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), preferring, and the response to these agonists was down-regulated by acetylcholine and up-regulated by nitric oxide. In ricin-treated tissue, cholinergic and nitridergic modulation was lost; in the presence of atropine and nitridergic modulation was lost; in the presence of atropine and N-nitro-L-arginine methyl ester, or tetrodotoxin, the response to stimulation of noncholinergic nerves and tachykinin agonists. According to rank order potencies, the rabbit ileal circular muscle was neurokinin (NK), preferring, and the response to these agonists was down-regulated by acetylcholine and up-regulated by nitric oxide. In ricin-treated tissue, cholinergic and nitridergic modulation was lost; in the presence of atropine and N-nitro-L-arginine methyl ester, or tetrodotoxin, the response to stimulation of noncholinergic nerves and tachykinin agonists.

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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, Met(O2)10] substance P; β-Ala[NKA], [β-Ala]neurokinin A; L-NAME, N-nitro-L-arginine methyl ester.
and NK2 agonists were altered during inflammation. Although it is becoming apparent that NK2 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 contractile response of circular muscle strips to EFS was also elevated. Finally, we examined whether this may result from altered tachykinergic responsiveness by determining the receptor subtypes involved in the response to EFS.

**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; Sjogren 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 after 5 hr, a time period that allowed development of ileitis, abnormal myoelectric activity (Sjogren et al., 1994) and increased response to EFS (Goldhill et al., 1995). Animals were killed with an overdose of pentobarbital. The loop was opened along its length, gently flushed of luminal contents with cold-oxygenated modified KBS and prepared for contractility studies.

**Contractility studies.** Muscle strips (~1 × 0.4 cm) with mucosa removed were cut in the axis of the circular muscle and attached to isometric tension transducers in 10-ml organ baths and continuously bathed with modified KBS at 37.5 ± 0.5°C. Tissues were allowed to equilibrate at L0 (the length at which no tension could be measured) for 20 min. The strips were then progressively stretched to L0, which was determined as the length at which maximum active force was generated in response to acetylcholine (0.5–1 mM). Strips were then allowed to equilibrate for an additional 20 min before the following studies were performed.

**Effect of inflammation on circular muscle response to tachykinins.** We investigated the effect of inflammation on the response to NK1 and NK2 agonists. The NK1 agonist used was the natural agonist SP and its structural analog [SAR]SP. The NK2 agonists used were the natural agonist NKA and its structural analog β-Ala[NKA]. Concentration-response curves were constructed on separate muscle strips for each of these agonists in vehicle- and ricin-treated tissues. Agonists were applied at 10–15-min intervals, with at least three changes of KBS between concentrations, to prevent desensitization. Studies were performed in the presence or absence of tetrodotoxin (1 μM) to distinguish between neural and non-neural effects of ricin treatment.

**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 antagonist atropine (1 μM) and the nitric oxide synthase inhibitor L-NNAME (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-nitricergic 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-NNAME (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.

**Nature of the noncholinergic response to EFS.** SP, which preferentially binds to NK1 receptors, is proposed to mediate non-cholinergic 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-NNAME 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 Lo to reduce the effect at this concentration (Hagan et al., 1991). 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).

**Solutions and drugs.** KBS contained (in mM) 118.5 NaCl, 4.75 KCl, 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 agglutininα) was dissolved in distilled water at the beginning of every experiment. Atropine sulfate and L-NNAME 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 (Belmont, CA) and dissolved in 0.01 N acetic acid and stored as a stock solution of 100 μM. GR 82,334 ([β-Pro3,spiroγ-lactam]Leu10,Trp14]physalaemin4−11); RBI, Natick, MA was dissolved in 0.01 N acetic acid and stored as a stock concentration of 5 mM.
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 occurs 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 nitridergic 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. 1b and 4). The 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 noncholinergic, non-nitridergic 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 value 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).

TABLE 1
EC₅₀ values for the responses of control and ricin-treated tissue to tachykinins

<table>
<thead>
<tr>
<th></th>
<th>SP</th>
<th>[Sar]SP</th>
<th>NKA</th>
<th>[β-Ala]NKA</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<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>
</tr>
</tbody>
</table>

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 NK₁ selectivity (Hellstrom et al., 1994; Maggi et al., 1992; Muller et al., 1988), and the guinea pig, NK₂ 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
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 mechanism appears 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
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. It should be noted that the data are the same as those in figure 1 but replotted to facilitate direct comparison between vehicle- and ricin-treated tissue.

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.

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 NK3 receptor in the control of intestinal smooth muscle, changes in NK3 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 NK2 antagonist CP 99,345, suggesting that NK2 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 intestine is normal. 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-
flammation and is likely to contribute to the altered intestinal function observed under these conditions.

References


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