Calcitonin Gene-Related Peptide Mediates the Protective Effect of Sensory Nerves in a Model of Colonic Injury

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ABSTRACT

Recently we demonstrated that sensory denervation with the neurotoxin capsaicin significantly worsened the inflammation in an acute and chronic model of experimental colitis, which suggests a protective role of sensory nerve fibers during gut inflammation. Because we could demonstrate that sensory neuropeptides like Calcitonin gene-related peptide (CGRP) and substance P (SP) are released from sensory nerve fibers during intestinal inflammation, both are strong candidates as mediators for the protective effect of sensory neurons. In this study we investigate the role of CGRP and SP during experimental colitis in the rat by use of receptor antagonists against CGRP (CGRP 8–37, 1 µg/h continuous subcutaneous infusion), SP (RP67580, a NK-1 receptor antagonist, 3 mg/kg i.p.) and an immunoneutralizing CGRP-antibody. A mild colitis was induced by a rectal enema containing trinitrobenzenesulfonic acid. The severity of inflammation increased markedly after 7 days in the CGRP receptor antagonist and CGRP-antibody group compared with the vehicle group as determined by a macroscopic damage score (10.4 ± 1.2 and 9.6 ± 1.6 vs. 6.2 ± 2.1) by a histologic ulceration score (82 ± 8% and 73 ± 6% vs. 42 ± 23%) and by myeloperoxidase activity (19.2 ± 6.8 and 18.1 ± 5.9 vs. 8.6 ± 5.3 U/mg tissue protein), respectively. Treatment with the specific SP receptor antagonist did not significantly alter the severity of colitis at 7 days compared with the control group. These data suggest that CGRP exerts mucosal protection during chronic experimental colitis.

Neuropeptide release in peripheral tissues in response to noxious stimuli is a well established efferent function of sensory neurons. In various tissues like the eye, the skin and the joints, sensory neuropeptides can mediate proinflammatory reactions termed “neurogenic inflammation” (Levine et al., 1984; Haegermark et al., 1978; Bill et al., 1979). Sensory neuropeptides also have potent protective properties against different kinds of injury. In the stomach, sensory neuropeptides protect the mucosa against multiple aggressive factors (Holzer et al., 1991a, b, 1995; Holzer, 1995).

Ablation of sensory nerve fibers by chronic treatment with the neurotoxin capsaicin significantly worsened the inflammation in an acute (Reinshagen et al., 1994) and a subacute model of experimental colitis (Reinshagen et al., 1996). These findings suggested a protective role of sensory nerves, containing several neuropeptides during inflammation in the colon. Because several neuropeptides are colocalized in the sensory neurons of the gastrointestinal tract (Costa et al., 1986), it was not clear which set of sensory neuropeptides is responsible for the proposed protective function of sensory nerves during inflammation in the gut. In the acute immune-complex-colitis model of the rabbit we could show that SP and CGRP are released from sensory nerve fibers in the gut wall during the time course of colitis (Éysselein et al., 1992).

Therefore, the aim of this study was to clarify the function of the sensory neuropeptides CGRP and SP during experimental chronic colitis with specific receptor antagonists and an immunoneutralizing CGRP antibody.

Materials and Methods

Induction of Colitis by TNB and Tissue Preparation

Male Wistar rats (250 g) were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (2 mg/kg) given intramuscularly. The distal colon was cleaned carefully with a small balloon catheter before the enema. Then TNB (30 mg/kg) in 50% ethanol was instilled into the rectum via a plastic feeding tube (8 F) in a volume of 0.7 ml, followed by 1.5 ml of air to induce mild colitis. Animals were sacrificed after 1 week by CO2-asphyxiation. The distal colon was dissected, the macroscopic damage score was determined (see below) and colonic tissue was taken for histology and MPO assay.

Experimental Design

Treatment with CGRP8–37. A mini-osmotic pump (Alza Corp., Palo Alto, CA) was implanted subcutaneously on the back of the rat, receiving...
between the shoulders, the day before the experiment \((n = 6)\). CGRP\(_{8-37}\) (Peninsula Laboratories Inc., Belmont, CA) dissolved in saline was released continuously at a rate of 1 \(\mu\)g/h beginning 6 h before induction of colitis and throughout the entire experiment. Comparable doses of systemic CGRP\(_{8-37}\) effectively blocked endogenous CGRP function on mucosal blood flow in the stomach (Holzer et al., 1989; Li et al., 1992).

**Treatment with CGRP mAb (4901).** Three milligrams per kilogram RP67580 4901 was injected intraperitoneally on days 9, 7, 5, 3 and 1 before induction of colitis \((n = 6)\). This regimen for immunoneutralization effectively inhibited the effect of exogenous rat CGRP on somatostatin release and gastric acid secretion in the stomach (Peskar et al., 1993; Wong et al., 1993).

**Treatment with RP67580 (specific nonpeptide SP receptor antagonist).** Three milligrams per kilogram RP67580 (Rhone-Poulenc Rorer, Collegeville, PA) was injected intraperitoneally 1 h before and 2, 4 and 6 days after induction of colitis \((n = 6)\). RP67580 is a specific and potent nonpeptide SP antagonist that acts selectively and competitively on NK-1 receptors. The dose of 3 mg/kg i.p. was chosen because it effectively blocked plasma extravasation induced by SP in the hind paw of the rat (Garrett et al., 1991). The inflammatory response after instillation of one drop of 0.1 N NaOH in the rat eye is mediated primarily by SP (Bynke et al., 1983) and provokes rapid wiping movements. In rats pretreated with 3 mg/kg RP67580, compared with those treated with the vehicle \((n = 6\) rats in each group), the wiping movements were decreased significantly from 13.0 ± 3 wiping movements (vehicle) to 4.2 ± 0.7 wiping movements (RP67580) 24 h after the injection of 3 mg/kg RP67580 \((P < .001)\).

**Control groups.** A mini-osmotic pump (Alza Corp., Palo Alto, CA) was implanted subcutaneously \((n = 6)\) and saline was released continuously at a rate of 1 \(\mu\)l/h starting 6 h before induction of colitis and then throughout the experiment. A second control group was injected with 2 mg i.p. of a control anti-KLH monoclonal antibody on days 9, 7, 5, 3 and 1 before induction of colitis \((n = 6)\). Both control groups were combined for statistical evaluation because no statistically significant differences were observed between them.

**Macrosopic Damage Score**

The distal 4 cm of the colon were evaluated macroscopically by two investigators in a blinded fashion with the macroscopic damage score previously described (Reinshagen et al., 1996). A scale of 0 to 6 was used to quantitate percent edema, erosions and ulcerations in this area. Edema: 0 = no edema; 1 = <50%, 2 >50% and <75%; 3 = ≥75%; erosions: 0 = no erosions; 1 = <25%; 2 = ≥25% and <50%; 3 = ≥50% and <75%; 4 = ≥75%; ulcerations: 0 = no ulcerations; 3 = ≥25%; 4 = ≥25% and <50%; 5 = ≥50%; and, <75%; 6 = ≥75%.

The individual scores for each percent edema, erosions and ulcerations were added and the sum was used as a value for the macroscopic damage in the colon.

**Histologic Quantitation of Colitis**

Histologic evaluation was performed on representative sections from each colon as described previously (Reinshagen et al., 1996). The amount of ulcerations in 2-cm strips of inflamed colon were determined by three different investigators in a blinded fashion and expressed in percent ulcerations of the examined area.

**Protein Measurements in Colonic Extracts**

Tissue protein content was measured in the colonic extracts with the methods described by Bradford (1976). MPO measurements in colonic tissues were related to tissue protein.

**Determination of MPO in Colonic Tissue Extracts**

Colonic tissue was rinsed after dissection with cold 0.1 M PB and homogenized with a Tekmar rotary tissue homogenizer in 5 volumes of hexadecl trimethyl ammonium bromide buffer (0.5% hexadecl trimethyl ammonium bromide in 50 mM phosphate buffer, pH 6.0), sonicated for 20 sec, and centrifuged for 10 min at 2400 rpm at 4°C. The supernatant was shock-frozen and thawed three times, then centrifuged for 10 min at 2400 rpm at 4°C. Supernatant \((100 \mu l)\) was mixed with 1 ml of 0.22% aqueous Guaiacol (Sigma, St. Louis, MO) and 2 ml of 10 mM PB, pH 6.0. Twenty microliters of 0.3% peroxidase was added to start the reaction. Absorbance at 470 nm was recorded with a Beckman spectrophotometer (RU 86) at 15-sec intervals for 2 min. As a standard, 100 \(\mu l\) of horseradish peroxidase \((0.975 U/ml\), Sigma, St. Louis, MO) was combined with 1 ml of Guaiacol, 2 ml of 10 mM PB, pH 6.0 and 5, 10 or 20 \(\mu l\) of 0.1% peroxide were added to start the reaction. The absorbance change at 470 nm for 1 \(\mu l\) of peroxide/min was calculated from the standard curve which equals 1 unit of MPO activity. Protein concentrations were measured in the supernatant according to Bradford (1976), and results were expressed as units of MPO per mg tissue protein.

**Statistical analysis.** Results are expressed as means ± S.E. The Mann Whitney \(U\) test for unpaired data was used to evaluate statistically significant \((P < .05)\) differences.

**Results**

**Effect of CGRP\(_{8-37}\), CGRP-mAb and RP 67580 on Colitis**

**Macroscopic damage score.** The macroscopic damage score increased significantly by 67% after treatment with CGRP\(_{8-37}\) 1 week after induction of colitis, compared with the control group that received only saline from the mini-osmotic pumps (fig. 1). Animals treated with the neutralizing...
CGRP-mAb showed a significant increase (54%) in the macroscopic damage score compared with the control group that received the control anti-KLH monoclonal antibody. Treatment with the specific SP antagonist RP67580 did not change the macroscopic damage score significantly when compared with the control group (see fig. 1).

**Index of mucosal ulcerations determined by histology.** The percentage of mucosal ulcerations was increased significantly in the animals treated with CGRP 8–37 (by 95% compared with the respective control group) and the neutralizing CGRP-mAb (by 73% compared with the respective control group). Mucosal ulcerations in the RP67580 group were not altered significantly compared with the control group (see fig. 2).

**MPO activity.** Seven days after induction of colitis, the MPO activity was increased significantly in the CGRP 8–37 (by 123% compared with the respective control group) and the neutralizing CGRP-mAb (by 110% compared with the respective control group). Again MPO activity was not changed significantly in the RP67580 group compared with the control group (see fig. 3).

**Histology.** After 1 week of mild TNB colitis there was a pronounced disruption of the epithelium and distortion of the glandular structures with only mild infiltration of inflammatory cells into the submucosa and no inflammatory cells in the muscle layers (fig. 4a). After treatment with the SP antagonist the histological appearance was similar to appearance after vehicle treatment (fig. 4b); whereas, in the groups treated with the CGRP receptor antagonist (fig. 4c) or the immunoneutralizing CGRP antibody (fig. 4d), there was a complete loss of the glandular structure with deep infiltration of inflammatory cells into the submucosal layers and the muscle layers of the gut.

**Discussion**

It was not known previously whether the protective effect of sensory neurons in experimental colitis was caused by the release of CGRP or SP. Our data show that blockade of endogenous CGRP by a specific receptor-antagonist or immunoneutralization results in increased mucosal damage in the TNB model of experimental colitis. Blockade of endogenous SP action by blocking NK-1 receptors did not change the experimental colitis in that model after 1 week. These data suggest that CGRP, but not SP, is the responsible mediator protecting the mucosa in this model of experimental colitis.

Ablation of sensory nerves by chronic pretreatment with the neurotoxin capsaicin increased the severity of experimental colitis in the acute immunocomplex-colitis model of the rabbit (Reinshagen et al., 1994) and the rat TNB colitis model used in this study (Reinshagen et al., 1996). These studies suggest that the presence of sensory neuropeptides in sensory neurons is relevant to mucosal protection. Capsaicin pretreatment damages small fiber sensory neurons containing a variety of sensory neuropeptides that are colocalized in different populations of sensory neurons (Costa et al., 1986). Therefore, it was not possible to determine from the capsaicin-
studies which sensory neuropeptide or which combination of sensory neuropeptides are responsible for the assumed protective mucosal effect. Recently, we showed that CGRP and SP are released during acute experimental colitis in the rabbit (Eysselein et al., 1991), which makes them possible candidates as mediators for mucosal protection.

It has been shown that CGRP protects the mucosa against acid back-diffusion (Holzer et al., 1991a; Li et al., 1992a) and damage caused by indomethacin or acetylsalicylic acid (Maggi et al., 1987) in the stomach. This effect by CGRP is caused by its potent vasodilatory capability to induce mucosal hyperemia. This hyperemic effect of CGRP has been shown to be NO-dependent because it can be inhibited by blockade of NO synthesis (Holzer et al., 1995).

CGRP was found to inhibit edema-promoting actions of inflammatory mediators like histamine or leukotriene B_4 (Raud et al., 1991) in the rat paw and in the human skin.

Exogenous parenteral administration of CGRP reduced the incidence and degree of indomethacin- or acetylsalicylic acid-induced gastritis in the rat (Maggi et al., 1987), and in the same group, induced a decrease of colon wet weight. However, no change in the percentage of damage in the colon was reported when CGRP was injected subcutaneously before induction of experimental colitis (Evangelista et al., 1993). If CGRP elicits other mucosa-protective mechanisms than the well-characterized hyperemic response is not known. Other potential CGRP effects could be the stimulation of mucus production, bicarbonate secretion or modulation of local growth factor action in the acute and chronic phase of inflammation.

SP primarily seems to have a proinflammatory mediator function in the gastrointestinal tract. It is well known that SP mediates an inflammatory response called neurogenic inflammation in the eye (Bill et al., 1979), the skin (Haegermark et al., 1978) and the joints (Levine et al., 1984). In the inflamed gut the role of SP is characterized by its abundant interaction with inflammatory cells and cytokines (for review see Sharkey, 1992).

SP exerts its biological actions using a high-affinity interaction with the SP or NK-1 receptor, although at higher concentrations it also interacts with NK-2 and NK-3 receptors (Vigna et al., 1994). Pharmacological blockade of NK-1 receptors, as performed in this study, also partially inhibits the function of neurokinin A and neurokinin B. Neurokinin A and neurokinin B have been shown recently to exert their biological actions in some tissues, like the rat spinal cord, through the NK-1 receptor with affinities similar to the receptor (0.5 nM) as SP (for review see Maggi et al., 1997).

In contrast to the findings in this study, McCafferty et al. (1994) showed that at a very early time after induction of colitis (6h), SP blockade ameliorated the experimental inflammation, which also suggests a proinflammatory action of SP in colonic inflammation.

CP-96,345, another SP antagonist, inhibited intestinal inflammation in the rat induced by Clostridium difficile toxin but did not alter the response to cholora toxin (Pothoulakis et al., 1994). CP-96,345 decreased inflammation in a model of jejunal infection with Trichinella spiralis (Kataeva et al., 1994), but SP was not involved in the regulation of granulocyte infiltration in experimental ileitis induced by TNBs (Miller et al., 1993).

LY-303870, another nonpeptide NK-1 antagonist, suppressed the initiation of inflammation in the spontaneous cotton-top tamarin model of colitis (Wood et al., 1996). Altogether these data point to the conclusion that SP, acting via NK-1 receptors, mediates in the acute phase of colonic inflammation a primarily proinflammatory effect, whereas CGRP seems to have a primarily anti-inflammatory function in the colon.

This hypothesis is supported by recent studies in patients with Crohn’s disease by Mantyh et al. (1995) showing an up-regulation of SP receptor density not only in inflamed tissue but also in noninflamed gut tissue of these patients.

It can be concluded that the balance of inflammatory and anti-inflammatory neuropeptides might be crucial in conserving hemostasis in the gut, and that imbalance of this system might contribute to the pathogenesis of inflammatory bowel disease.

References


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