percoll were collected, washed twice with PBS, and resuspended in RPMI complete medium. Aliquots of cell suspensions were aspirated, and the number of cells was measured on a hemacytometer. Trypan blue exclusion experiments in the end of each purification step were performed to estimate the viability of the cells. Ninety-five percent of these cell preparations were characterized as epithelial cells as assessed by microscopy and absence of staining with mucus. Ileal fragments were incubated (30 min at 37°C) in HBSS containing 50 mM Tris and 10 mM MgCl2 (pH 7.4) followed by rapid filtration through a glass fiber filter (Whatman GF/C) pre-soaked for 2 h in 0.1% polyethylenimine. Filters were then washed three times with 5 ml of ice-cold buffer, and SP binding was estimated by measuring radioactivity in the filters by gamma radiation spectrometry in a Nuclear Enterprises NE 1600 gamma counter at a counting efficiency of 70%. Specific binding was estimated by subtracting nonspecific binding from total binding. Data were collected in triplicate, and results are the means ± SE of two to three experiments per time point.

RESULTS

Toxin A Causes Increased NK-1R mRNA Expression in Rat Ileum

Northern blot analysis using a 588-bp probe encoding for the NK-1R mRNA (~2.5 kb band) indicated increased NK-1R mRNA expression in rat ileal tissue after toxin A administration. As shown in Fig. 1A, the NK-1R was slightly expressed in mucosal scrapings of buffer-treated ileal loops and highly expressed in the mucosal scrapings obtained 30, 60, and 120 min after toxin A administration. A semiquantitative analysis of NK-1R mRNA levels showed that toxin A increased the relative abundance of NK-1R mRNA 2.5-fold (P < 0.05, Fig. 1B) 30 min after ileal administration of toxin A. Sixty minutes after toxin A injection, NK-1R mRNA levels were increased twofold compared with controls but this difference was not statistically significant. Two hours after toxin A injection, NK-1R mRNA levels were higher compared with controls and with the 30- and 60-min exposure to toxin A (Fig. 1B).

Hybridization of control ileal tissues (30 min after injection of buffer) with an antisense full-length riboprobe encoding for the entire sequence of the NK-1R mRNA shows very little signal present (Fig. 2C). NK-1R mRNA is markedly increased 30 min after exposure of rat ileum to toxin A (Fig. 2A) compared with 30-min exposure to buffer (Fig. 2C). Particularly notable is the presence of intense signal in intestinal epithelial cells, including villus and crypt cells, with high levels of expression in the base of the crypt (Fig. 2, A, B, and E, and Fig. 3A). Signal is also present in the submucosa, the intestinal lamina propria, and the muscularis layer. Higher magnification (not shown here) revealed that the signal on the muscularis layer was present primarily in the muscularis externa and that specific labeling was also present in enteric ganglionic cells of the myenteric plexus. Two hours after toxin A exposure, there is a further increase in the NK-1R mRNA signal (Fig. 2B) compared with 30-min toxin A exposure (Fig. 2A). The abundance of NK-1R mRNA is dramatically increased in ileal tissues 3 h after toxin A administration (Fig. 2E) compared with 3-h exposure to buffer (Fig. 2F). Hybridization of toxin A-exposed tissue with a sense probe encoding for the NK-1R mRNA shows
very few grains that localize over cells (Fig. 3B) compared with the antisense probe (Fig. 3A).

Pretreatment of Rats With NK-1R Antagonist CP-96345 Prevents Increase in NK-1R mRNA Expression After Exposure to Toxin A

We next examined whether pretreatment of rats with the NK-1R antagonist CP-96345 influenced NK-1R mRNA expression in response to toxin A. Rats were injected with either saline or CP-96345 10 min before exposure of ileal loops to toxin A, and after 2 h ileal tissues were processed for in situ hybridization using the riboprobe for NK-1R mRNA. Figure 2D shows a reduction in the abundance of NK-1R mRNA present in tissue from rats treated with the antagonist compared with tissue from animals not treated with the antagonist (Fig. 2B).

Toxin A Causes Increased Specific $^{125}$I-SP Binding to Membranes From Purified Intestinal Epithelial Cells

Because our in situ hybridization results showed intense NK-1R mRNA signal in ileal epithelial cells early after toxin A administration (Fig. 2), we next examined whether SP binding activity in intestinal epithelial cells is also increased. Our results (Fig. 4) show that membranes from purified epithelial cells 4 h after injection with buffer show very little $^{125}$I-SP binding, which was entirely nonspecific. However, there is increased SP binding to membranes from intestinal epithelial cells 15 min after toxin A administration compared with control membranes. Furthermore, ~70% of SP binding could be displaced by an excess of unlabeled SP, indicating that SP binding is specific. Specific SP binding to epithelial cell membranes continued...
increase was noted 4 h after toxin A injection. Toxin A injection is specific. Rat ileal loops were exposed to toxin A for 2 h and then processed for in situ hybridization using antisense (A) and sense (B) [35S]UTP-labeled riboprobes detecting NK-1R mRNA. Note relative absence of specific signal for SP receptor mRNA in toxin A-exposed tissues hybridized with the sense riboprobe. Results are representative of 4 similar experiments using different rats for each condition.

We report herein that exposure of rat ileum to purified toxin A causes marked upregulation of NK-1Rs in rat intestinal mucosa in both intestinal epithelial cells and cells in the lamina propria in response to ileal administration of C. difficile toxin A. Whereas in control tissues NK-1R mRNA and protein are detectable at low levels, rapid increases in the abundance of NK-1R message (Figs. 1 and 2) and protein (Fig. 5) are noted even before secretory and inflammatory changes to this toxin are measurable at 2–4 h (4). The functional importance of NK-1Rs in this model of intestinal inflammation is underscored by previous experiments showing that pharmacological blockade with specific NK-1R antagonists significantly reduces fluid secretion and intestinal inflammation in this model (22, 31). Recent studies also indicate that mice genetically deficient in the NK-1R are almost protected from the secretory and inflammatory changes, as well as from epithelial cell damage induced by toxin A (7), providing direct evidence for the importance of these receptors in C. difficile toxin A enteritis. The pathways by which NK-1R upregulation occurs during the course of toxin A enteritis have not been elucidated in our study. An interesting possibility is that inflammatory mediators, such as macrophage inflammatory protein 2 (5), released from intestinal epithelial cells after intraluminal toxin A administration, may upregulate NK-1R expression in intestinal epithelial cells.

The major finding in our study is the increased expression of NK-1R mRNA and protein localized on intestinal epithelial cells after exposure of rat ileum to toxin A as shown by in situ hybridization (Fig. 2) and immunohistochemistry (Fig. 5). Binding of 125I-SP to plasma membranes of purified intestinal epithelial cells was also increased after toxin A administration (Fig. 4). Whether this increase in 125I-SP binding is due to SP binding to NK-1 or other SP receptor subtypes (NK-2 or NK-3) cannot be ascertained from our binding experiments. Previous results indicated the presence of NK-2 receptors in canine intestinal epithelial cells (15). Our results also confirm several previous studies that provided indirect evidence for the presence of SP receptors in epithelial cells of the small intestine (2, 3, 13, 33). Our in situ hybridization results showing the presence of NK-1R mRNA on ileal epithelial cells (Fig. 2) also agree with the findings of Cooke et al. (11) on the localization of NK-1R mRNA in colonic epithelial cells. Recent studies on the localization of immunoreactive NK-1Rs in the gastrointestinal tract of the rat and the guinea pig show the presence of NK-1Rs on neurons...
and interstitial cells and the absence of immunoreactivity on epithelial cells (28, 35), confirming our findings that in the control ileum there are very few detectable NK-1Rs at the mRNA and protein level (Figs. 1, 2, and 5). It is only after initiation of the inflammatory response that there is a rapid increase in SP receptors that is easily detectable in the intestinal epithelial cells (Figs. 2 and 5).

Our results further indicate that there are cells within the lamina propria and the submucosa of the rat ileum expressing NK-1Rs during toxin A enteritis (Figs. 2 and 5). The presence of functional SP receptors has been previously indicated in cells of the intestinal lamina propria, including macrophages (4) and mucosal mast cells (38). Using an antiserum directed against the carboxy terminus of the rat NK-1R, Mantyh et al. (22) showed increased endocytosis of the NK-1R in enteric neurons 3 h after exposure of rat ileal loops to C. difficile toxin A, indicating the presence of functional SP receptors in intestinal neurons in response to toxin administration.

Administration of a NK-1R antagonist before injection of ileal loops with toxin A reduced the signal for the NK-1R mRNA in response to the toxin (Fig. 2). These results indicate that a SP-dependent pathway regulates NK-1R mRNA in enterocytes. We recently reported a time-dependent increase in SP content and SP mRNA levels in lumbar dorsal root ganglia during toxin A enteritis (4). Mantyh et al. (22) also showed that the increased NK-1R endocytosis in enteric neurons in response to toxin A was inhibited by pretreatment of rats with capsaicin or prior exposure of animals to the specific nonpeptide NK-1R antagonist CJ-11974. Furthermore, lamina propria macrophages, activated as a result of toxin A enteritis, themselves secrete sufficient SP to enhance the release of tumor necrosis factor-alpha in an autocrine or paracrine fashion (4). Thus results from this and previous studies indicate that during toxin A enteritis SP released from primary sensory neurons and lamina propria macrophages mediates its effects by upregulating SP receptors in intestinal epithelial cells and enteric neurons (22) and other cells of the intestinal lamina propria, including lamina propria macrophages (4).

The presence of NK-1Rs in intestinal epithelial cells and their rapid upregulation during toxin A enteritis raises interesting new questions as to the function of these cells and in particular the participation of SP receptors in mediating these functions during intestinal inflammatory responses. The recent report by Wang et al. (36) of a thyrotropin-releasing hormone-thyroid-stimulating hormone network in the intestinal mucosa mediated by thyrotropin-releasing hormone receptors in enterocytes and intraepithelial lymphocytes is in keeping with this broader view of the function of intestinal epithelial cells and local immune cells in the intestine. One possibility is that NK-1Rs in intestinal epithelial cells play a proinflammatory role during...
intestinal inflammation by way of release of inflammatory mediators in response to SP. We recently demonstrated that SP can stimulate the release of the proinflammatory cytokine interleukin-8 from human U373 MG astroglia cells, indicating a possible proinflammatory role for SP in these cells (8). However, another aspect of SP function that should be considered in this context is that SP may also have a protective effect in intestinal epithelial cell function in a manner similar to that described for pulmonary epithelial cells (34, 42).

We acknowledge support from National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-47343 to C. Pothoulakis and a Fellowship Grant to I. Castagliuolo from the Crohn’s and Colitis Foundation of America.

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Received 30 July 1997; accepted in final form 2 March 1998.

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