

Botulinum Neurotoxin A Blocks Cholinergic Ganglionic Neurotransmission in the Dog Heart

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ABSTRACT—There is no data about whether botulinum neurotoxin inhibits the parasympathetic ganglionic neurotransmission in the heart, although botulinum toxin as a clinical drug inhibits the release of acetylcholine at the neuromuscular junction. Therefore, we investigated whether botulinum toxin (type A) injected into the sinoatrial (SA) fat pad inhibits decreases in heart rate induced by stimulation of the preganglionic parasympathetic nerves in the heart of the anesthetized dog. Stimulation of the parasympathetic nerves in the SA fat pad (SAP stimulation) prolonged the atrial interval but not the atrioventricular (AV) interval, and cervical vagus nerve stimulation (CV stimulation) prolonged both atrial and AV intervals. After botulinum toxin (20 or 25 mouse units) was injected into the SA fat pad, it gradually inhibited the prolongation of the atrial interval evoked by SAP and CV stimulations but not the prolongation of the AV interval evoked by CV stimulation. Conditioning successive stimulation of the cervical vagus nerves accelerated the inhibition by botulinum toxin of the chronotropic response to CV stimulation. These results indicate that selective injection of botulinum toxin into the SA fat pad blocks bradycardia mediated by parasympathetic ganglionic activation in the dog heart.

Keywords: Botulinum toxin, Bradycardia, Cholinergic neurotransmission, Intracardiac parasympathetic ganglia

The botulinum neurotoxins are produced by *Clostridium botulinum* and cause the clinical syndrome of botulism, a flaccid paralysis of the skeletal muscles. The botulinum toxins are the most potent toxins known and seven different neurotoxins, serotype A, B, C₁, D, E, F and G, have been classified. These toxins are zinc-binding metalloendopeptidases (1). They disrupt cholinergic neurotransmission by blocking the exocytotic release of acetylcholine stored in synaptic vesicles (2). Botulinum toxins are used in the clinical treatment of several spastic disorders (3, 4) and the experimental treatments (5–7). Those clinical and experimental treatments have been performed on the neuromuscular junctions. Although botulinum toxin injection into the coronary artery of mammals has been reported to have direct cardiac effects (8), there are no reports of the effects of botulinum toxin on the parasympathetic ganglia of the heart. Serotype A among these toxins is the most popular type used as a therapeutic agent of several spastic disorders (3, 4).

Heart rate is decreased by acetylcholine released from the parasympathetic nerve terminals that are activated by the impulses mediated through the parasympathetic ganglia in the heart. In humans (9) as well as dogs (10–12), almost all parasympathetic inputs that influence the heart rate pass through the parasympathetic ganglionic cells in the sinoatrial (SA) fat pad that overlies the right atrial junction of the right pulmonary veins in the heart. Stimulation of the parasympathetic neural elements in the SA fat pad decreases atrial rate and may cause sinus arrest in the dog heart (12). These results suggest that the regulation at the ganglionic neurotransmission of the parasympathetic neural transduction mechanism is one of the methods for the parasympathetic neural regulation of the heart. Acetylcholinesterase inhibitors, muscarinic receptor antagonists and post-receptor mechanism regulators, i.e., a G-protein regulator and a cyclic GMP inhibitor, have been developed to regulate bradycardia at the post-ganglionic neurotransduction mechanism. Therefore, we tested whether selective injection of botulinum toxin A into the SA fat pad blocks bradycardia induced by cervical vagus nerves in the anesthetized dog heart.

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MATERIALS AND METHODS

The animal experiments were approved by the Shinshu University School of Medicine Animal Studies Committee and animals were obtained through the Animal Laboratory for Research of Shinshu University School of Medicine.

Preparations

Fifteen mongrel dogs, which weighed 15–28 kg, were anesthetized with pentobarbital sodium (30 mg/kg, i.v.); supplemental doses of pentobarbital sodium were given as necessary to maintain stable anesthesia. A tracheal cannula was inserted, and intermittent positive-ventilation (tidal volume of 20 ml/kg and frequency of 15 strokes/min) was started. The chest was opened transversely at the fourth intercostal space. Each cervical vagus nerve was ligated tightly and crushed at the neck, and each stellate ganglion was crushed with a tight ligature at its junction with the ansa subclavia. These maneuvers remove almost all tonic neural activity to the heart (13).

Two bipolar electrodes were placed on the base of the epicardial surface of the right atrial appendage and the epicardial surface of the right ventricle to record the electrical activity. Atrial interval and atrioventricular (AV) intervals (AV conduction time) were measured and displayed on a thermo-writing rectigraph (WT685T; Nihon Kohden, Tokyo). Systemic arterial blood pressure was also measured via the right femoral artery.

Two bipolar silver electrodes, which had 2-mm inter-electrode distance, were used to stimulate intracardiac parasympathetic neural elements (12). One was placed on the fat pad overlying the right atrial side of the juncture of the right pulmonary veins (SA fat pad): we refer to this electrical stimulation of the intracardiac parasympathetic nerves to the SA node region as SAP stimulation. The other was placed on the fat pad (AV fat pad) at the junction of the inferior vena cava and left atrium: we refer to this electrical stimulation of the intracardiac parasympathetic nerves to the AV node region as AVP stimulation. Both electrodes were connected to an electrical stimulator (SEN7103, Nihon Kohden). We used a steady stimulation with 10-V pulse amplitude, 0.01–0.05-ms pulse duration and a frequency of 10–30 Hz for 20 s. This stimulation intensity was subthreshold for activation of pacemaker cells and cardiac muscle cells. SAP stimulation was adjusted to prolong the atrial interval by 300 ms (a 50 beats/min decrease) or more and AVP stimulation was adjusted to prolong the AV interval 30 ms or more. To stimulate preganglionic parasympathetic efferent nerves to the heart, two fine copper needle electrodes were inserted into each cervical vagus complex: we refer to such electrical stimulation as CV stimulation. Cervical vagus nerve fibers were stimulated with 10 V, 0.01–0.02 ms, and a frequency of

5–20 Hz to induce similar prolongations of the atrial interval and AV interval that were induced by SAP stimulation and AVP stimulation, respectively.

Protocols

We studied the effects of botulinum neurotoxin A injected into the SA fat pad on the increases in atrial interval induced by SAP stimulation and CV stimulation and the increases in AV interval induced by AVP stimulation and CV stimulation for 7 h in three groups, the control group ($n = 4$), the botulinum toxin treated group without the successive cervical vagus stimulation ($n = 5$) and the toxin treated group with the successive vagus stimulation ($n = 4$). Thirty minutes after the determination of the control cardiac responses to each electrical stimulation, botulinum neurotoxin A was injected into the SA fat pad at a dose of 20 or 25 mouse units in a volume of 0.2 ml saline. Then, we studied the effects of botulinum toxin on the cardiac responses to each 20-s-stimulation with 1-h interval for 7 h. In the botulinum toxin treated group with the conditioning vagus stimulation, to determine whether conditioning successive vagus stimulation accelerates the inhibitory effects of botulinum toxin on the negative cardiac responses to parasympathetic nerve stimulation, we stimulated the cervical vagus nerves at a frequency of 2 Hz with 0.1-ms pulse duration and 10 V during experiments. Three minutes before the test stimulation, we stopped the successive vagus stimulation and then we determined the cardiac responses to each parasympathetic nerve stimulation.

To test the inhibitory effects of botulinum toxin on the magnitude of the negative cardiac responses to parasympathetic stimulation, we further investigated the effects of a stimulation frequency of the vagus stimulation on the inhibitory effects of botulinum toxin in 4 dogs. We changed the stimulation frequency from 2 to 5, 10 and 30 Hz with 0.01-ms pulse duration and 10 V. Each 20-s stimulation with 30-s interval was given to the vagus nerves with 1-h interval for 7 h. During the experiments, we stimulated the cervical vagus nerves at a frequency of 2 Hz with 0.1-ms pulse duration and 10 V. Three minutes before the test stimulation, we stopped the successive vagus stimulation and then we determined the cardiac responses to each parasympathetic nerve stimulation.

Toxin

Botulinum toxin, botulinum neurotoxin serotype A, was kindly supplied by Dr. Shunji Kozaki (Osaka Prefecture University, Osaka).

Statistical analyses

All data are shown as the means \pm S.E.M. Fifty percent inhibition times (IT_{50}) were determined for each time-response curve. An analysis of variance with Bonferroni's

test was used for the statistical analysis of multiple comparisons of data. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Chronotropic and dromotropic responses to SAP stimulation, AVP stimulation and CV stimulation (Table 1)

Stimulation of both sides of the cervical vagus nerve complexes (CV stimulation) prolonged both atrial and AV intervals as shown in Table 1. On the other hand, stimulation of the selective parasympathetic ganglia in the SA

fat pad (SAP stimulation) prolonged the atrial interval (sinus cycle length) but not the atrioventricular interval. Stimulation of the selective parasympathetic ganglia in the AV fat pad (AVP stimulation) increased the AV interval but not the atrial interval.

Effects of botulinum toxin A injected into the SA fat pad on the negative chronotropic and dromotropic responses to CV stimulation and discrete parasympathetic stimulation

After an injection of the botulinum neurotoxin A into the SA fat pad at a dose of 20 or 25 mouse units in a volume of 0.2 ml saline in the open-chest anesthetized dogs, it

Table 1. Changes in atrial interval (AAI) and atrioventricular interval (AVI) in response to stimulation of the cervical vagal nerve fibers (CV stimulation), stimulation of the parasympathetic nerve fibers in the SA fat pad (SAP stimulation) and stimulation of the parasympathetic nerve fibers in the AV fat pad (AVP stimulation) in the open-chest anesthetized dogs

Stimulation	Number of animals	Before stimulation		Stimulation	
		AAI (ms)	AVI (ms)	Increase in AAI (ms)	Increase in AVI (ms)
CV stimulation					
control	n = 4	481 ± 41.8	143 ± 8.7	516 ± 67.6	61 ± 11.6
botulinum, VS (−)	n = 5	482 ± 18.5	140 ± 7.7	440 ± 49.4	44 ± 7.0
botulinum, VS (+)	n = 5	434 ± 16.2	127 ± 10.9	442 ± 99.7	44 ± 8.3
SAP stimulation					
control	n = 4	489 ± 41.6	143 ± 8.6	516 ± 33.9	−2 ± 3.5
botulinum, VS (−)	n = 5	476 ± 17.5	141 ± 5.5	456 ± 71.3	1 ± 2.3
botulinum, VS (+)	n = 4	435 ± 16.1	129 ± 9.4	455 ± 114.3	−6 ± 3.4
AVP stimulation					
control	n = 4	487 ± 41.5	143 ± 8.4	0	48 ± 7.0
botulinum, VS (−)	n = 4	478 ± 23.2	144 ± 4.7	0	40 ± 4.4
botulinum, VS (+)	n = 4	443 ± 11.1	129 ± 9.7	0	63 ± 6.9

Data of the control group (control), botulinum toxin treatment without conditioning vagus stimulation (botulinum VS (-)), and botulinum toxin treatment with conditioning vagus stimulation (botulinum VS (+)) are shown as the means ± S.E.M. in each stimulation experiment.

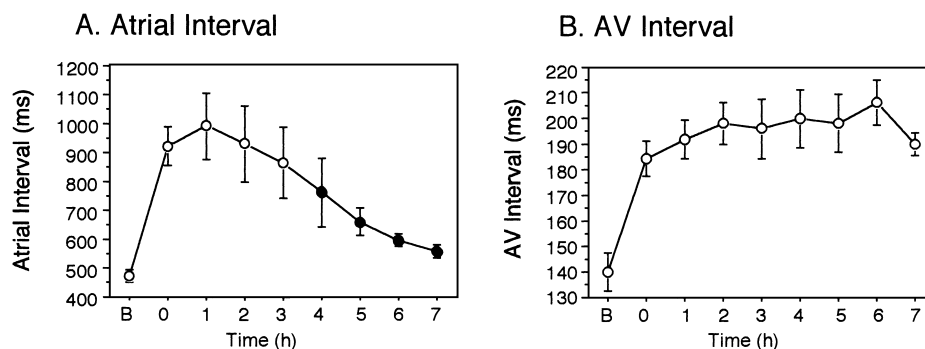


Fig. 1. Inhibition by botulinum toxin of the increase in atrial interval (A) but not the increase in atrioventricular (AV) interval (B) evoked by cervical vagus nerve stimulation in 5 anesthetized dogs. B, control atrial or AV interval before stimulation of the cervical vagus nerve fibers. Time 0 shows the atrial and AV intervals evoked by cervical vagus stimulation before the botulinum toxin injection into the SA fat pad. Circles show mean values of the data and closed circles show the significant (*P* < 0.05) changes from the control value. Vertical bars show S.E.M.

gradually inhibited the prolongation of the sinus cycle length in response to CV stimulation (Fig. 1A), but not the prolongation of the AV conduction for 7 h (Fig. 1B).

The inhibition by botulinum toxin at the used doses of the negative chronotropic response to vagus stimulation developed gradually (Figs. 1 and 2A). Botulinum toxin suppressed the chronotropic response to vagus stimulation 7 h after toxin treatment. The 50% inhibition time was 3.77 ± 0.55 h (Table 2). When we successively stimulated the CV nerves at a frequency of 2 Hz (i.e., conditioning stimulation) during the experiments, the inhibition by botulinum toxin of the chronotropic response to CV stimulation was accelerated significantly ($P < 0.001$, Fig. 2A). Its 50%

inhibition time was 1.56 ± 0.22 h and it is shorter ($P < 0.05$) than that of the non-conditioning stimulation groups. However, the botulinum toxin injected into the SA fat pad did not affect the negative dromotropic response to vagus stimulation in either experimental group (Fig. 2B).

Botulinum toxin A treated into the SA fat pad inhibited the negative chronotropic response to SAP stimulation (Fig. 2C). The 50% inhibition time (1.63 ± 0.38 h) by toxin in the conditioning stimulation group was shorter ($P < 0.05$) than that (3.34 ± 0.73 h) in the non-conditioning stimulation group. The 50% inhibition times by botulinum toxin in the CV stimulation group were not significantly different from those in the SAP stimulation group. On the other

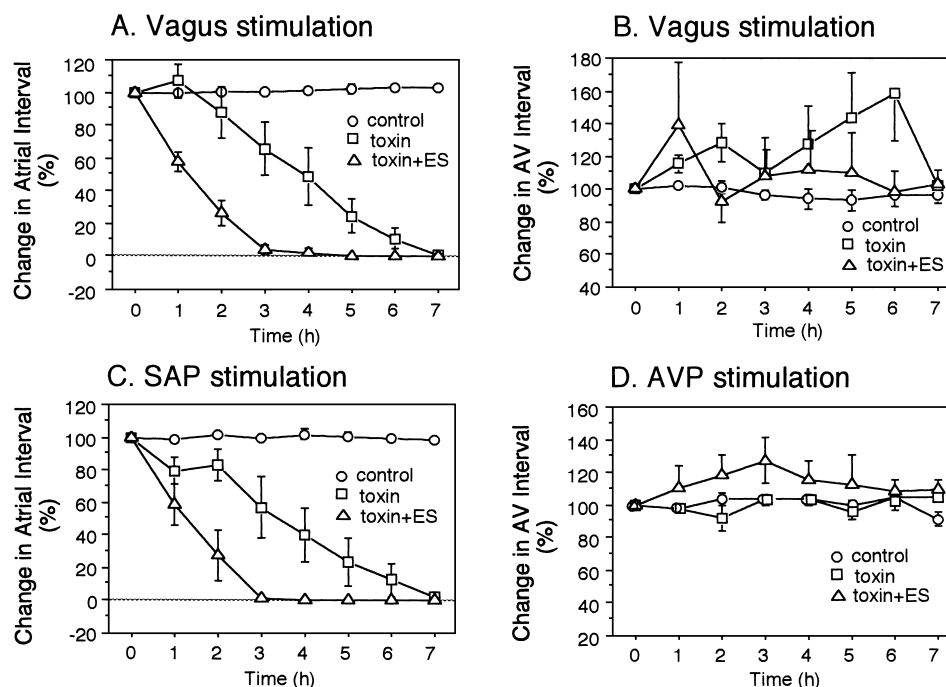


Fig. 2. Effects of botulinum toxin on the negative chronotropic (A) and dromotropic (B) responses to cervical vagus nerve stimulation, the negative chronotropic responses to SA fat pad parasympathetic nerve stimulation (C), and the negative dromotropic responses to AV fat pad parasympathetic nerve stimulation (D) in the anesthetized dog. Circles, control; squares, botulinum toxin alone; triangles, botulinum toxin with conditioning electrical stimulation (ES). Vertical bars show S.E.M. The number of animals is shown in Table 1.

Table 2. Fifty percent inhibition times (IT_{50}) by botulinum toxin A of the negative chronotropic responses to stimulation of the cervical vagus nerve fibers (CV stimulation) and stimulation of the parasympathetic nerve fibers in the SA fat pad (SAP stimulation) in open-chest anesthetized dogs

Stimulation	Number of animals	IT_{50} (h)
CV stimulation without the conditioning stimulation	n = 5	3.77 ± 0.55
CV stimulation with the conditioning stimulation	n = 4	$1.56 \pm 0.22^*$
SAP stimulation without the conditioning stimulation	n = 5	3.34 ± 0.73
SAP stimulation with the conditioning stimulation	n = 4	$1.63 \pm 0.38^*$

* $P < 0.05$ vs the IT_{50} of the data without the conditioning stimulation group.

hand, botulinum toxin injected into the SA fat pad did not inhibit the negative dromotropic responses to AVP stimulation in the conditioning or non-conditioning stimulation group (Fig. 2D).

In 4 dogs treated with saline into the SA fat pad, the negative electrical cardiac responses to each stimulation did not change significantly for more than 7 h in each experiment (Fig. 2).

Effects of botulinum toxin on the magnitude of the negative chronotropic responses to CV stimulation

Stimulation of the vagus nerves at a frequency of 2, 5, 10 and 30 Hz with 0.01 – 0.03-ms pulse duration and 10 V for 20 s increased the sinus cycle length by 43 ± 6.3 , 68 ± 6.3 , 130 ± 26.8 and 275 ± 38.8 ms, respectively, in 4 anesthetized dogs. Botulinum toxin treatment inhibited the negative chronotropic responses to vagus stimulation at a frequency of 2 to 30 Hz similarly with time during conditioning stimulation. There is no significant difference among the 50% inhibition times of the negative chronotropic responses by the toxin. The 50% inhibition times by the toxin of the responses to vagus stimulation at 2, 5, 10 and 30 Hz were 1.66 ± 0.31 , 1.44 ± 0.31 , 1.27 ± 0.30 and 1.39 ± 0.34 h, respectively, in 4 dogs in which cervical vagus nerves were stimulated at 2 Hz successively during the experiments.

DISCUSSION

In the present study, we first demonstrated that botulinum neural toxin A inhibited a decrease in sinus rate in response to CV nerve stimulation in anesthetized dogs, when botulinum toxin had been injected into the SA fat pad selectively and suggest that the botulinum toxin can inhibit the parasympathetic ganglionic neurotransmission in the dog heart in situ as did it the neuromuscular junction in patients. Thus, we speculate that botulinum toxin will be developed for clinical use to treat the vaso-vagal syncope, parasympathetic related atrial fibrillation, and other diseases that are worsened by parasympathetic activation.

Botulinum toxins are zinc-binding metalloendopeptidases (1). Botulinum toxin A disrupts cholinergic neurotransmission by blocking the exocytotic release of acetylcholine stored in synaptic vesicles (2). Recently Kozaki et al. (14) suggested that ganglioside GT1b functions as a component of the receptor complex for the botulinum toxin A on synaptic transmission of rat synaptic cervical ganglion neurons. It is thought that on exocytosis of synaptic vesicles, botulinum toxin A binds the receptor sites and internalizes into the presynaptic cytosol by endocytosis. In the present study, botulinum toxin injected into the SA fat pad abolished the negative chronotropic responses to stimulation of the cervical vagus nerves or to stimulation of the

SA fat pad parasympathetic neural elements without affecting atrioventricular conduction time (Figs. 1 and 2). Parasympathetic nerve fibers selectively innervate either the sinoatrial or the atrioventricular nodal regions, and those nerve fibers pass through discrete epicardial fat pads in dog hearts (10, 11, 15, 16), and similarly in human hearts (9). In each fat pad, there are many parasympathetic ganglionic cells as well as parasympathetic nerve fibers (15 – 17). Therefore, we suggested that botulinum toxin inhibits the parasympathetic ganglionic neurotransmission in the dog heart. We also speculate that we can regulate bradycardia at the ganglionic neurotransmission in the heart. If the chance of exocytosis is more frequent, internalization of botulinum toxins would be accelerated. As shown in Fig. 2, successive conditioning cervical vagus stimulation at 2 Hz accelerated the inhibition by botulinum toxin of the bradycardia induced by vagus stimulation.

In the present study, we investigated a single dose of botulinum toxin because of the limitation of experimental animals, long time course of the drug effects, and the amount of botulinum toxin. A previous report demonstrated that the greater the concentration of the toxin, the shorter the time for inhibition (18). Botulinum toxin A blocks cholinergic transmission in a dose-dependent manner in mammalian hearts (19). Thus, we need further studies of the dose-dependent effects of the toxin to determine the useful doses for the long term or clinical usage. We have reported only short time (7 h) efficacy of botulinum toxin in the dog heart. Thus, we need to investigate the long term effect of the toxin on the cardiac function using the 24-h Holter electrocardiogram. We also should estimate the effects of the botulinum toxin on the organ or tissues neighboring the injection site, although the toxin did affect the heart rate without other cardiovascular responses determined in the present study.

As an experimental therapeutics, to decrease the basal sphincter of Oddi pressure, local injection of the botulinum toxin into the papilla of Vater with endoscopy was performed (20). Using thoracoscopy, if injection of botulinum toxin into the epicardial fat pad could be performed, vagally induced syncope may be prevented. Moreover, as botulinum toxin blocks ganglionic cholinergic neurotransmission, such local injection of botulinum toxin into stellate ganglia will cause preganglionic sympathetic nerve denervation. This may prevent the patients with certain ventricular arrhythmias from sudden death by reducing the sympathetic nerve activities to the heart.

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