Tachykinergic Neurotransmission Is Enhanced in Small Intestinal Circular Muscle in a Rabbit Model of Inflammation

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ABSTRACT

Previous electrophysiological studies have shown that tachykinin-mediated excitatory junction potentials are enhanced in a rabbit model of inflammatory bowel disease. The present study extends these findings by investigating the contractile response to stimulation of noncholinergic nerves and tachykinin agonists. According to rank order potencies, the rabbit ileal circular muscle was neurokinin (NK)1 preferring, and the response to these agonists was down-regulated by acetylcholine and up-regulated by nitric oxide. In ricin-treated tissue, cholinergic and nitricergic modulation was lost; in the presence of atropine and L-NAME, the response to stimulation of noncholinergic nerves and tachykinin agonists was enhanced. The noncholinergic response to nerve stimulation was predominantly mediated by NK1 receptors, and the enhanced response of ricin-treated tissue to NK1 agonists probably contributes to the increased response to electrical field stimulation observed under these conditions. Increased tachykinin response and loss of control of this response by acetylcholine and nitric oxide are likely to have profound effects on intestinal motility and could contribute to some of the symptomology of inflammatory bowel disease.

Tachykinins are important excitatory neurotransmitters in the enteric nervous system and are involved in the coordination of gastrointestinal motility (Bartho et al., 1983; Bartho and Holzer, 1985). This family of peptides includes the products of two genes, the PPT I gene, which produces SP and NK-A (Nawa et al., 1983), and the PPT II gene, which produces NK-B (Kotani et al., 1986), a tachykinin that is present in very small quantities in the intestinal tract (Tateishi et al., 1990). These tachykinins preferentially bind to NK1, NK2, and NK3 receptors, respectively. Each of these receptors has been localized to circular muscle cells in the small intestine (Hellstrom et al., 1994), although it is of note that biological effects of NK3 activation have been predominantly attributed to a neural site of action (Croci et al., 1995).

Tachykinins are strongly implicated in IBD, a chronically debilitating condition associated with abnormal intestinal motility, which may contribute to cramping, abdominal pain and diarrhea. Tachykinin levels are increased in the colonic submucosa and mucosa of patients with ulcerative colitis (Goldin et al., 1989; Koch et al., 1987). Immunocytochemical studies have shown increased tachykinin levels around epithelial cells in the colon of ulcerative colitis patients and the colonic musculature in Crohn’s disease (Mazumdar and Das, 1986). In addition to increased tachykinin levels, up-regulation of tachykinin receptors on intestinal blood vessels has been reported in ulcerative colitis (Mantyh et al., 1988).

We recently developed an experimental model of IBD produced by the intraluminal administration of the cytotoxic plant lectin ricin into the rabbit ileum. Ricin caused mucosal inflammation, epithelial damage and increased myoelectric activity (Sjogren et al., 1994). These changes represent a general response to acute inflammation because the hapten trinitrobenzene sulfonic acid evoked similar alterations (Sjogren et al., 1994). In vitro, EFS of enteric nerves resulted in larger noncholinergic EJPs in ricin-treated circular muscle than in controls (Goldhill et al., 1995), and this was suggested to contribute to the increased myoelectric activity in vivo. SP autodesensitization reversed the increased response to EFS (Goldhill et al., 1995), prompting us to speculate that altered tachykinin-mediated neurotransmission contributes to altered neuromuscular control during inflammation. We have suggested that altered neuromuscular control may contribute to the intestinal cramping and diarrhea associated with IBD.

In the present study, we further investigated the hypothesis that tachykinin-mediated neurotransmission is altered during intestinal inflammation. Our first aim, therefore, was to determine whether contractile responses to specific NK1, NK2, and NK3 agonists was enhanced. The noncholinergic response to nerve stimulation was predominantly mediated by NK1 receptors, and the enhanced response of ricin-treated tissue to NK1 agonists probably contributes to the increased response to electrical field stimulation observed under these conditions. Increased tachykinin response and loss of control of this response by acetylcholine and nitric oxide are likely to have profound effects on intestinal motility and could contribute to some of the symptomology of inflammatory bowel disease.

ABBREVIATIONS: CL, confidence interval; NK, neurokinin; L-NAME, N-nitro-L-arginine methyl ester; IBD, inflammatory bowel disease; SP, substance P; EFS, electric field stimulation; EJP, excitatory junction potential; KBS, Krebs-bicarbonate-saline; PPT, preprotachykinin; [SAR]SP, [Sar9,Met11(O2)]substance P; β-Ala[NKA], [β-Ala9]neurokinin A; L-NAME, N-nitro-L-arginine methyl ester.
and NK3 agonists were altered during inflammation. Although it is becoming apparent that NK3 receptors play a role in the contraction of intestinal smooth muscle, this subtype was not studied due to the relative unavailability of pharmacological tools, especially antagonists. It was unclear from earlier studies (Goldhill et al., 1995) whether increased EFS-evoked EJPs resulted in an increased contractile response because electrophysiological responses are known to be uncoupled from smooth muscle contraction in inflammation (Goldhill et al., 1994; Snape et al., 1980). Thus, the second aim of the present study was to confirm that the noncholinergic, non-nitric-oxide-mediated neurotransmission (Goldhill et al., 1995), thus allowing the specific investigation of noncholinergic excitation. Trains of EFS were applied at 20-min intervals to prevent desensitization.

Nature of the noncholinergic response to EFS. SP, which preferentially binds to NK1 receptors, is proposed to mediate noncholinergic excitation in the guinea pig small intestine (Taylor and Bywater, 1986), but this has not been confirmed in the rabbit. To establish whether NK1 receptors mediate noncholinergic excitation in the rabbit ileum, the response to maximal frequency EFS (10 Hz) was determined after a 20-min pretreatment with atropine and L-NAME and the specific NK1 antagonist GR 82,334 (10 μM) or in a paired piece of tissue with its vehicle, 0.01 N acetic acid. Previous reports have shown this antagonist to have a maximal and specific effect at this concentration (Hagan et al., 1991), and we have shown it to reduce the contractile response to SP (5 nM) by >70% in rabbit ileal circular muscle under control and inflamed conditions (94±4% vs. 70±19% in control and ricin-treated tissue respectively; n = 4).

Data analysis. Maximum increases in muscle tone in response to tachykinin addition or EFS were obtained through visual analysis of chart recorder outputs. Responses to tachykinins are expressed as absolute tension development. EFS data were expressed as a percentage of the response to 1 mM acetylcholine added at L0, to reduce the variation of this data. The response to this concentration of acetylcholine was not altered by ricin in this series of experiments. The response to EFS (10 Hz) in the presence of GR 82,334 was expressed as a percentage of the response after incubation in vehicle. Values are given as mean ± S.E.M. The number of animals (n) is shown in parentheses. Tachykinin concentration responses were fitted to sigmoid curves (GraphPAD, San Diego, CA.), and EC50 values (with 95% CLs) were determined from these curves. Differences between frequency or concentration-response curves were assessed statistically using multivariate analysis of variance, with adjustments made for multiple comparisons. In cases in which curves were significantly different to one another, maximal responses were compared statistically using Student’s t test. The effect of GR 82,334 was assessed statistically using Student’s t test. A value of P <.05 was considered significant in each case.

Solutions and drugs. KBS contained (in mM) 118.5 NaCl, 4.75 KC1, 2.54 CaCl2, 1.19 NaH2PO4, 1.19 MgSO4, 25.0 NaHCO3 and 11.0 glucose. This solution was gassed with 95% O2/5% CO2 to give a pH of 7.3 to 7.4. All drugs were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated. Ricin toxin (Ricinus communis agglutinin) was dissolved in distilled water at the beginning of every experiment. Atropine sulfate and L-NAME were both dissolved in distilled water as stock solutions of 10 and 100 mM, respectively. SP, [SAR]SP, NKA and [β-Ala]NKA were obtained from Peninsula Laboratories (Belmont, MA) and dissolved in 0.01 N acetic acid and stored as a stock solution of 100 μM. GR 82,334 ([L-Pro<sup>3</sup>spiro-γ-lactam]Leu<sup>10</sup>,Trp<sup>11</sup>physalaemin<sub>12-11</sub>); RBI, Natick, MA) was dissolved in 0.01 N acetic acid and stored as a stock concentration of 5 mM.

Methods

Induction of inflammation. Male New Zealand White rabbits were divided into a control vehicle-treated group and a ricin-treated group. Acute ileitis was induced with ricin as previously described (Goldhill et al., 1995; Snape et al., 1994). Briefly, animals were anesthetized with intramuscular xylazine (9 mg/kg) and ketamine (50 mg/kg) and maintained with intravenous pentobarbital (15 mg/ml). A midline incision was made, a ligated terminal ileal loop (10 cm in length) was constructed in each animal and 1 ml of ricin (1 mg/ml) or vehicle was injected into the lumen. The loop was removed immediately after 5 hr, a time period that allowed development of ileitis, abnormal myoelectric activity (Sjogren et al., 1995) whether increased noncholinergic excitation in the guinea pig small intestine (Taylor and Bywater, 1986), but this has not been confirmed in the rabbit. To establish whether NK1 receptors mediate noncholinergic excitation in the rabbit ileum, the response to maximal frequency EFS (10 Hz) was determined after a 20-min pretreatment with atropine and L-NAME and the specific NK1 antagonist GR 82,334 (10 μM) or in a paired piece of tissue with its vehicle, 0.01 N acetic acid. Previous reports have shown this antagonist to have a maximal and specific effect at this concentration (Hagan et al., 1991), and we have shown it to reduce the contractile response to SP (5 nM) by >70% in rabbit ileal circular muscle under control and inflamed conditions (94±4% vs. 70±19% in control and ricin-treated tissue respectively; n = 4).

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Effect of acetylcholine and nitric oxide on the response to tachykinins. Previous electrophysiological studies (Goldhill et al., 1995) showed that increased noncholinergic excitation in ricin-treated tissue was tempered by muscarinic receptors and/or nitric oxide. Therefore, concentration-response curves to NK1 and NK2 agonists were constructed after the cumulative addition of the muscarinic agonist atropine (1 μM) and the nitric oxide synthase inhibitor L-NAME (0.1 mM), to investigate whether modulation of tachykinin response by acetylcholine or nitric oxide was altered during inflammation. These concentrations were the same as those in previous studies (Goldhill et al., 1995).

Effects of ricin treatment on noncholinergic, non-nitric-oxide-mediated responses to EFS. In this series of experiments, muscle strips were passed through a pair of ring electrodes (2-mm diameter) and stimulated for 10 sec by square-wave pulses (0.5-msec duration; supramaximal voltage) at 1 to 10 Hz. Pulses were delivered to the electrodes from a Grass S88 stimulator (Grass Instruments, Quincy, MA). Preliminary studies showed that EFS-evoked changes were maximal at 10 Hz and abolished by the neural blocker, tetrodotoxin (1 μM), demonstrating that responses were due to neural stimulation. Stimulation was performed in the presence of atropine (1 μM) and L-NAME (0.1 mM). These concentrations have been used to abolish cholinergic and nitric oxide-mediated neurotransmission (Goldhill et al., 1995), thus allowing the specific investigation of noncholinergic excitation. Trains of EFS were applied at 20-min intervals to prevent desensitization.
Results

Response of circular muscle to tachykinins. Under control conditions, the rank order of potency of the tachykinin agonists was SP = [Sar]SP > NKA >> [β-Ala]NKA in both vehicle- and ricin-treated tissues (table 1), suggesting that this tissue is NK1 preferring. To determine whether alterations in the response to NK1 and NK2 receptor activation during inflammation, the remainder of the present study was devoted to investigation of the contractile response to the specific agonists [Sar]SP and [β-Ala]NKA. This avoids interpretational problems associated with the use of the natural agonists, SP and NKA, which display considerable overlap with respect to their binding to NK1 and NK2 receptors.

Effect of acetylcholine and nitric oxide on the response of vehicle-treated tissue to tachykinins. The addition of atropine significantly altered the response of vehicle-treated tissue to the selective NK1 agonist [Sar]SP (P < .05) (fig. 1a). This did not correspond to a change in potency (3.0 nM, 95% CL = 2.5–3.6 vs. 3.2 nM, 95% CL = 1.8–5.6 nM; n ≥ 6 in absence and presence of atropine respectively), but the maximal response was significantly increased (P > .05). These increases in responsiveness were reversed by further addition of the nitric oxide synthase inhibitor L-NAME, so the response to NK1 stimulation in the presence of atropine and L-NAME was not significantly different than that in the absence of drugs (fig. 1a). In contrast to [Sar]SP, the response to [β-Ala]NKA was unaffected by atropine or L-NAME (fig. 1c).

Effect of ricin treatment on cholinergic and nitric-ergic control of tachykinin response. After ricin treatment, neither atropine nor atropine and L-NAME significantly (P > .05) altered the concentration response to [Sar]SP or [β-Ala]NKA (figs. 1, b and d).

Effect of ricin treatment on noncholinergic, non-ni-tridergic response to tachykinins. In the presence, but not the absence, of atropine and L-NAME, ricin treatment significantly altered the response to both [Sar]SP and [β-Ala]NKA (fig. 2). In both cases, this effect corresponded to a small but not significant increase in the maximum response and an increase in potency. Ricin decreased the EC50 values of [Sar]SP from 5.4 nM (95% CL = 5.2–5.4 nM) to 1.9 nM (95% CL = 1.6–2.3 nM) and that of [β-Ala]NKA from 114.8 nM (95% CL = 112.2–114.8 nM) to 58.9 nM (95% CL = 58.5–59.3 nM). To confirm that this increased responsiveness was not related to activation of noncholinergic, non-nitridergic nerves the response of vehicle- and ricin-treated to both [Sar]SP and [β-Ala]NKA was compared in the presence of tetrodotoxin. Figure 3 shows that under these conditions, ricin-treated tissue was still hyperresponsive to tachykinergic stimulation. As in the presence of atropine and L-NAME, after neural blockade, ricin-treated tissue was more sensitive

than vehicle-treated tissue to [Sar]SP (0.19 nM (95% CL = 0.16–0.23 nM) vs. 3.09 nM (95% CL = 1.68–5.89 nM), respectively) and to [β-Ala]NKA/[Sar]SP (6.49 nM (95% CL = 4.79–8.91 nM) vs. 53.70 nM (95% CL = 52.48–54.95 nM), respectively).

Response to EFS. In the presence of atropine and L-NAME, EFS evoked a frequency-dependant contractile response, reaching a maximum at 10 Hz (fig. 4). The addition of the NK1 antagonist GR 82,334 reduced the maximal response of vehicle-treated tissue to EFS by 81.5 ± 11.69% (n = 4; P < .01), showing that NK1 receptors play a major role in the noncholinergic response to EFS.

Effect of ricin on the response to EFS. Because the responsiveness to NK1 specific agonists is enhanced during inflammation, and these receptors mediate a large portion of the response to EFS, it was reasonable to speculate that ricin would increase the response to EFS. The present study shows this to be the case (fig. 4), thus extending our earlier electrophysiological studies that showed that in the presence of atropine and L-NAME, the myoelectric response to EFS was increased by ricin (Goldhill et al., 1995).

Discussion

In a previous electrophysiological study, we reported that acute inflammation increased the magnitude of EFS-evoked noncholinergic EJPs (Goldhill et al., 1995) and speculated that this may contribute to cramping and abdominal pain during intestinal inflammation. Because this effect was reversed by SP autodesensitization, it was suggested that an altered response to or release of tachykinins contributes to this phenomenon. Data from the present study support this hypothesis, demonstrating that acute inflammation increases the response to NK1 and NK2 agonists under noncholinergic conditions, that the rabbit circular muscle is NK1 preferring and that these receptors play a major role in mediating the response to EFS. The enhanced response to NK1 agonists may therefore contribute to the increased contractile response to EFS also seen under these condition in the present study.

SP and NKA are derived from the same PPT I gene (Nawa et al., 1983) which explains why both of these peptides are found in the same synaptic vesicles (Deacon et al., 1987). SP and NKA bind preferentially to NK1 than NK2 receptors; however, there is considerable interspecies differences with respect to the balance between these subtypes. The circular muscle of the human, dog and rat small intestine show NK2 selectivity (Hellstrom et al., 1994; Maggi et al., 1992; Muller et al., 1988), and the guinea pig, NK1 selectivity (Maggi et al., 1994). In the course of the present study, the responsiveness of healthy rabbit circular muscle to the tachykinins was characterized, and we report that the rank order of potencies for the various tachykinin agonists is similar to that of the

### TABLE 1

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<thead>
<tr>
<th></th>
<th>SP</th>
<th>[Sar]SP</th>
<th>NKA</th>
<th>[β-Ala]NKA</th>
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<tr>
<td>Control</td>
<td>6.3 (5.4–7.2)</td>
<td>3.0 (2.5–3.6)</td>
<td>17.4 (13.2–22.4)</td>
<td>74.1 (72.4–74.1)</td>
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<tr>
<td>Ricin</td>
<td>5.2 (4.0–6.8)</td>
<td>7.4 (6.3–8.7)</td>
<td>17.4 (16.2–18.2)</td>
<td>83.2 (81.3–83.2)</td>
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EC50 values with 95% confidence values given in parentheses from ≥6 replicates. Note that all values are for studies performed in the absence of atropine or L-NAME.
guinea pig small intestinal circular muscle (Maggi et al., 1994).

The mechanism of action of SP is well understood in the guinea pig ileum. Contractile effects of SP on longitudinal muscle are mediated, in part, by cholinergic nerves (Regoli et al., 1984) because the effects of SP are inhibited by atropine. In contrast, the NK₁-mediated response to EFS is reduced by acetylcholine, suggesting presynaptic inhibition (Wiklund et al., 1993). Tachykinergic neurotransmission is also regulated by nitric oxide. The most commonly reported effect of nitric oxide is to reduce tachykinin neurotransmission (Wiklund et al., 1993), although under certain conditions, nitric oxide appears to contract intestinal smooth muscle by releasing tachykinins (Bartho and Lefebvre, 1994). Regulation of tachykinin neurotransmission in rabbit ileal circular muscle has not been previously described. The effect of atropine and L-NAME on the response to [Sar]SP suggests that under healthy conditions, acetylcholine reduces tachykinergic contraction, whereas nitric oxide enhances contraction. The mechanisms of these effects are beyond the scope of this study. In contrast, one of the most important findings of the present study is that these regulatory mechanisms appear to be lost during inflammation.

The tachykinins have previously been implicated in functional alterations observed during inflammation. Mucosal/submucosal levels of SP are increased in the colon of ulcerative colitis (Goldin et al., 1989; Koch et al., 1987). SP levels are also increased by ~60% in the muscle layer of the ileum from Crohn’s disease patients (Koch et al., 1987). Moreover, NK₁ receptor numbers are also reported to be increased in IBD (Mantyh et al., 1988). In a guinea pig model of IBD, trinitrobenzene sulphonic acid increased the density of SP staining in the small intestinal myenteric plexus (Miller et al., 1993). Despite considerable evidence that NK₁ receptors are involved in the pathophysiology of intestinal inflammation, the present study is the first to show that tachykinergic control of contraction is altered during inflammation. This alteration occurs at two levels. First, as described above, the interactions between nitric oxide and acetylcholine, and NK₁ excitation are absent during inflammation, and thus the regulation of tachykinergic neurotransmission may be lost under these conditions. Second, we have shown that under...
noncholinergic conditions, or in the presence of TTX, the contractile responses to both [Sar]SP and the NK2 agonist [β-Ala]NKA, are increased during inflammation. It is of note that although evidence is starting to accumulate that implicates the NK2 receptor in the control of intestinal smooth muscle, changes in NK2 responsiveness were not investigated in the present series of experiments and therefore deserve future study. The mechanism by which increased responsiveness to tachykinins occurs is unclear but appears to involve an increase in sensitivity as the response curves to both [Sar]SP and [β-Ala]NKA were shifted to the left. It is not clear whether this reflects increased receptor sensitivity or postreceptor modification, and binding studies are required to resolve this issue. Whatever the exact mechanism, changes in the receptor/second-messenger system are nonneural in nature as differences between vehicle- and ricin-treated tissue were observed in the presence of tetrodotoxin. Alternatively, increased tachykinin sensitivity could result from down-regulation of the neutral endopeptidase, the enzyme responsible for tachykinin breakdown. This has previously been demonstrated in the rat small intestine after Trichinella spiralis infection (Hwang et al., 1993). This explanation, however, cannot fully explain the present data as [β-Ala]NKA is insensitive to enzyme metabolism (Patacchini et al., 1989).

In the guinea pig ileum, in the presence of atropine and nitric oxide synthase inhibition the response to EFS was abolished by the NK1 antagonist CP 99,345, suggesting that NK1 receptors mediate most of the noncholinergic excitatory response to nerve stimulation (Wiklund et al., 1993). The same appears to be true in the rabbit ileum, as in the present study we show that GR82,334 almost abolished the response to EFS. Because the noncholinergic response to EFS is predominantly NK1 mediated and NK1-mediated excitation is enhanced under noncholinergic, non-nitridergic conditions during inflammation, the response to EFS should be enhanced under these conditions if transmitter release in uninjured. It was necessary to test this hypothesis directly, however, as it has previously been shown that transmitter release is impaired in Trichinella-infected rats (Collins et al., 1989). Even if this is the case in ricin-evoked inflammation, we show in the present study that in agreement with our previous electrophysiological data (Goldhill et al., 1995), the contractile response to EFS is enhanced during inflammation.

In summary, we demonstrated that (1) inflammation abolishes cholinergic down-regulation and nitridergic up-regulation of the NK1 response, and (2) inflammation increases circular muscle responsiveness to NK1 and NK2 agonists, which could contribute to the heightened response to noncholinergic nerve stimulation. Consequently, we speculate that the response to those stimuli that evoke tachykinin-mediated contractions alone will be augmented during inflammation. Also, the loss of cholinergic and nitridergic modulation of tachykinergic neurotransmission would be expected to result in an altered response to stimuli that corelease acetylcholine and/or nitric oxide with the tachykinins. However, before specific defects can be more accurately predicted, a greater understanding of neural pathways and their response to different stimuli is necessary. What is clear, however, is that such changes are likely to impair the ability of the intestine to adapt to its ever-changing luminal conditions during in-

Fig. 2. Concentration-response curves to [Sar]SP and [β-Ala]NKA in vehicle-treated tissue (●, n ≥ 6) and in ricin-treated tissue (○, n ≥ 6). Each of the response curves were performed in the presence of atropine (1 μM) and L-NAME (1 mM). These pairs of concentration-response curves were compared statistically using a multivariate analysis of variance. † P < .05 vs. vehicle-treated tissue.

Fig. 3. Concentration-response curves to [Sar]SP and [β-Ala]NKA in vehicle-treated tissue (●, n ≥ 6) and in ricin-treated tissue (○, n ≥ 6). Each of the response curves were performed in the presence of tetrodotoxin (1 μM). These pairs of concentration-response curves were compared statistically using a multivariate analysis of variance. † P < .05 vs. vehicle-treated tissue.

Fig. 4. Effect of EFS on the response of vehicle-treated tissue (●, n = 5) and ricin-treated tissue (○, n = 7). EFS was performed for 10 sec (0.5-msec pulse width, supramaximal voltage) in the presence of atropine (1 μM) and L-NAME (1 mM). These pairs of frequency-response curves were compared statistically using a multivariate analysis of variance. † P < .05 vs. vehicle-treated tissue.
flammation and is likely to contribute to the altered intestinal function observed under these conditions.

References


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