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Inhibitory Effects of Botulinum Toxin Type A on Pyloric Cholinergic Muscle Contractility of Rat

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Abstract

Botulinum toxin type A (BTX-A) selectively cleaves synaptosomal-associated protein of 25 kDa (SNAP-25) and results in inhibition of the fusion of synaptic vesicles containing neurotransmitters with the presynaptic membrane to undergo exocytosis and release. The aim of this study was to investigate whether BTX-A inhibited the pyloric smooth muscle contractility induced by acetylcholine (ACh) after BTX-A-mediated cleavage of SNAP-25 antagonized by toosendanin (TSN). Three groups of rat pyloric muscle strips were studied *in vitro*. All strips were allowed to equilibrate for 52 min under a basal loading tension of 1 g in Krebs solution and spontaneous contractile waves were recorded as their own controls before adding each drug. According to experimental protocols, 100 μM ACh, 1 μM atropine, 29.6 μM TSN and 10 U/ml BTX-A was added, respectively. BTX-A directly inhibited pyloric spontaneous contraction and ACh-induced contractile response. Addition of 10 U/ml BTX-A still inhibited pyloric smooth muscle contractility following incubation of TSN, while subsequent administration of 100 μM ACh had no effect. BTX-A inhibits pyloric smooth muscle contractility in our study suggesting BTX-A inhibits not only ACh release from cholinergic nerves but also muscarinic cholinergic muscular transmission.

Key Words: acetylcholine, botulinum toxin type A, pyloric smooth muscle, synaptosomal-associated protein of 25 kDa, toosendanin

Introduction

Botulinum toxin (BTX) is a biological exotoxin produced from the gram-positive anaerobic bacterium *Clostridium botulinum*. It is well established that the principal target of BTX is the cholinergic nerve ending of neuromuscular junctions in skeletal muscles, where inhibition of acetylcholine (ACh) release by BTX results in neuromuscular blockade and paralysis (5, 13). Seven distinct serotypes of BTX have been identified and designated types A, B, C, D, E F and G (8, 34). One subtype is BTX type A (BTX-A), which consists of a heavy chain (HC, ~100 kDa) and a light chain (LC, ~50 kDa), linked by a single disulfide bound and non-covalent forces (21). HC is responsible for binding of toxin to reserve terminal and for internalization of LC to cytosol. LC is a zinc-dependent endopeptidase and specially cleaves a synaptosomal-associated protein of 25 kDa (SNAP-25) at the neuromuscular junction, thereby preventing the fusion of synaptic vesicles exocytosis and subsequent neurotransmitter release (1, 11). Based on this mechanism, BTX-A has been successfully used to treat sialorrhoea, temporomandibular disorder, bruxism, focal dystonia, muscle spasm, and muscle hypertrophy (2, 3, 9, 17, 19, 26).

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Gastroparesis is a disorder in which the stomach takes too long to empty its contents owing to increased gastric outlet resistance with pyloric sphincter dysfunction or pylorospasm, and a complication of diabetes, diabetic gastroparesis often occurs (23). This pylorospasm might cause a delay in gastric emptying and result in gastroparetic symptoms. In gastrointestinal smooth muscle, botulinum toxin appears to reduce cholinergic transmission by inhibiting ACh release, as shown *in vitro* (22) and *in vivo* (31) studies. It has been shown that intrapyloric BTX-A injections might relax the pylorus and facilitate gastric emptying in patients with diabetes (7, 15), idiopathic (25) and post-surgical gastroparesis (28). The pylorus is innervated by intrinsic neurons of the gastrointestinal nervous system (GNS) and by extrinsic parasympathetic, sympathetic and sensory nerves (30). The intrinsic neurons of GNS play an exclusive role in regulation of pyloric function after denervation *in vitro*. SNAP-25, the substrate for botulinum toxin, is also present in gastrointestinal smooth muscle, suggesting an additional site for botulinum toxin (13).

The aim of this study was to determine the effects of botulinum toxin on pyloric smooth muscle *in vitro*, when BTX-A-mediated cleavage of SNAP-25 was selectively blocked by toosendanin ($C_{30}H_{38}O_{11}$, FW = 574, TSN), a potential antibotulismic agent which has been demonstrated to antagonize BTX-A-mediated cleavage of SNAP-25 (33, 39). Our findings also provide valuable information for botulinum toxin detection on smooth muscle and for synaptic protein inhibitor screening assays used to develop botulinum toxin antagonists.

Materials and Methods

Pyloric Muscle Strip Preparation

Adult Sprague–Dawley rats (Experimental Animal Center, Lanzhou University, Lanzhou, Gansu, PRC) weighing 200–250 g were housed individually in cage with *ad lib* food and water in a 12-h light-dark cycle (light 07:00–19:00 h) room at 21 ± 1°C temperature and 50% relative humidity for 7 days before experiments. Experimental procedures were approved by the Institutional Animal Care and Use Committees of Gansu Province Medical Animal Center and Lanzhou University and carried out in accordance with European Communities Council Directive of 24 November 1986 (86/609/EEC). All performances were undergone to minimize animal suffering and only the number of animals necessary to produce reliable scientific data was used. The stomach with pylorus and proximal duodenum were removed after rat was sacrificed by CO_2. An approximately 10 mm × 2 mm pyloric circular muscle strip was rapidly separated from stomach asphyxiation. The prepared strip was carefully rinsed and suspended by a string in an incubation bath containing 5 ml Krebs bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 12.2 mM glucose, oxygenated with 95% O_2-5% CO_2, pH7.4) at constant 37°C. One terminal of strip was attached to a muscular force transducer (JH-2, Space Medico-Engineering Institute, Beijing, PRC) onto the Mac Lab (model BL-420E, TM, Chengdu, Sichuan, PRC) for isometric tension recording. Muscle strips were allowed to equilibrate under a basal loading tension of 1g for 52 min and their spontaneous contractile waves were regularly recorded as their own controls prior to using drugs.

Experimental Protocols

Pyloric muscle strips were divided into three groups to study *in vitro*. In the first group, 1 μM atropine (n = 5, Sigma, St. Louis, MO, USA) or 10 U/ml BTX-A (n = 5, Lanzhou Institute of Biological Products, Lanzhou, Gansu, PRC) was respectively added into two incubation baths for 4 h after initial response to 100 μM ACh (Sigma) for 10 min. In the second group (n = 10), 100 μM ACh was added for 4 h after initial response to 10 U/ml BTX-A or 1μM atropine for 30 min. In the third group (n = 5), 10 U/ml BTX-A was added after initial response to 29.6 μM TSN (Key Laboratory of Neurobiology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, PRC) for 20 min, and 30 min later, 100 μM ACh subsequently added for 4 h.

Data Analysis

Contractile responses to drugs were measured as the maximal contractile response after treatment. Each preparation served as its own control with the tension, frequency and amplitude of contraction in Krebs solution compared respectively with those of contraction induced by ACh, TSN, BTX-A and atropine. Data were expressed as means ± standard error of the mean (SEM) of results obtained from 5 to 20 muscle strips and statistically analyzed using one-way analysis of variance (ANOVA), and *post hoc* Fisher's least significant difference (LSD) test by SPSS 17.0 for Windows. In all statistical com-parisons, the criterion for significance was set at $P < 0.05$.

Results

BTX-A Inhibited Pyloric Muscle Contractions Induced by ACh

In the first group, addition of 100 μM ACh into

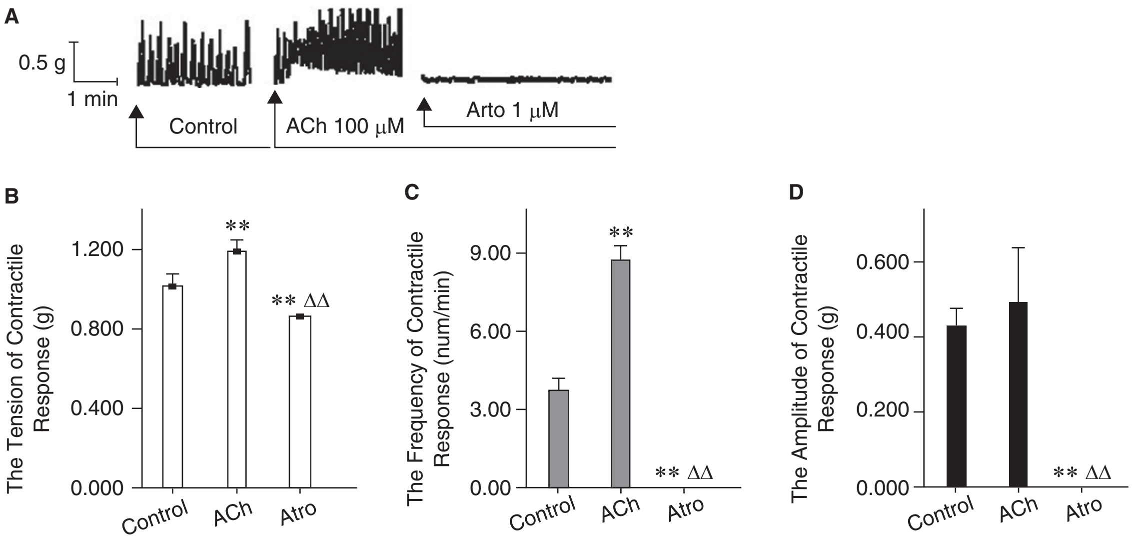

Fig. 1. Effects of ACh and subsequent atropine on rat pyloric isolated smooth muscle strips. A: The pyloric contractile graph showed that pyloric muscle contracted spontaneously in Krebs solution, 100 μM ACh induced a more intensive contractile responses and subsequent addition of 1 μM atropine inhibited completely ACh-induced muscle contractile response. B, C and D: The tension, frequency and amplitude of the pyloric contractile response to ACh and then atropine additions. $^{**}P < 0.001$ *vs.* control; $^{\Delta\Delta}P < 0.001$, atropine *vs.* ACh. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test (Ach, acetylcholine; Atro, atropine.)

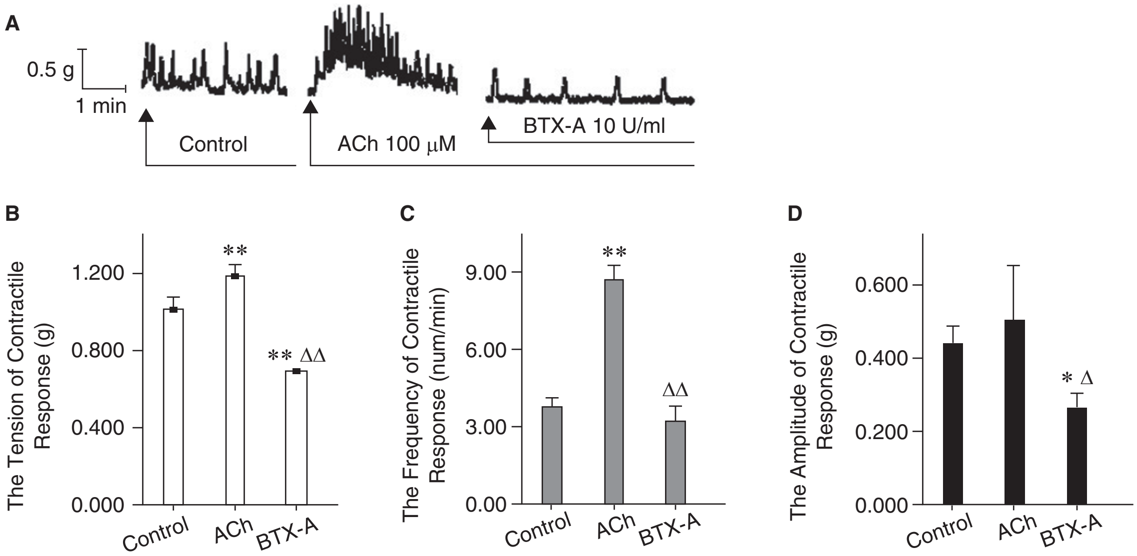

Fig. 2. Effects of ACh and subsequent BTX-A on rat pyloric isolated smooth muscle strips. A: The pyloric contractile graph showed that pyloric muscle contracted spontaneously in Krebs solution, 100 μM ACh induced a more intensive contractile responses and subsequent addition of 10 U/ml BTX-A inhibited ACh-induced muscle contractile response. B, C and D: The tension, frequency and amplitude of the pyloric contractile response to ACh and then BTX-A additions. $^{*}P < 0.05$, $^{**}P < 0.001$, *vs*. control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.001$, BTX-A *vs*. ACh. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

Krebs solution significantly enhanced pyloric muscle contractile tension (from 1.020 ± 0.025 to 1.188 ± 0.031 g, $P < 0.001$; Fig. 1, A and B; Fig. 2, A and B) and frequency (from 3.75 ± 0.13 to 8.75 ± 0.25 num/min, $P < 0.001$; Fig. 1, A and C; Fig. 2, A and C), but not contractile amplitude (from 0.431 ± 0.021 to 0.493 ± 0.067 g, $P = 0.769$; Fig. 1, A and D; Fig. 2, A and D) in preparations. Subsequent addition of 10 U/ml BTX-A suppressed ACh-induced contractile responses including tension (from 1.188 ± 0.031 to 0.702 ± 0.004 g, $P < 0.001$; Fig. 2, A and B), frequency (from 8.75 ± 0.25 to 3.25 ± 0.25 num/min,

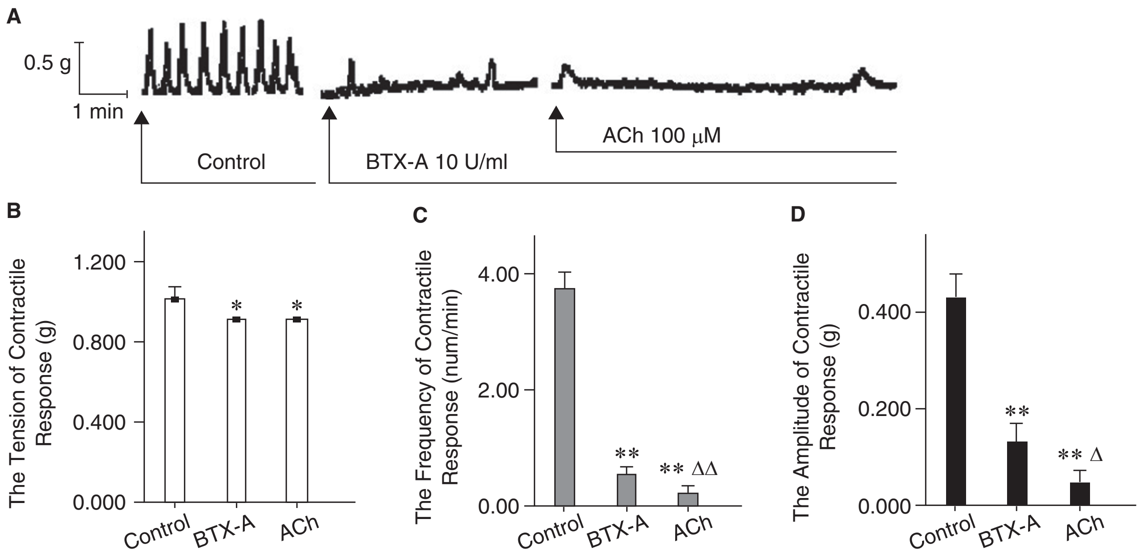

Fig. 3 Effects of BTX-A and ACh on rat pyloric isolated smooth muscle strips. A: The pyloric contractile graph showed that pyloric muscle contracted spontaneously in Krebs solution, 10 U/ml BTX-A incubation suppressed the pyloric muscle contraction and further addition of 100 μM ACh did not induce muscle contractile response. B, C and D: The tension, frequency and amplitude of the pyloric contractile response to BTX-A and then ACh additions. $^{*}P < 0.05$, $^{**}P < 0.001$, *vs*. control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.001$, ACh *vs*. BTX-A. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

$P < 0.001$; Fig. 2, A and C) and amplitude (from 0.493 ± 0.067 to 0.262 ± 0.019 g, $P = 0.014$ Fig. 2, A and D). Atropine 1 μM comparably suppressed ACh-induced contractile responses. Nevertheless, the inhibition of atropine was almost complete (Fig. 1).

ACh Did Not Agitate Any More Muscle Contractions following BTX-A Treatment

In the second group, BTX-A at 10 U/ml (Fig.3) directly inhibited pyloric muscle spontaneous contractions, including contractile tension (from 1.020 ± 0.025 to 0.919 ± 0.008 g, $P = 0.006$), frequency (from 3.75 ± 0.13 to 0.56 ± 0.06 num/min, $P < 0.001$) and amplitude (from 0.431 ± 0.021 to 0.132 ± 0.019 g, $P < 0.001$), and the addition of ACh, 30 min later, did not agitate any more pyloric muscle contraction (Fig. 3).

BTX-A Inhibited Pyloric Muscle Contractions following TSN Treatment

In the third group, the addition of TSN 29.6 μM in initial period of 20 min had no influence on pyloric muscle spontaneous contractility, subsequent BTX-A addition still decreased pyloric muscle contractile tension from 0.963 ± 0.008 to 0.913 ± 0.006 g, $P < 0.001$ (Fig. 4, A and B) and amplitude from 0.412 ± 0.032 to 0.225 ± 0.021 g, $P < 0.001$ (Fig. 4, A and D). The inhibitory effect of BTX-A was continuous on pyloric smooth muscle so that ACh did not excite muscle contractility (Fig. 4).

Discussion

The pyloric muscle spontaneous contraction is mediated by endogenous GNS *in vitro*. Our current results has demonstrated that ACh induced strong contractile responses in pyloric smooth muscle strips and subsequent atropine almost suppressed this effects (Fig. 1). When BTX-A instead of atropine was added, its inhibitory effect on ACh-induced contractile responses was similar to atropine and its action appeared relatively weak (Fig. 2). This suggests that the suppression of spontaneous contraction is due to the inhibition of ACh release from cholinergic nerves. However, exogenous ACh contracted the muscle by acting on smooth muscle muscarinic receptors of post-synaptic membrane directly, because the ACh-induced contractile responses were inhibited by atropine. The experimental protocol in the study involved initial incubation of muscle strips with BTX-A and further addition ACh in order to identify that the presynaptic action of BTX-A wheth-er affected ACh post-synaptic action. After BTX-A incubation, the paralysed muscle strips were not agitated by exogenous ACh (Fig. 3), which suggested that BTX-A could inhibit or block the bind of ACh with cholinergic muscarinic receptors.

Our results have further shown that the 10 U/ml of BTX-A directly inhibited cholinergic smooth muscle contractility. There appears to be two mechanisms for BTX-A to decrease pyloric spontaneous contraction *in vitro*. Firstly, BTX-A inhibits ACh release

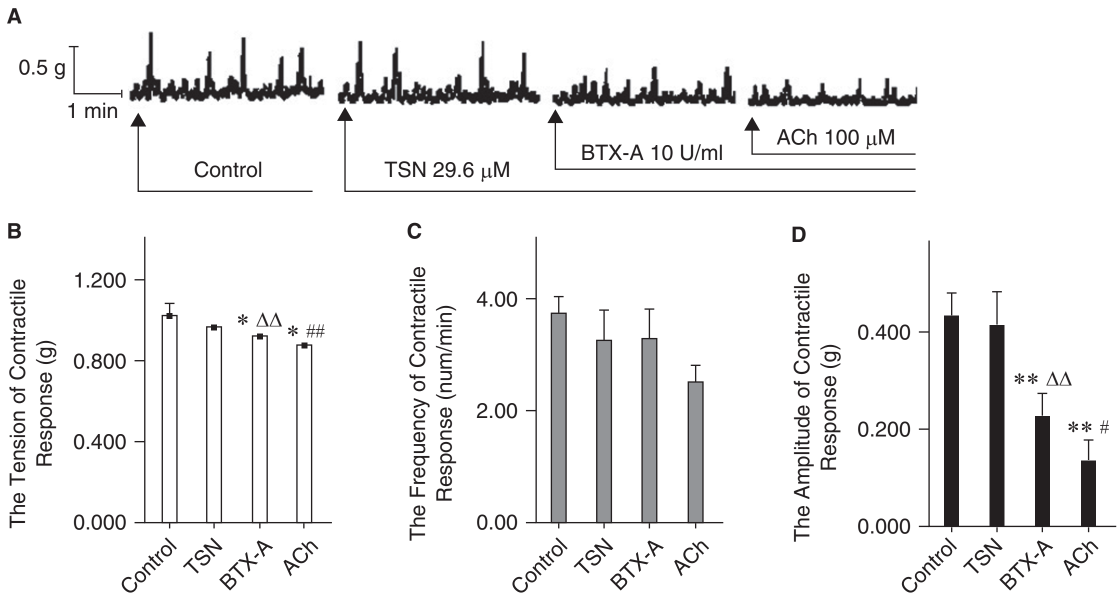

Fig. 4 Effects of BTX-A and then ACh on rat pyloric isolated smooth muscle strips after TSN incubation. A: The pyloric contractile graph showed that pyloric muscle contracted spontaneously in Krebs solution, 29.6 μM TSN incubation did not influence pyloric muscle spontaneous contractility, 10 U/ml BTX-A subsequent addition still inhibited pyloric muscle contractile tension and amplitude and 100 μM ACh did not enhance pyloric muscle contractions. B, C and D: The tension, frequency and amplitude of the pyloric contractile response to TSN, BTX-A and then ACh additions. $^{*}P < 0.05$, $^{**}P < 0.001$, *vs.* control; $^{\Delta\Delta}P < 0.001$, BTX-A *vs*. TSN; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, ACh *vs*. BTX-A. Statistics were analyzed by one-way ANOVA and followed by Fisher's LSD test.

from cholinergic nerves in striated muscle in a classical way (12). A four-step mechanism consisting of binding, internalization, translocation and cleaving soluble N-ethylmaleimide sensitive fusion protein (NSF) accessory protein receptor (SNARE) protein is the accepted view to explain BTX-A intoxication (4, 27, 29). The HC of BTX can bind to the synaptic membrane and entire molecule then internalizes into the synaptic terminal by receptor-mediated endocytosis. The LC of BTX interferes with SNAP-25, which leads to the failure of the ACh-containing vesicles to fuse with the plasma membrane and therefore inhibition of ACh exocytosis (6, 16, 38). On the other hand, in gastrointestinal smooth muscle, BTX-A could also appear to alleviate muscle contraction by interacting with several other neuronal signaling pathways such as those triggered by substance P (SP), glutamate, and calcitonin gene related peptide (5, 24). Our recent study has demonstrated that pyloric intrasphincteric injection of BTX-A *in vivo* inhibited pyloric myoelectrical slow activity in amplitude, spike activity and SP release in rats (10). In fact, our current study showed that the action of BTX-A in noncholinergic pathway (shown in Fig. 4) seemed to be incomplete and weaker than it done in the classical cholinergic pathway (shown in Fig. 3). These data suggest that the neurontoxic effect of BTX on smooth muscle acts through not only ACh but also neuropeptides such as SP.

TSN, a triterpenoid derivative extracted from the bark of *Melia toosendan* Seib et Zucc, has been recently demonstrated to be an effective antibotulismic agent by interfering with the cleavage of SNAP-25 by BTX-A (33, 39). After TSN treatment, the synaptosomes resist BTX-A-mediated cleavage of their SNAP-25 due to a direct inhibition of endopeptidase activity of the LC of BTX-A (39). Our further studies showed that BTX-A still decreased pyloric muscle strip contractility after the incubation of TSN, the onset of inhibitory effect was persistence so that the subsequent addition of ACh did not cause contractile response. Recently, several studies have suggested the possibility of the mechanisms of action of BTX-A. BTX-A inhibits both ACh- (12) and SP-(32) induced pyloric smooth muscle contractility in a concentration and time-dependent manner. Especially, at higher concentrations, BTX-A (10 U/ml) directly inhibits smooth muscle contractility as evidenced by the decreased contractile response to ACh (12). In addition, SP, coexisting with ACh and encephalin (19, 20) in gastrointestinal smooth muscle, has been identified a novel role that appears to be important for the maintenance of muscular responsiveness to the principal excitatory neurotransmitter, ACh (18). So, reduction of SP, together with the inhibitory characteristics of concentration- and time-dependent effects of BTX-A, might be the reason that paralysed muscle strips could not be agitated by exogenous ACh. It has also been suggested that BTX-A affects smooth muscle contractility *via* cholinergic muscarinic muscular transmission, possibly at the receptor level or on intracellular pathways.

In summary, BTX-A directly inhibited pyloric spontaneous contraction *in vitro* suggesting inhibition of ACh release from cholinergic nerves. BTX-A also decreased pyloric muscle strips contractility after the incubation of TSN, an antagonist to BTX-A-mediated cleavage of SNAP-25. The onset of inhibitory effects was so continual that the addition of ACh could no longer induce contractile response. These evidences suggest that BTX-A inhibits not only ACh release from cholinergic nerves but also muscarinic cholinergic muscular transmission.

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