Inhibitory effects of botulinum toxin on pyloric and antral smooth muscle

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James, Arlene N., James P. Ryan, and Henry P. Parkman. Inhibitory effects of botulinum toxin on pyloric and antral smooth muscle. Am J Physiol Gastrointest Liver Physiol 285: G291–G297, 2003. First published March 26, 2003; 10.1152/ajpgi.00296.2002.—Botulinum toxin injection into the pylorus is reported to improve gastric emptying in gastroparesis. Classically, botulinum toxin inhibits ACh release from cholinergic nerves in skeletal muscle. The aim of this study was to determine the effects of botulinum toxin on pyloric smooth muscle. Guinea pig pyloric muscle strips were studied in vitro. Botulinum toxin type A was added; electric field stimulation (EFS) was performed every 30 min for 6 h. ACh (100 μM)-induced contractile responses were determined before and after 6 h. Botulinum toxin caused a concentration-dependent decrease of pyloric contractions to EFS. At a low concentration (2 U/ml), botulinum toxin decreased pyloric contractions to EFS by 43 ± 9% without affecting ACh-induced contractions. At higher concentrations (10 U/ml), botulinum toxin decreased pyloric contraction to EFS by 75 ± 7% and decreased ACh-induced contraction by 79 ± 9%. In conclusion, botulinum toxin inhibits pyloric smooth muscle contractility. At a low concentration, botulinum toxin decreases EFS-induced contractile responses without affecting ACh-induced contractions suggesting inhibition of ACh release from cholinergic nerves. At higher concentrations, botulinum toxin directly inhibits smooth muscle contractility as evidenced by the decreased contractile response to ACh.

TREATMENT OF SYMPTOMATIC GASTROPARESIS is primarily with prokinetic agents that increase antral contractility, improve antroduodenal coordination, and accelerate gastric emptying. Increased gastric outlet resistance due to pyloric sphincter dysfunction or pylorospasm has been described, primarily in diabetic gastroparesis (16). This pylorospasm might also cause a delay in gastric emptying and result in gastroparetic symptoms. Botulinum toxin injection into the lower esophageal sphincter (LES) decreases LES tone and improves symptoms in achalasia (20). Botulinum toxin injection into the pyloric sphincter is reported to improve gastric emptying and reduce dyspeptic symptoms in patients with gastroparesis (7, 14, 17).

Classically, in striated muscle, botulinum toxin inhibits ACh release from cholinergic nerves (10). Botulinum toxin A selectively cleaves the synaptosomal-associated protein (SNAP)-25, leading to the inability of synaptic vesicles containing neurotransmitters to undergo exocytosis and release of neurotransmitters (2). In gastrointestinal smooth muscle, botulinum toxin appears to also reduce cholinergic transmission by inhibiting ACh release, as shown in in vitro (1, 15) and in vivo (21, 23) studies. SNAP-25, the substrate for botulinum toxin, is also present in gastrointestinal smooth muscle, suggesting an additional site for botulinum toxin (15). The aim of this study was to determine the effects and mechanism of action of botulinum toxin on pyloric and antral smooth muscle.

MATERIALS AND METHODS

Pyloric and Antral Muscle Strip Preparation

Guinea pigs (Covance Research Products, Denver, PA) weighing ~400 to 450 g were used for this study. This study was approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine. The stomach with pylorus and proximal duodenum were removed after the guinea pig was killed by CO2 asphyxiation. The stomach was opened along the longitudinal axis and rinsed with Krebs bicarbonate buffer (in mM: 120 NaCl, 4.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 22 NaHCO3, 1.2 NaH2PO4, and 11.5 glucose, oxygenated with 95% O2-5% CO2, pH 7.4).

The mucosa was removed from the antrum and pylorus. Antral and pyloric circular muscle strips were prepared and suspended in 10-ml organ baths containing Krebs bicarbonate buffer (temperature 37°C). Generally, from each guinea pig, two antral muscle strips and two pyloric muscle strips were used. Muscle tension was measured along the circular muscle direction by using an isometric force transducer (model FT.03C, Grass Instruments, AstroMed, W. Warwick, RI). Muscle strips were suspended between platinum electrodes placed adjacent and parallel to the long axis of the muscle strip. Electrodes were connected to an electric stimulator (model SD9, Grass Instruments). After a 30-min equil-
ibration period, the preparations were stretched until the contractile force to 100 μM ACh was maximal.

Experimental Protocols

Muscle strips were studied in vitro for their contractile responses to electric field stimulation (EFS) and ACh. EFS with 2–16 Hz, 100 V, 0.5-ms pulse width duration (square wave), and 60-s train duration parameters was used to activate the intrinsic nerves. Nω-nitro-ω-arginine methyl ester (L-NAME; 100 μM) was added to inhibit nitric oxide-mediated relaxation. After initial control responses to EFS and ACh, 100 μM botulinum toxin type A (Allergan, Irvine, CA) was added in concentrations of 2, 4, or 10 U/ml. EFS (4 Hz, 60 s) was performed every 30 min for 6 h in the continued presence of botulinum toxin. ACh (100 μM)-induced contractile responses were also determined before and 6 h after botulinum toxin incubation. Control muscle strips were also studied with the same protocol but without the addition of botulinum toxin. In additional studies, the contractile responses to substance P (Sigma, St. Louis, MO) were performed before and after 6 h of incubation with botulinum toxin in a similar protocol as described above for ACh and EFS.

Data Analysis

Contractile responses to ACh, substance P, and EFS were measured as the maximal contractile response after stimulation. Data are expressed as means ± SE of results obtained from 4 to 15 muscle strips. One- and two-way ANOVA with factors of time after botulinum toxin administration and/or concentration of botulinum toxin and Student’s t-test with Bonferroni correction were used to determine whether the effects of botulinum toxin on EFS, ACh, and substance P-induced contractions were significantly different from the control responses. Each preparation served as its own control with the amplitude of contraction after incubation with botulinum toxin compared with the amplitude of contraction in Krebs solution immediately preceding the addition of botulinum toxin.

RESULTS

Pylorus

General observations: effects of EFS, ACh, and substance P. In normal Krebs solution, EFS produced frequency-dependent contractile responses in 28 of 28 pyloric muscle strips. The addition of 100 μM L-NAME increased the EFS-induced contractile response to 4 Hz from 2.3 ± 0.2 to 3.1 ± 0.2 g (P < 0.001). Pyloric contractions to EFS were completely inhibited by 1 μM atropine or 1 μM TTX indicating activation of cholinergic nerves (Fig. 1).

ACh caused concentration-dependent contractions of pyloric muscle strips (Fig. 2). ACh-induced contractions were completely inhibited by atropine but not by TTX, indicating a direct smooth muscle effect of ACh on pyloric smooth muscle (Fig. 1).

Substance P also caused concentration-dependent contractions of pyloric muscle strips (Fig. 2). The contractile effect of substance P was dose dependent with

![Fig. 1. Contractile effects of electric field stimulation (EFS) and ACh on guinea pig pyloric muscle strips. A: effect of Nω-nitro-ω-arginine methyl ester (L-NAME) and TTX. In normal Krebs solution (a), EFS produced a frequency-dependent contractile response with frequencies of 2, 4, 8, and 16 Hz; the response to 100 μM ACh is shown right. Note the change of scale after the 4-Hz EFS stimulation. The addition of 100 μM L-NAME (b) increased the contractile response to EFS. The addition of TTX to L-NAME (c) abolished the contractile response to EFS without abolishing the contractile response to ACh. B: effect of atropine on pyloric contractions to EFS and ACh. The addition of atropine to L-NAME inhibited the contractile response to both EFS and ACh (a). Small relaxations are seen during EFS in the presence of atropine and L-NAME (b).](http://ajpgi.physiology.org/)

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increasing amplitude of contractions from concentrations of 10^{-9} M to 3 \times 10^{-5} M (Fig. 2). The contractile response to substance P 10^{-6} M was 0.082 ± 0.055 kg/cm^2 compared with that of substance P (10^{-5} M, 0.120 ± 0.068 kg/cm^2) and to that of ACh (10^{-6} M, 0.206 ± 0.067 kg/cm^2).

Effect of botulinum toxin and atropine. Botulinum toxin caused a time-dependent and concentration-dependent decrease of pyloric contractions to 4 Hz EFS (Figs. 3 and 4). The inhibitory effects of botulinum toxin on EFS-induced contractions were gradual and slowly progressive (Fig. 4A). There was a significant concentration-related effect of botulinum toxin to inhibit EFS-induced contractions in the pylorus (two-way ANOVA with factors of concentration and duration of incubation in botulinum toxin: F = 20.458; P < 0.001). Each of the concentrations (2, 4, and 10 U/ml) of botulinum toxin inhibited EFS-induced contractions over time (2 U/ml: F = 3.996, P = 0.054; 4 U/ml: F = 12.749, P = 0.001; 10 U/ml: F = 45.35, P = 0.001). At a low concentration (2 U/ml), botulinum toxin after 6-h incubation decreased pyloric contractions to EFS by 43 ± 9% (P < 0.01) without affecting 100 μM ACh-induced contractions (2 ± 5% inhibition) (Figs. 3A and 4). At higher concentrations (10 U/ml), botulinum toxin decreased pyloric contraction to EFS by 75 ± 7% (P < 0.01) and decreased 100 μM ACh-induced contraction by 79 ± 9% (P < 0.01) (Figs. 3B and 4). For comparison, the muscarinic receptor antagonist, 1 μM atropine, nearly totally abolished the contractile response to EFS and ACh over the entire 6-h incubation period (Fig. 4).

In subsequent experiments, the effect of botulinum toxin was determined on EFS and substance P-induced contractions of the pylorus. Botulinum toxin (10 U/ml) decreased the pyloric contractile responses to EFS by 24 ± 11% (P = 0.05) after 6-h incubation in botulinum toxin but had no effect on substance P (1 μM)-induced contractions (7 ± 13% inhibition) (Fig. 5). In additional
experiments, the effect of botulinum toxin was tested to a higher concentration of substance P, namely 10 μM. Botulinum toxin (10 U/ml) decreased EFS-induced pyloric contractions by 25 ± 2% (P < 0.01) after a 6-h incubation in botulinum toxin but did not affect the contraction to 10 μM substance P (128 ± 32% of control; P > 0.10).

**Antrum**

**General observations.** In normal Krebs solution, EFS produced contractile responses in 13 of 22 (59%) antral muscle strips and a relaxation in 9 of 22 (41%) antral smooth muscle strips. The addition of 100 μM L-NAME caused a contractile response in all antral muscle strips averaging 2.4 ± 0.3 g. Antral contractions to EFS in the presence of L-NAME were completely inhibited by 1 μM atropine or 1 μM TTX, indicating activation of cholinergic nerves. ACh-induced contractions were inhibited by atropine but not TTX, indicating a direct smooth muscle effect of ACh on antral smooth muscle.

**Effect of botulinum toxin A and atropine.** Botulinum toxin had similar inhibitory effects on antral smooth muscle as in the pylorus (Figs. 6 and 7). There was a significant concentration-related effect of botulinum toxin to inhibit EFS-induced contractions in the antrum (two-way ANOVA with factors of concentration and duration of incubation in botulinum toxin: F = 9.318; P < 0.001). The concentrations of botulinum toxin that significantly inhibited EFS-induced contractions over time were with 4 U/ml (F = 16.371; P < 0.001) and 10 U/ml (F = 21.865; P < 0.001) but not with the lowest concentration of 2 U/ml (F = 0.102; P = 0.751). There was no significant inhibition with botulinum toxin (2 U/ml) compared with the time-dependent control muscle strips (Fig. 7A). At 4 U/ml, botulinum toxin decreased antral contractions to EFS by 60 ± 14% (P < 0.05) without affecting ACh-induced contractions (P = 0.05) (Figs. 6 and 7). At 10 U/ml, botulinum toxin decreased antral contractions to EFS by 61 ± 21% (P < 0.05) and decreased ACh-induced contractions by 49 ± 25% (P = 0.05) (Fig. 7). For comparison, the muscarinic receptor antagonist, 1 μM atropine, nearly totally abolished the contractile response to EFS throughout the 6-h time period (Fig. 7).

The inhibitory effects on the antrum tended to be less pronounced than in the pylorus, but direct statistical comparison of this did not reach statistical signif-

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**Fig. 4.** Effect of incubation of Botulinum (BT) on pyloric muscle strip contractility. A: time-dependent decrease in the pyloric contractile response to EFS. This inhibitory effect was also concentration-dependent with increasing inhibition from 2 to 10 U/ml. Shown for comparison is the near-total inhibitory response seen with atropine (ATR). There was a significant concentration-related effect of BT in inhibiting ACh-induced contractions in the pylorus by using two-way ANOVA with factors of time and concentration of BT (F = 20.458, P < 0.001). Each of the concentrations of BT inhibited EFS-induced contractions over time: 2 U/ml: F = 3.996, P = 0.054; 4 U/ml: F = 12.749, P = 0.001; 10 U/ml: F = 45.35, P = 0.001. B: effect of BT on ACh-induced pyloric contractions. There was no effect on 2 and 4 U/ml BT on the contractile response to ACh. In contrast, the highest concentration, 10 U/ml, caused a 79 ± 9% inhibitory effect on ACh-induced contractions. Shown also is the near-complete inhibition seen with atropine. There was a significant concentration-related effect of BT in inhibiting ACh-induced contractions in the pylorus by using one-way ANOVA with a factor of concentration of BT (F = 4.578, P = 0.013). Only the concentration of 10 U/ml BT was significantly different from control (F = 7.57, P = 0.019).

**Fig. 5.** Effect of Botulinum toxin on pyloric contractions to EFS and substance P (SP, 1 μM). The addition of 10 U/ml Botulinum toxin caused a progressive decrease in EFS-induced contractions. At the end of the 6-h Botulinum incubation, the substance P (1 μM)-induced contractile response is similar to the control substance P response at the beginning of the tracing. Note that for the EFS contractions there is a scale change.

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EFFECT OF BOTULINUM TOXIN ON THE PYLORUS

At 2 U/ml, botulinum toxin reduced the EFS-induced contractions in the pylorus by 43 ± 10% whereas in the antrum, by only 22 ± 27% (P = 0.484). Among all the concentrations (0, 2, 4, and 10 U/ml), two-way ANOVA for the percent inhibition at the end of 6-h incubation with factors concentration of botulinum toxin and location (antrum vs. pylorus) was not significant (F = 0.052; P = 0.820).

DISCUSSION

Our study has shown that botulinum toxin inhibits pyloric smooth muscle contractility. There appears to be two mechanisms for botulinum toxin to decrease pyloric sphincter contractility: inhibition of ACh release and direct inhibition of smooth muscle contractility. At a low concentration, botulinum toxin decreases EFS-induced contractile responses without affecting ACh-induced contractions, suggesting inhibition of ACh release from cholinergic nerves. At higher concentrations, however, botulinum toxin appears to directly inhibit smooth muscle contractility as evidenced by the decreased contractile response to ACh.

The experimental protocol used in this study involved incubation of the muscle strips to botulinum toxin for a prolonged period (6 h). The inhibitory effects of botulinum toxin on EFS-induced contractions were not immediate; the onset of the inhibitory effect was gradual and slowly progressive. This time-dependent effect may be related to either the mechanism of action of botulinum toxin or diffusion of the toxin into the tissue. The inhibitory effects were also concentration dependent in the concentration range of 2 to 10 U/ml. At the end of 6 h, the contractile effect to EFS was inhibited by 43% with 2 U/ml botulinum toxin and by 75% with 10 U/ml. By comparison, atropine that blocks muscarinic cholinergic receptors nearly completely abolished EFS-induced contractions. Studies in hippocampal slices have also shown that the inhibitory effect of botulinum toxin A is time and concentration dependent (19).

At a low concentration, botulinum toxin decreased EFS-induced contractile responses of the pylorus with out affecting ACh-induced contractions. This suggests botulinum toxin inhibits ACh release from cholinergic nerves. Classically, in striated muscle, botulinum toxin inhibits ACh release from cholinergic nerves (10). Neurotransmitter release, most notably ACh, involves fusion of the synaptic vesicles with the plasma mem-

Fig. 6. Effect of Botox on antral contractions to EFS and ACh. The addition of 4 U/ml of Botox caused a progressive inhibitory effect on EFS-induced contractions. At the end of 6-h incubation, ACh was given and the response was similar to the control ACh response at the beginning of the tracing.

Fig. 7. Effect of incubation of Botox (BT) on antral muscle strip contractility. A: there was time-dependent decrease in the antral contractile response to EFS. This inhibitory effect was seen with the concentrations of 4 and 10 U/ml, but not with the lower concentration of 2 U/ml. Shown for comparison is the near-total inhibitory response seen with atropine (ATR). There was a significant concentration-related effect of Botox to inhibit EFS-induced contractions in the antrum by using two-way ANOVA with factors of time and concentration of Botox (F = 9.318, P < 0.001). Concentrations of Botox that significantly inhibited EFS-induced contractions over time were: 4 U/ml: F = 16.371, P < 0.001; 10 U/ml: F = 21.865, P < 0.001, but not the lowest concentration of 2 U/ml (F = 0.102, P = 0.751). B: effect of Botox on ACh-induced antral contractions. There was a trend for Botox to cause a concentration-dependent decrease in ACh-induced antral contractions (F = 2.991, P = 0.053) by using one-way ANOVA with a factor of concentration of Botox. The concentration of Botox (10 U/ml) was significantly different from control (F = 11.094, P = 0.005). Shown also is the near-complete inhibition of ACh-induced contractions seen with atropine.
brane and extrusion of the ACh out of the neuron into the synapse. SNAP-25 is a protein implicated in the fusion of neuronal presynaptic vesicles with the plasma membrane (6). Botulinum toxin is the neurotoxin produced by the bacteria *Clostridium botulinum* (26). It consists of a heavy protein chain linked by a disulfide bond to a lighter protein chain. The heavy chain can bind to the synaptic membrane and the entire molecule is then taken up into the synaptic terminal by receptor-mediated endocytosis. The light chain interferes with SNAP-25. When botulinum toxin cleaves SNAP-25, the ACh-containing vesicles can no longer fuse with the plasma membrane, and exocytosis of ACh is inhibited (9, 14). In gastrointestinal smooth muscle, it is also thought that botulinum toxin reduces cholinergic transmission by inhibiting ACh release. This has been previously suggested in vitro studies with the LES (21) and the sphincter of Oddi (23) and now in the present in vitro study with the pyloric sphincter. Other studies (15, 24) have suggested botulinum toxin may have effects on other nerves besides cholinergic nerves. In rabbit hippocampal slices, although the release of ACh is most sensitive, botulinum A toxin incubation also inhibited noradrenalin and 5-HT release (19). In guinea pig uterine artery, botulinum toxin appears to affect EFS-induced contractility by several mechanisms including nearly abolishing contractions due to ACh release, reducing neuropeptide-mediated contractions but without an affect on nitric oxide-mediated relaxations (18). Other studies in gastrointestinal smooth muscle have suggested that botulinum toxin has no effect on nonadrenergic, non-cholinergic, inhibitory response of the guinea pig fundus (22) and the LES (4).

Our studies showed that higher concentrations of botulinum toxin (10 U/ml) directly inhibit cholinergic smooth muscle contractility as evidenced by the decrease contractile response to ACh. ACh, in this study, acts primarily on smooth muscle muscarinic receptors to directly contract the muscle, because the contractions to ACh were inhibited by atropine, but not by TTX. This suggests high concentrations of botulinum toxin may have a direct inhibitory effect on smooth muscle contractility. Our further studies showed that botulinum toxin decreased contractile responses to ACh but not to substance P, which contracts the pylorus predominantly by a direct effect on smooth muscle. This differential effect of botulinum toxin on ACh but not substance P-induced contractions may potentially offer some insight into the mechanism of botulinum toxin because it appears to be a specific effect on muscle contractility that would decrease contractility to a variety of agonists. High concentrations of botulinum toxin affect smooth muscle contractility to cholinergic muscarinic muscular transmission, possibly at the receptor level or on intracellular pathways. The exact cellular mechanism of botulinum toxin inhibiting ACh-induced contractions was not detailed in this study. Interestingly, SNAP-25, the substrate for botulinum toxin, has also been found to be present in gastrointestinal smooth muscle (12). This suggests an additional site for botulinum toxin in regulating muscle contractility, besides the classical effect of acting on presynaptic neurons to reduce ACh release (12). Botulinum toxin light chain administered intracellularly in smooth muscle cells has been shown to increase potassium channel currents (12). This would tend to hyperpolarize the muscle membrane and result in a decrease in muscle tone. SNAP-25 may have an additional role in inhibiting calcium channels (11, 12, 25). Both of these would decrease ACh-induced contractions. It is not known whether smooth muscle cells have the membrane pathways to internalize the toxin to act on intracellular SNAP-25, as are present on neurons (13).

Further studies are needed to reconcile the apparent neurotransmitter (ACh)-specific effect of botulinum toxin on the pylorus as shown in this study with the putative effects on potassium and calcium channels shown in other studies.

What is the clinical corollary of these in vitro studies? Delayed gastric emptying in diabetic gastroparesis is associated with antral hypomotility, increased pyloric tone, and pylorospasm. Increased gastric outlet resistance due to pyloric sphincter dysfunction or pylorospasm has been described, primarily in diabetic gastroparesis (5, 16) and may be, in part, responsible for the delay in gastric emptying and resultant symptoms. Botulinum toxin injected into the pylorus in patients with gastroparesis might relax the pylorus and facilitate gastric emptying. Several small open-label studies have shown this in diabetic gastroparesis (7, 14) and in idiopathic gastroparesis (17). Intrasphincteric injection of botulinum toxin has been shown to reduce pyloric pressure waves and pylorospasm in diabetic gastroparesis, but had no effect on antral contractility (5, 8).

Our study showed that botulinum toxin, in addition to decreasing pyloric contractility, also decreased antral smooth muscle contractility in vitro. Diffusion of botulinum toxin into the antrum or a misplaced injection might occur when attempting to inject the pylorus by using an endoscopic sclerotherapy needle. The inhibitory effect of botulinum toxin on EFS-induced antral contractility extrapolated to the in vivo condition might cause antral hypomotility and worsen gastric emptying. Postprandial antral contractility, as measured by antral manometry, correlates with gastric emptying of a solid meal (3). In our in vitro study, the pylorus tended to be more sensitive than the antrum to the inhibitory effect of botulinum toxin; however, direct comparison of this failed to reach statistical significance.

We studied the effects of botulinum toxin on both antral and pyloric muscle strips. It is also possible that the observed direct smooth muscle effect of the toxin may be peculiar to an in vitro system. Our experiments used time controls with Krebs incubation but did not use a protein control. Furthermore, we did not investigate whether the effect of botulinum toxin is temperature dependent. A natural extension of our in vitro studies is to investigate whether these inhibitory effects of botulinum toxin occur in vivo by injecting the
pylorus, in a similar manner to that performed clinically, and measuring changes in pyloric sphincter contractility.

In summary, botulinum toxin inhibits pyloric and antral smooth muscle contractility. At a low concentration, botulinum toxin decreases EFS-induced contractile responses without affecting ACh-induced contractions suggesting inhibition of ACh release from cholinergic nerves. At higher concentrations, botulinum toxin directly inhibits smooth muscle contractility as evidenced by the decreased contractile response to ACh.

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