In vitro evidence that rabbit distal colonic muscularis mucosae has a Clostridium difficile toxin A receptor

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In vitro evidence that rabbit distal colonic muscularis mucosae has a Clostridium difficile toxin A receptor. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G402–G409, 1998.—In the rabbit ileum Clostridium difficile toxin A causes inflammation and mucosal damage via a specific glycoprotein receptor that contains α-D-galactose. In rabbit colon toxin A also causes inflammation, and this is associated with increased myoelectric activity and eicosanoid production. The present in vitro study was undertaken to determine if a toxin A receptor on one or more layers of colonic smooth muscle could mediate the motor effects of this agent. Toxin A (20–100 µg/ml) was without effect on longitudinal and circular muscle but had two different effects on the muscularis mucosae. Initial exposure to the toxin caused increased numbers of spontaneous contractions and a small, atropine- and tetrodotoxin-, and indomethacin-resistant increase in resting tone. More importantly, however, 30-min exposure to toxin A resulted in attenuated muscularis mucosae responses to acetylcholine and KCl. Both the small excitatory and the larger inhibitory effects of toxin A were abolished by pretreatment with the lectin BS-1, which binds to toxin A receptors, but not by the nonreceptor-binding lectin DBA. These data strongly suggest that toxin A causes significant motor effects on the distal colonic muscularis mucosae via a receptor-mediated mechanism. These mechanical data were supported by the presence of histologically demonstrable toxin A and BS-1 binding sites on the muscularis mucosae but not on either the longitudinal or circular muscle layers, both of which were unresponsive to the toxin. By depressing muscularis mucosae function and, ultimately, mucosal movement as a result of toxin A production, C. difficile may promote its own proliferation, thus further contributing to the development of antibiotic-associated colitis.

antibiotic-associated colitis; contraction; circular muscle; immunohistochemistry; lectin; longitudinal muscle

IT HAS BEEN KNOWN for many years that bacterial overgrowth can affect the motility of the human intestine (33), and it has been subsequently shown using the rabbit that many bacterial strains produce toxins that are capable of mediating this effect (14, 19, 31). Of particular interest in this respect is Clostridium difficile, which produces two toxins, namely, toxin A (an enterotoxin) and toxin B (a cytotoxin). C. difficile has now been identified as the causative agent in antibiotic-associated pseudomembranous colitis (1). In the rabbit small intestine in vivo, C. difficile toxin A causes histological damage and elicits a secretory response (18, 20). In addition, it recently has been demonstrated in vivo that when C. difficile toxin A is administered to rabbit ileal loops it ultimately causes a depolarization of the circular muscle layer which is associated with an increased excitability and exaggerated responses to agonists (9). However, when naive rabbit ileal muscle is exposed to toxin A in vitro it is without effect (9).

There is now considerable evidence to suggest that the effects of C. difficile toxin A on the rabbit ileum are mediated via its first binding to a specific epithelial cell receptor, which has been characterized subsequently as an α-galactose- and N-acetylgalcosamine-containing glycoprotein receptor coupled to a pertussis toxin-sensitive G protein (28). Additionally, both in vivo and in vitro studies have shown that in combining with its receptor toxin A binds specifically to carbohydrates containing the trisaccharide Galα1,3Galβ1,4GlcNAc (4, 35).

In contrast, relatively little is known of the effects or mechanisms of action of C. difficile toxin A in the rabbit colon. From the available literature the rabbit colon appears to be considerably more resistant to the effects of this toxin than is the ileum, requiring at least a 10 times greater concentration to elicit secretion or tissue damage (2, 17, 20).

In humans, the pathophysiological consequences of C. difficile proliferation are most commonly observed in the colon, and in vitro both toxin A and toxin B have been shown to alter the electrophysiological characteristics of the human colonic epithelium (30). Because there is now quantitative in vivo evidence that C. difficile has the ability to damage the epithelium of the rabbit distal colon while at the same time altering its motor function (2), the aim of the present study was to investigate in vitro potential receptor-mediated motor effects of C. difficile toxin A on each of the three muscle layers that comprise this region of the intestine.

METHODS

Male New Zealand White rabbits were euthanized with pentobarbital (60 mg/kg ip). After laparotomy the distal 5 cm of colon immediately proximal to the pelvic brim were located and removed. This segment was opened along its mesenteric border and rinsed in warm Krebs solution to remove residual fecal material. Preparations were then pinned out, mucosal surface down, in a Sylgard (Dow Corning)-coated 7-in. petri dish in oxygenated Krebs solution. Full-thickness segments 1 or 3 cm × 3 mm in the longitudinal axes and 1 cm × 3 mm in the circular axes were excised. The muscularis propria of each strip was separated from the mucosa, muscularis mucosae, and submucosa by sharp dissection. Strips of longitudinal and circular muscle 1 cm × 3 mm were mounted in 1.0-ml organ baths at 37 ± 0.5°C. One end of the tissue was connected to a stationary mounting point on the bottom of the
bath, and the other was connected to a Grass FT03D force-displacement transducer under a tension equivalent to a 1-g load. Based on length-tension studies (21) this load stretched the muscles to approximately L₀, the optimal length for the generation of active tension.

Strips of muscularis mucosae with mucosa attached were prepared using the "sutured edge" technique originally described by Percy and Christensen (26). Briefly, longitudinally oriented strips of mucosa, muscularis mucosae, and submucosa 3 cm in length were tied in the middle with 5-0 surgical thread and folded, mucosal surfaces inward, so that they were one-half their original length. The oral and aboral ends, now side by side, were tied together to form a loop. Because curling of the tissue tends to expose the mucosal surface rather than the submucosal aspect, the vertical edges of the preparation were sutured at four points with 7-0 surgical thread. This holds the preparation flat and ensures that the submucosa and muscularis mucosae are fully exposed to the bathing medium, but it does not compromise its ability to contract.

These strips were mounted in 1.0-ml organ baths at 37 ± 0.5°C, and one end of the tissue was connected to a stationary mounting point on the bottom of the bath. The other end was connected to a Grass FT03D force-displacement transducer under a tension equivalent to a 0.75-g load. It has been shown previously that under these conditions this muscle is stretched to approximately L₀ (24). Responses from all experiments were recorded on a SensorMedics R611 dynograph recorder.

Responses to pharmacological agents. After a 30-min equilibration period the viability of each tissue preparation was assessed by first exposing it to a maximum concentration of acetylcholine (10⁻³ M). After a 15-min recovery period a cumulative concentration-response curve to acetylcholine (10⁻³ to 10⁻⁶ M) was constructed. All contractile responses were expressed as a percentage of each tissue's initial maximum response to acetylcholine. The effects of toxin A on the responses of the muscularis mucosae to pharmacological agents were assessed after 30 min of continuous exposure to the toxin at the stated concentrations. The amounts of toxin A utilized in these studies were based on preliminary experiments in which we studied concentrations ranging from 1 to 100 µg/ml. All drugs were added to the bath in volumes not exceeding 1% of the total bath volume.

Preparation of toxin A. Toxin A was prepared from filtered cultured supernatants of C. difficile strain 10463 and was purified using a modification of the method of Sullivan et al. (32) as described by Pothoulakis et al. (28).

Toxin A was biotinylated using the biotin derivative NHS-LC-Biotin, by a modification of the method described by Hnatowitch et al. (13). Toxin A in 50 mM bicarbonate buffer (pH 8.0) was incubated with NHS-LC-Biotin in a 1:20 mole-to-mole ratio of toxin A to biotin for 2 h. Unreacted reagent was removed by dialysis against 50 mM Tris·HCl buffer (pH 7.4). Biotinylated toxin A was indistinguishable from native toxin A with respect to both cytotoxic and enterotoxigenic activity when assayed using rabbit intestinal loops, IMR 90 fibroblasts, and rat basophilic leukemia cells (6). Additionally, native and biotinylated toxin A were identical when compared by SDS-PAGE electrophoresis according to the methods described by Laemmli (15), followed by immunoblotting using streptavidin biotinylated horseradish peroxidase and 4-chloro-1-naphthol as a substrate (6).

Histology. Staining was performed on 5-µm sections of Formalin-fixed, paraffin-embedded sections of longitudinal muscle, circular muscle, and muscularis mucosae. Sections were stained with hematoxylin and eosin and examined for possible morphological changes. All immunohistochemical staining steps were performed at room temperature, using PBS (pH 7.4) as the wash medium. Slides were rehydrated with xylene and graded alcohol and then treated with 3% hydrogen peroxide in methanol for 10 min. After being washed with PBS, slides were incubated with 1% fetal bovine serum in PBS for 20 min and were then blotted. Slides were treated with BS-1 (5 µg/ml; a lectin derived from Bandeiraea simplicifolia) for 30 min and with biotinylated toxin A (200 µg/ml) for 1 h. This step was followed by three washes with PBS. Streptavidin-peroxidase (10 µg/ml) was then added for 30 min. After three further washes with PBS, slides were treated with diaminobenzidine for 6–10 min, washed extensively with water, counterstained with methyl green, dehydrated, and then mounted. Control positive slides were obtained by staining normal rabbit ileum with biotinylated toxin A and staining normal colonic epithelium with BS-1. Control negative slides were obtained by using nonbiotinylated toxin A. Slides were examined for biotinylated toxin A and BS-1 reactivity in a blinded fashion without knowledge of the source.

Drugs and solutions. All experiments investigating contractile function were performed in a Krebs solution of the following composition (in mM): 118.5 NaCl, 4.75 KCl, 2.54 CaCl₂, 1.19 NaH₂PO₄, 1.19 MgSO₄, 25 NaHCO₃, 11 glucose, 0.02 choline gassed with a mixture of 95% O₂-5% CO₂.

Acetylcholine chloride (Sigma, St. Louis, MO) was dissolved in a 5% NaH₂PO₄ solution and serially diluted with Krebs solution taken to pH 4.0 by the addition of 0.1 N HCl. Atropine sulfate, streptavidin-peroxidase conjugate, the biotinylated and native forms of the lectin BS-1 from Bandeiraea simplicifolia, the lectin DBA from Dolichos biflorus, tetradotoxin, and indomethacin were all obtained from Sigma.

Statistical analysis. Where appropriate, data were analyzed using a paired Student’s t-test (before and after treatment) or, where more than two treatments were compared, by ANOVA with Bonferroni correction. In all cases P < 0.05 was considered significant. Throughout the text n is the number of animals used for that particular portion of the study.

RESULTS

Spontaneous contractile activity. All preparations of longitudinal muscle, circular muscle, and muscularis mucosae exhibited spontaneous contractile activity throughout the experimental period. The frequencies of these were longitudinal muscle, 7.08 ± 0.55 contractions per minute (n = 15); circular muscle, 11.13 ± 0.67 contractions per minute (n = 16); and muscularis mucosae, 8.54 ± 0.22 contractions per minute (n = 43). When compared by ANOVA with Bonferroni correction each of these values was significantly different from the other two (longitudinal muscle P < 0.001 vs. circular muscle and P < 0.05 vs. muscularis mucosae; circular muscle P < 0.001 vs. muscularis mucosae).

Toxin A had no effect on the spontaneous contractile frequency of either the longitudinal or the circular muscle at concentrations of 20 and 100 µg/ml. In contrast, both concentrations of toxin A significantly increased the frequency of spontaneous contractions in the muscularis mucosae (Fig. 1).

In a second series of experiments muscularis mucosae preparations were pretreated for 30 min with either the lectin BS-1 (100 µg/ml), which binds terminal α-galactose residues (11), or the lectin DBA (100
µg/ml), which preferentially binds to N-acetyl-α-D-galactosamine residues (11). Neither BS-1 nor DBA at these concentrations and exposure times had a significant effect on muscularis mucosae spontaneous contractions (Fig. 2). After incubation with and in the continuous presence of DBA, toxin A (100 µg/ml) still caused a significant increase in spontaneous contractile frequency. However, when BS-1 was used in this protocol toxin A was without a significant effect (Fig. 2).

Responses to pharmacological stimuli. Toxin A (20 µg/ml) did not significantly alter the baseline tone or the responses of the longitudinal muscle (n = 5), circular muscle (n = 6), or muscularis mucosae (n = 5) to acetylcholine (10⁻⁹–10⁻³ M; not shown).

On addition to the bathing medium, toxin A at a concentration of 100 µg/ml had no immediate effect on any muscle layer. However, it induced a “latent contraction” of the muscularis mucosae within 5 min (Fig. 3). This latent contraction was relatively small in amplitude (8.28 ± 1.19 mN, n = 13) compared with a maximal acetylcholine-induced contraction in the same preparations (24.25 ± 2.23 mN, n = 13). In contrast, toxin A-induced contractions were not observed in either the longitudinal or the circular muscle layer. Toxin A-induced latent contractions occurred in tissues pretreated with either atropine (10⁻⁶ M, 30-min exposure, n = 6) or tetrodotoxin (10⁻⁶ M, 5-min exposure, n = 6). When indomethacin (10⁻⁶ M, 30-min exposure) was used, toxin A-induced contractions took 5–8 min to develop but were not significantly reduced in amplitude (n = 5). In tissues pretreated with BS-1 (100 µg/ml) toxin A-induced contractions were absent, whereas DBA (100 µg/ml) treatment was ineffective in this respect.

In addition to causing a latent contraction, toxin A (100 µg/ml) significantly depressed the maximum response of the muscularis mucosae to acetylcholine, while again having no significant effect on the responses of either the longitudinal or the circular muscle layers (Fig. 4).

The inhibitory effect of toxin A on the responses of the muscularis mucosae to acetylcholine was unaffected after a 30-min pretreatment with and in the continuous presence of DBA (100 µg/ml). However, in tissues pretreated with BS-1 (100 µg/ml), toxin A-induced depression of the acetylcholine-induced responses was absent (Fig. 5). Neither DBA (n = 5) nor BS-1 (n = 5) alone at these concentrations had a significant effect on the muscularis mucosae responses to acetylcholine (not shown).
To investigate further the nature of the inhibitory effect of toxin A on the muscularis mucosae, its ability to affect contractile responses to an 80 mM K\textsuperscript{+} challenge was investigated. As with acetylcholine, the responses of the muscularis mucosae to K\textsuperscript{+} were significantly depressed following a 30-min exposure to toxin A (100 µg/ml) (Fig. 6). BS-1 (100 µg/ml) alone had no significant effect on muscularis mucosae contractions to K\textsuperscript{+} but abolished the toxin A-induced depression of this response. DBA (100 µg/ml), however, significantly potentiated the responses of the muscularis mucosae to K\textsuperscript{+} alone and therefore its effects on toxin A-induced inhibition could not be assessed.

**DISCUSSION**

The results of the present study demonstrate that C. difficile toxin A has two distinct motor effects on the muscularis mucosae of the rabbit distal colon. The first is an excitation that is characterized by a concentration-dependent increase in the frequency of spontaneous contractile events, followed by a small but significant latent contraction. The second, and most prominent, motor effect is a decrease in the ability of the muscle to contract in response to pharmacological stimuli such as acetylcholine and a high K\textsuperscript{+} solution. These phenomena...
were resistant to pretreatment of the tissue with conventional pharmacological agents to exclude neuronal involvement, muscarinic receptors, and prostaglandin production. These observations together suggest that C. difficile toxin A has a direct action on the muscularis mucosae itself.

Evidence was also obtained which supports the hypothesis that the motor effects of C. difficile toxin A on the rabbit distal colonic muscularis mucosae are receptor-mediated. First, the effects of toxin A on this tissue were attenuated or abolished by the lectin BS-1, which has previously been shown to preferentially bind to the toxin A receptor on rabbit small intestinal brush-border membranes (28). In contrast, the nonreceptor binding lectin DBA was ineffective in this respect. Second, when examined utilizing biotinylated toxin A or BS-1, positive staining was observed in both the epithelium and the muscularis mucosae but not in the longitudinal or circular muscle layers. These observations correlate with the lack of effect of toxin A on either layer of the muscularis propria in vitro and with its ability to cause epithelial damage in this region in vivo (2) and to alter the motor properties of the muscularis mucosae in vivo. Whereas an apparently direct action of C. difficile toxin B on rabbit ileal circular muscle in vitro has been reported (7), toxin A is without effect on this muscle under the same experimental conditions and has only indirect actions in vivo (9). Thus the data from the present study represent the first quantitative and qualitative evidence for the presence of a C. difficile toxin A receptor on an intestinal smooth muscle preparation.

Fig. 6. Toxin A (100 µg/ml)-induced depression of 80 mM K+-induced contractions of muscularis mucosae was absent in tissues pretreated with BS-1 (100 µg/ml). Note that BS-1 at this concentration did not by itself significantly affect responses of muscularis mucosae to K+.

Data are expressed as percentage of each tissue's maximum response to acetylcholine and are means and SE of ≥6 observations. Statistical significance is represented as * P < 0.05.

Fig. 7. Comparison of rabbit distal colonic muscularis mucosae and epithelial C. difficile toxin A binding sites stained with the biotinylated lectin BS-1. Longitudinal and circular muscle were negative in this respect (not shown). Note darker staining in mucosa and muscularis mucosae regions, but that muscle staining is lighter than epithelial staining. Magnification ×10.
Utilizing the present experimental protocols we were unable to detect any measurable effect of toxin A on either the longitudinal or circular muscle of the rabbit distal colon in vitro. This would at first appear to conflict with our previous study, in which we showed that luminal application of toxin A to the same colonic region in vivo causes a significant and protracted increase in myoelectric activity in the circular muscle layer (2). The in vivo response to the binding of toxin A to epithelial receptors takes at least 1.5 h to become maximal, persists for several hours, and is thought to be due to the production of motility-altering inflammatory mediators, such as prostaglandin E$_2$ and leukotrienes C$_4$, D$_4$, and E$_4$ (2). The predominantly inhibitory effect of toxin A on the muscularis mucosae preparation used in the present study results from binding of the toxin to a receptor located on the muscle itself, occurs within minutes, and is essentially indomethacin-insensitive. Based on these observations the contrasting actions of toxin A on the musculature of the colon in vivo and in vitro can be explained by considering the location of the receptor mediating the responses under discussion.

In addition to smooth muscle, the muscularis mucosae preparation used in this study is composed of mucosal epithelium, lamina propria, submucosa, and attendant submucosal nerves. The lamina propria is known to be an important source of prostaglandin production (16), and it is known that in vitro, in response to certain stimuli, muscularis mucosae contractions can be attributed solely to de novo synthesis of arachidonic acid metabolites (23). However, as noted previously, in the presence of an effective concentration of indomethacin (23) that by itself had no actions on the muscularis mucosae, the latent contraction to toxin A was delayed but was neither inhibited nor attenuated. This suggests that the excitatory component of the motor response to toxin A may involve a process that is accelerated by enhanced cyclooxygenase activity but that ultimately does not require arachidonic acid metabolite production to produce a contraction.

One possible eicosanoid-dependent mechanism for muscularis mucosae excitation that could explain the latent contraction seen in the present study is a prostaglandin-induced stimulation of excitatory submucosal motor nerves. There are two important observations that would initially appear to support such a phenomenon. First, it is known that in both the rabbit and the rat distal colon the motor effects of prostaglandin F$_2$ on the circular muscle layer are significantly altered by tetrodotoxin (21, 22). Second, it has recently been shown in rat small intestine in vivo that agents inhibiting neuronal function can attenuate the secretory effects of toxin A (3). In the case of the rabbit distal

Fig. 8. Immunohistochemical evidence for *C. difficile* toxin A binding sites on rabbit distal colonic muscularis mucosae obtained utilizing biotinylated toxin A. Moderate binding is indicated by darker staining outlining individual cells of muscularis mucosae. Longitudinal and circular muscle were negative in this respect but are not shown here because, under these conditions, photographs of negative staining are essentially blank. Magnification ×20.
colon, however, it has previously been demonstrated that the excitatory innervation of the muscularis mucosae is entirely cholinergic (27). Thus, because the excitatory effects of toxin A on this tissue were both tetrodotoxin- and atropine-resistant, alterations in neuronal function would appear unlikely to be the mechanism underlying its action on this tissue. The atropine resistance of this phenomenon also makes it improbable that an indirect action such as inhibition of cholinesterase is the mechanism underlying toxin A-induced muscularis mucosae excitation. In the rabbit distal colon exposure of the muscularis mucosae to a low concentration of eserine has been shown to significantly increase the frequency of spontaneous contractions (27). However, the acetylcholine dependence of this effect also renders it atropine sensitive, whereas, as noted previously, toxin A-induced excitability is unaffected by this muscarinic antagonist.

It could also be argued that excitatory mediators released from cells such as neutrophils and mast cells could participate in the toxin A-induced latent excitatory response. Such cells are always present to some extent in the region of the rabbit distal colonic submucosa, muscularis mucosae, and epithelium (e.g., see Ref. 10). Whereas a role for excitatory mediators being released from such cells cannot be excluded based on the present data, it should be noted that in vitro exposure of the rabbit distal colonic epithelium itself to a high concentration of C. difficile toxin A does not alter basal short-circuit current (5), an effect that would be anticipated if the toxin was eliciting the release of significant quantities of excitatory endogenous mediators in this tissue (10). Moreover, because the toxin A-induced excitation and the inhibition were both attenuated by the lectin BS-1, this suggests that they are each mediated via a single common binding site or receptor located on the muscularis mucosae itself.

One additional possibility considered as an explanation for these observations was that BS-1 inhibits the actions of toxin A by binding to the toxin itself rather than by acting at a receptor. However, because toxin A is not a glycoprotein and lacks the Galβ1-3Galα1-4GlcNAc structure to which BS-1 binds, this would appear unlikely. Furthermore, when examined in Western blot experiments, no evidence was obtained for binding between these two proteins (Pothoulakis, unpublished observations).

In the rabbit ileum in vitro, C. difficile toxin B causes the cells of the circular muscle layer to become depolarized while at the same time attenuating the muscle’s response to applied stimuli such as carbachol (7). The basis of these phenomena has not been explained, but it was suggested that alterations in Ca2+ movement might be involved (7, 8). In view of the similarities between the motor effects of toxin B in the ileum and toxin A on the colonic muscularis mucosae it is possible that both act, at least in part, through a Ca2+-dependent mechanism. In support of this hypothesis is the known ability of C. difficile toxin A to promote the release of intracellular Ca2+ in human granulocytes (29). In gastrointestinal smooth muscle release of intracellular Ca2+ would be associated with an initial increase in motor activity and, if the released Ca2+ was not resequenced, this would be reflected in a decrease in the ability of the muscle to respond to subsequent pharmacological stimuli. This proposed pattern of events correlates well with the observed behavior of the colonic muscularis mucosae exposed to C. difficile toxin A. In addition, the decreased response to a K+ stimulus also supports this suggestion by demonstrating that an alteration in the excitation-contraction coupling process takes place at a level beyond the cell membrane.

One question that remains to be answered is the significance of these observations to the pathophysiology of antibiotic-associated colitis resulting from the proliferation of C. difficile in the colon. Although the muscularis mucosae is the muscle layer closest to the colonic lumen, the large molecular weight of C. difficile toxin A suggests that it would be contained on the apical side of the epithelium and would not have access to this site. However, the presence of circulating antibodies to toxins A and B in human subjects (34) suggests that transepithelial movement of these toxins may take place. One possible mechanism by which this could be achieved is the known ability of both toxin A and toxin B to alter the barrier function of intestinal epithelial cells in culture by altering the permeability of tight junctions (11, 12). Alternatively, toxin A may gain access to the submucosal aspect of the epithelium after toxin-induced mucosal damage, a characteristic of the latter stages of the disease process.

The function of the muscularis mucosae is thought to be related to control of the intestinal luminal surface area, thus indirectly facilitating or attenuating the processes of absorption and secretion. However, it has also been suggested that muscularis mucosae-induced mucosal movement may act to prevent bacterial adherence and/or overgrowth (25). Depression of muscularis mucosae motor function by a bacterial toxin would have the potential to set up a vicious cycle whereby bacterial adherence is enhanced, leading to the production of more toxin and thus further depressing mucosal movement and promoting additional bacterial proliferation and toxin production. The presence of a toxin A receptor on the muscularis mucosae suggests therefore that it may make a significant contribution to the processes that lead to the development of C. difficile-mediated antibiotic-associated diarrhea and colitis.

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