Alterations in capsaicin-evoked electrolyte transport during the evolution of guinea pig TNBS ileitis

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The aim of this study was to examine the actions of capsaicin-sensitive nerves in the intestine during the establishment and resolution of intestinal inflammation. We studied capsaicin-evoked secretion in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ileitis in the guinea pig; a model of chronic inflammation (26, 27, 29). Our previous studies (35) had established the neural secretomotor pathway activated by capsaicin-sensitive nerves in this tissue. They demonstrated that capsaicin selectively stimulates capsaicin-sensitive nerves in the submucosa to release neurotransmitter that activates cholinergic and noncholinergic secretomotor neurons. These neurons, in turn, innervate mucosal enterocytes evoking chloride secretion. This indicates that the synthesis and release of neurotransmitters from capsaicin-sensitive nerves is upregulated during inflammation and that this results in enhanced effector responses and faster resolution of the inflammatory response. Other studies, however, have demonstrated that substance P (SP) immunoreactivity in capsaicin-sensitive neurons falls as inflammation progresses (27), implying that the activity of capsaicin-sensitive nerves may decrease over time. Together, these data suggest that the effluent function of capsaicin-sensitive nerves may vary during the evolution of the inflammatory response.

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formation enabled the responses to capsaicin to be followed during inflammation and identification of the specific site involved (i.e., neural or enterocyte) when changes occurred. Unlike other studies (2), inflamed tissue was taken from nonulcerated segments of intestine to ensure that results of transport studies were not altered by ulcerated epithelium.

**MATERIALS AND METHODS**

Guinea pigs (Hartley; 180–200 g) were obtained from Charles River Laboratories (Trois Rivieres, Quebec, Canada) and were maintained on standard laboratory chow and tap water ad libitum. All protocols were approved by the Queen’s University Animal Ethics Committee, and procedures involving animals followed the guidelines of the Canadian Council on Animal Care.

**Induction of ileitis.** Guinea pigs were given a preanesthetic tranquilizer (Innovar-vet: 0.5 ml/kg im) and anesthetized with pentobarbital sodium (Somnotol: 15 mg/kg sc). After a midline laparotomy, a distal section of ileum was exposed and 0.5 ml saline (sham surgical controls) or 0.5 ml TNBS (30 mg/ml in 50% EtOH) were injected intraluminally 4 cm from the ileocecal junction, as previously described (26, 27). The ileum was replaced into the abdominal cavity, and the incision was sutured. Animals were killed immediately after surgery, at 8 h, or 1, 3, 5, 7, 14, or 30 days following surgery, and tissue was obtained for Ussing-chamber studies and histological evaluation.

**Ussing-chamber studies.** Animals were rendered immediately unconscious with a single blow to the head and killed by carotid and cervical transection. An 8-cm ileal segment, originating from a site 4 cm proximal to the ileocecal junction, was removed and placed in a cold, oxygenated (5% CO2-95% O2) modified Krebs buffer solution containing (in mM) 115.0 NaCl, 2.0 KH2PO4, 2.4 MgCl2, 1.3 CaCl2, 8.0 KCl, and 25.0 NaHCO3. Segments were scored along the mesenteric border, and the muscularis externa was removed. Submucosal-mucosal preparations were mounted in standard Ussing chambers (World Precision Instruments, Sarasota, FL) with 0.6-cm2 exposed surface area. Sites with grossly visible damage were excluded from these studies. The serosal side of the preparation was bathed by modified Krebs buffer containing 10 mM glucose, and the mucosal buffer contained 10 mM mannitol. The chambers were maintained at 37°C and oxygenated (5% CO2-95% O2). After a 10-min equilibration period, potential difference (PD) was recorded in the open-circuit condition and then clamped to 0 mV by applying a short-circuit current (Isc) using a voltage-clamp apparatus (EVC-4000, World Precision Instruments). The Isc was amplified via a 5110 oscilloscope and converted into a digital signal (TL-1 DMA interface, Axon Instruments, Foster City, CA) that was stored as a continuous measurement on a computerized data-acquisition system (Axotape, Axon Instruments). The Isc was monitored as an indicator of net sc was monitored as an indicator of net resistance was calculated using Ohm’s law. All drugs were added to the serosal side of the chamber, and 10 min elapsed between drug applications, during which time a stable baseline was reestablished. Standard electrical field stimulation (EFS; 100 V, pulse duration 0.5 ms, 10 Hz, 5s) was delivered with a Grass stimulator via two stainless steel posts mounted at the back of both chambers. Isc responses were expressed as the maximum change in Isc from baseline (μA/cm2).

Characterization of the capsaicin-sensitive neural circuit mediating electrolyte transport (35) in this model demonstrated the specific sites of action of capsaicin, SP, and carbachol (see Fig. 6A). On the basis of these findings, capsaicin was employed to selectively stimulate neuropeptide release from capsaicin-sensitive nerves, SP was used to stimulate submucosal secretomotor nerves, and carbachol, combined with TTX, was used to directly stimulate chloride secretion by the enterocyte.

In a separate series of experiments, jejunal tissue was also obtained 20 cm proximal to the site of TNBS ileitis for Ussing-chamber studies. In addition, after the findings in the initial studies at days 1, 3, 5, 7, and 30, which demonstrated that agonist-evoked responses were reduced at 7 days in TNBS animals, a further set of experiments was conducted to determine whether these findings persisted at a later time point, 14 days.

**Assessment of inflammation.** In a separate series of experiments, tissue was obtained from animals receiving either saline (sham surgical controls) or TNBS, as described in Induction of ileitis. A 10-cm segment of ileum was removed immediately proximal to the instillation site at 4 cm from the ileocecal junction. The first and last centimeter of the segment were placed in liquid nitrogen and stored at −80°C for subsequent myeloperoxidase (MPO) assay to quantify inflammation. In the remaining 8 cm, tissue samples from sites meeting the criteria for use in electrophysiological studies were examined for epithelial integrity. Briefly, tissue was cut along the mesenteric border, pinned out mucosa-side-up, and fixed in Carnoy’s, dehydrated, and wax-infiltrated using a standard protocol. Wax blocks were sectioned at 5 μm and stained with hemotoxylin and eosin. To assess mucosal integrity, sections from sham surgical controls (saline) and TNBS-treated animals were examined by an investigator who was blinded to the treatment received.

MPO activity was measured according to the procedure of Krawisz et al. (21). Briefly, frozen intestinal segments were placed into a solution (1 ml/50 mg tissue) of hexade cytrimethylammonium bromide (0.5%, wt/vol) in potassium phosphate buffer (50 mM, pH 6.0). Samples were homogenized on ice for 30 s, centrifuged for 30 min at 10,000 rpm and 4°C, and then incubated at 60°C for 2 h. After incubation, 100 μl of supernatant were added to 2.9 ml of 0.00005% H2O2 in an o-dianisidine dihydrochloride (Sigma, St. Louis, MO) solution in a cuvette, and the absorbance was measured at 460 nm. Results were expressed as units of MPO activity per 100 milligrams of tissue (wet weight), where 1 unit of MPO activity is the amount of enzyme required to split 1 μmol H2O2/min at 25°C.

**Drugs.** Capsaicin (Sigma) was dissolved in Tween 80, alcohol, and saline (80:10:10); SP (Peninsula Laboratories, Belmont, CA), tetrodotoxin (Sigma), and carbachol (Sigma) were dissolved in distilled water.

**Statistics.** These data were expressed as means ± SE. For each experiment, the basal PD, Isc, resistance, and the maximal change in Isc in response to secretagogues were averaged for each treatment group. Comparisons of agonist-evoked responses in sham surgical controls (saline) and TNBS treatment groups for separate time points were analyzed using two-tailed t-tests in initial studies at days 1, 3, 5, 7, and 30 and a one-tailed t-test in follow-up studies at 14 days. The level of significance was set at P < 0.05.

**RESULTS**

**General properties.** The properties of mucosal-submucosal in vitro preparations were examined from...
three different animal groups in this study. Control preparations (nonoperated controls) were obtained from animals that were killed without prior surgical intervention (day 0). Sham surgical controls (saline-treated preparations) and TNBS-treated preparations were obtained from killed animals that previously had undergone laparotomy at 8 h and 1–30 days before study with injection of either saline or TNBS into the terminal ileum, respectively (see MATERIALS AND METHODS).

Macroscopically, erythema of the mucosa and serosa was confined to the distal 8–10 cm. Mucosal ulceration was evident at and distal to the injection site in the TNBS groups on days 1, 3, 5, and 7 in most preparations. No ulceration was macroscopically evident in the proximal adjoining 10-cm segment taken for histological and electrophysiological study. Histological examination of sections of tissue confirmed the integrity of the epithelium at the selected sites (n = 4 for each group at 8 h of time; Fig. 1, days 1, 3, 5, 7, and 30). Both the sham surgical controls (saline) and TNBS had a significant mean increase in MPO activity at day 1 compared with nonoperated controls (Table 1). MPO activity in the TNBS group, however, was significantly greater than the sham surgical control group levels at days 1, 3, and 5.

Under basal resting conditions, a negative $I_{sc}$ was maintained in all preparations, consistent with net mucosal-to-serosal anion flux, as previously reported for in vitro ileal mucosal transport (31). The basal resistance, PD, and $I_{sc}$ of in vitro submucosal-mucosal preparations of the nonoperated controls, sham surgical controls (saline), and TNBS-treated animals are shown in Table 2. Some differences existed between groups, but these were not consistent within treatment groups. These changes likely reflect variation due to experimental technique, as described by others (30).

The addition of TTX (1 μM) to the serosal bath in nonoperated controls (day 0) caused a significant decrease in basal $I_{sc}$ (mean = $-62.3 \pm 6.7 \mu A/cm^2$) compared with vehicle (mean = $-0.4 \pm 0.01 \mu A/cm^2$, P < 0.05), as previously described (13). Compared with nonoperated controls (day 0), the TTX-evoked reduction in basal $I_{sc}$ effect in preparations from the TNBS group at day 7 was significantly less ($-32.9 \pm 10.0 \mu A/cm^2$, P < 0.05). The effect in the surgical control group (saline) at the same time point did not reach statistical significance ($-37.8 \pm 9.4 \mu A/cm^2$).

$I_{sc}$ response to capsaicin. Our previous studies have demonstrated that serosal application of capsaicin evokes a dose-dependent increase in $I_{sc}$ (35). These studies demonstrated that this increase in $I_{sc}$ results from selective activation of capsaicin-sensitive nerves with ensuing release of neuropeptides. These neuropeptides activate submucosal secretomotor neurons, that, in turn, innervate mucosal enterocytes (see Fig. 6A), causing chloride secretion (35). The $I_{sc}$ response, evoked by activation of this pathway, was biphasic with a brief initial phase (capsaicin EC$_{50}$ = 50 nM) followed by a large second phase (capsaicin EC$_{50}$ = 90 nM). In the current study, 50 nM capsaicin evoked a typical biphasic response in all nonoperated control and sham surgical control preparations (Fig. 2). Both phases of the response were unaltered by TNBS at 8 h after instillation. By day 1, however, the large second phase $I_{sc}$ response to capsaicin in TNBS-treated preparations was almost completely abolished compared with sham surgical controls (saline). When sham surgical controls (saline) and TNBS-treated groups were compared, there was also a significant decrease in the TNBS group on days 1–7 (Fig. 2; P < 0.05). There was also a trend toward a similar reduction at day 14, but this did not reach statistical significance. This inflammation-induced decrease in capsaicin-evoked $I_{sc}$ was lost at day 30. The first phase of the capsaicin-evoked response was markedly reduced at day 1 in the TNBS animals compared with sham surgical controls (saline). There was also a large reduction in the magnitude of the phase 1 responses in the TNBS compared with the
INFLAMMATION ALTERS CAPSAICIN-EVOKED SECRETORY RESPONSES

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Table 1. Changes in myeloperoxidase activity in TNBS and saline control animals

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5 ± 1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>139.3 ± 35.1</td>
<td>14.5 ± 2.5</td>
<td>15.9 ± 6.6</td>
<td>23.5 ± 7.4</td>
<td>4.6 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>TNBS</td>
<td>247.2 ± 25.1</td>
<td>29.8 ± 3.9</td>
<td>58.6 ± 4.3</td>
<td>25.5 ± 10.6</td>
<td>3.5 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

Values are units/100 mg tissue wet weight expressed as means ± SE; n = 5 for each group. *P = 0.026; †P = 0.003; ‡P = 0.006. Control, nonoperated animals; sham, operated animals (saline); TNBS, 2,4,6-trinitrobenzenesulfonic acid.

Table 2. Basal R, PD, and Isc in saline and TNBS animals

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>8 h</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
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<tbody>
<tr>
<td>PD, mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.9 ± 0.3</td>
<td>1.3 ± 0.0</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 1.0</td>
<td>1.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>TNBS</td>
<td>2.5 ± 0.1†</td>
<td>2.2 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>3.9 ± 0.4</td>
<td>2.0 ± 0.2*</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td>Isc, ±μA/cm²</td>
<td>74.4 ± 3.1</td>
<td>66.0 ± 4.2</td>
<td>76.8 ± 4.3</td>
<td>56.4 ± 4.8</td>
<td>57.3 ± 5.9</td>
<td>52.9 ± 4.9</td>
<td>92.8 ± 5.5</td>
<td>52.9 ± 4.9</td>
</tr>
<tr>
<td>R, Ω/cm²</td>
<td>99.7 ± 5.0</td>
<td>62.0 ± 6.9</td>
<td>98.1 ± 11.7</td>
<td>76.8 ± 6.1</td>
<td>108.7 ± 17.6</td>
<td>50.5 ± 13.0</td>
<td>55.2 ± 4.3*</td>
<td>55.2 ± 4.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for each group, *P < 0.03; †P < 0.05; ‡P < 0.001. R, resistance; PD, potential difference; Isc, short-circuit current. Control: 0 h, nonoperated animals; 8 h—Day 30, operated animals (saline).
altered neural function from changes occurring at the level of the enterocyte, because we had carefully defined the components of this neural pathway (see Fig. 6A) in previous studies (35) and the current study examined inflamed tissue with nonulcerated epithelium. To determine whether this effect was confined to alterations in capsaicin-sensitive nerves, we examined the effects of SP and carbachol-evoked responses (see Fig. 6A). These studies showed that the attenuated capsaicin-evoked secretion was confined to capsaicin-sensitive nerves during the first 5 days, because there was no concomitant alteration in SP- and carbachol-evoked electrolyte transport. However, by 7 days, carbachol-evoked responses in the presence of TTX were also significantly reduced, demonstrating changes at the level of the enterocyte (see Fig. 6C). These findings persisted for at least an additional 7 days. The reduction of SP responses, which occurred at these same time points, may also solely reflect changes at the level of the enterocyte but could also include alterations in submucosal secretomotor neurons. The reduction in TTX-sensitive baseline secretion (i.e., spontaneous submucosal secretomotor-evoked secretion) seen at 7 days may reflect this effect. Together, these studies demonstrate that both neural and nonneural secretory mechanisms are affected during the evolution of the ileitis.

There are several possible mechanisms that might underlie the inflammation-induced attenuation of capsaicin-sensitive nerve function. One possibility is that local release of inflammatory cytokines, such as interleukin (IL)-1β (14, 16, 24), may inhibit release of neurotransmitter. This inhibitory effect appears to affect multiple neural pathways, including cholinergic nerves, as suggested in preliminary studies (24). This action is an unlikely explanation of the capsaicin attenuation, given that the submucosal secretomotor pathways, which include cholinergic nerves (35), were unaffected. An alternative explanation is that tachyphylaxis occurs following supramaximal stimulation from cytokines, rendering the tissue hyporesponsive to additional stimuli. This is also unlikely, given that basal secretion did not differ between sham surgical controls (saline) and TNBS-treated animals and that SP and carbachol stimulation was initially unaffected. The most likely possibility is that inflammation results in constant stimulation of capsaicin-sensitive nerve terminals resulting in eventual depletion of neurotransmitter. This concept is directly supported by a related study that examined SP tissue levels and immunoreactivity in the same model of guinea pig TNBS ileitis and studies in the colon using an immune complex model (7, 25, 26). In the guinea pig TNBS-ileitis studies, SP immunoreactivity in the mucosa and submucosa was reduced by day 1 of inflammation and whole tissue SP content by day 3 (7, 25, 26). The reduced immunoreactivity was particularly marked in perivascular nerves, which are almost solely capsaicin-sensitive afferent nerves (8, 34). In a similar fashion, studies in the colon (7) have shown that inflammation reduces the SP content in whole thickness colonic extracts as early as 8 h. Together, these findings show that SP neurotransmitter content falls during the same time period that the capsaicin nerve-evoked responses are attenuated, strongly supporting the notion that the reduced secretory responses result from neurotransmitter depletion from the capsaicin-sensitive nerves.

The role of hypersecretion of the epithelium during inflammation within the small intestine has not been fully resolved. In the colon, diarrhea induced by TNBS colitis appears to be secondary to loss of electrolyte absorption attributable to epithelial damage and not altered electrolyte secretion (2). In the current study, the effects of TNBS-ileitis were examined at sites that were inflamed but the mucosa was intact. There was some variability in basal I_\text{sc} measurements, as shown in Table 2, but these did not display any pattern or distribution within the data that would suggest a physiological effect. Such variation is typical in Ussing chamber studies (30), and as a result, I_\text{sc} measurements are traditionally reported as ΔI_\text{sc}. Table 2 also

Fig. 2. Capsaicin-evoked short-circuit current (I_\text{sc}) is markedly attenuated by 24 h of inflammation. A: a representative trace showing that 50 nM capsaicin evoked a typical biphasic increase in I_\text{sc} in sham surgical control (saline) tissue, as previously described (34). A brief phase I response was followed by a prolonged phase II response. B: capsaicin-evoked phase II responses (open bars) were markedly attenuated by 24 h following installation of TNBS compared with sham surgical control (saline) animals (filled bars). This effect persisted until day 7 and returned to control values by day 30. Phase I responses exhibited a similar pattern (data not shown). Each point represents the mean ± SE for 9 experiments. *P < 0.05.
shows that baseline resistance was not reduced by the inflammation. This finding is consistent with previous reports (2) in which inflammation was shown to reduce intestinal secretion while the epithelial barrier was maintained. We also found that by day 7, epithelial electrolyte transport was hyporesponsive to secretagogues (see Fig. 3). Previous studies of TNBS colitis and mitomycin-C colitis have also shown the epithelium is hyporesponsive to secretagogues (2, 17). Our studies and others (2) suggest this effect is not due to a loss of epithelial integrity but rather, as previously suggested (17), may be due to a receptor-coupling defect or desensitization of receptor-linked secretory mechanisms.

Previous studies (15, 16) have shown that the acute local inflammatory response to TNBS can alter neural function in the myenteric plexus at both inflamed and noninflamed sites in the intestine. These actions appeared to be due to the local release of cytokines, which act at the site of inflammation and at the distant noninflamed sites (15). IL-1β has been shown to be released at the site of intestinal inflammation (18), and exogenous application of IL-1β in control tissue can mimic alterations in neural function seen during the inflammatory response (14). Furthermore, the suppression in neural function observed during the inflammatory response could be attenuated by infusion of...
sham surgical controls (saline). Although these values were significantly less than the TNBS group until day 7, they were greater than nonoperated controls until between days 7 and 30. This effect in the sham surgical controls (saline) presumably resulted from handling of the ileum during surgery. When this sham effect was removed (by subtracting from the TNBS-induced rise in MPO), it was evident that TNBS-induced MPO activity had fallen almost 100% by day 7. In contrast, MPO levels continued to rise by day 7 in previous ileitis studies (26) and remained elevated at day 30. However, this study and others reporting sustained elevations in MPO activity used a similar or greater concentration of TNBS in 50% ethanol (2, 3, 26, 27) in contrast to the 30% ethanol used in the present study. Ethanol-induced perturbation of the mucosa is the first step in the induction of inflammation, and thus increased concentrations of ethanol used in other studies may facilitate increased permeation of TNBS into the mucosa and produce both acute and chronic inflammation. Moreover, the ileal segment used in Ussing chamber studies and histological analyses was up to 8 cm removed from the site of TNBS administration. This site was selected based on reports (2) that TNBS disrupts the mucosal integrity at the site of instillation. Thus

IL-1-receptor antagonist, a selective antagonist of IL-1β (16). In the present study, TNBS-evoked ileitis also attenuated secretory responses at noninflamed sites in the proximal jejunum. These actions, however, were not confined to neural pathways, because when carbachol in the presence of TTX was employed to directly stimulate the enterocyte (see Fig. 5), these responses were dramatically attenuated. Together, these data suggest that cytokines can act at noninflamed sites to alter intestinal secretion through neural and nonneural mechanisms. Further studies are needed to determine whether this action is predominantly mediated by IL-1β or whether additional or alternative cytokines are involved.

In the present study, the time course and severity of the inflammatory response produced by TNBS differ from the characteristics of TNBS-induced ileitis reported previously (26). The determination of the duration of the TNBS-evoked rise in MPO in the current study was confounded by a parallel rise in MPO in the jejunum 20 cm proximal to the site of TNBS instillation. Myeloperoxidase (MPO) levels were normal in the jejunum in both sham surgical controls (saline) and TNBS-treated animals (see text). These data suggest that cytokines can act at noninflamed sites to alter intestinal secretion through neural pathways, because when Carbachol-evoked responses were also almost completely attenuated in TNBS-treated animals at both the site of inflammation in the ileum and at the noninflamed site in the jejunum compared with sham surgical controls (saline; open bars). Each bar represents the mean ± SE (n = 4; P < 0.01). Carbachol-evoked responses were also almost completely attenuated in the jejunum. Each bar represents the mean ± SE (n = 4; P < 0.01).

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the ileal segment used in this study might exhibit less inflammatory change compared with previous studies (27) in which the site of TNBS-induced damage was directly examined.

In summary, the findings of the current study demonstrate that the efferrent actions of capsaicin-sensitive nerves remain functional in the very early stages of inflammation (i.e., 8 h) but not during more established inflammation. Consequently, in models in which the mucosa is acutely challenged by a noxious agent, release of neurotransmitter, which appears to protect against mucosal damage (12), is limited to the time frame of the initial challenge. The loss of function during established inflammation may suggest that this defense mechanism can be functionally ablated by continued stimulation. This relationship may, however, not apply to all inflammatory settings, because SP expression in capsaicin-sensitive nerves is increased in infection models of intestinal inflammation (10, 30), suggesting different mechanisms may be involved.

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