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Presynaptic Effects of Botulinum Toxin Type A on the Neuronally Evoked Response of Albino and Pigmented Rabbit Iris Sphincter and Dilator Muscles

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Purpose: To investigate the effects of botulinum toxin type A (botulinum A toxin) on the autonomic and other nonadrenergic, noncholinergic nerve terminals.

Methods: The effects of botulinum A toxin on twitch contractions evoked by electrical field stimulation (EFS) were studied in isolated albino and pigmented rabbit iris sphincter and dilator muscles using the isometric tension recording method.

Results: Botulinum A toxin inhibited the fast cholinergic and slow substance P-ergic component of the contraction evoked by EFS in the rabbit iris sphincter muscle without affecting the response to carbachol and substance P. These inhibitory effects were more marked in the albino rabbit than in the pigmented rabbit. Botulinum A toxin (150 nmol/L) did not affect the twitch contraction evoked by EFS in the rabbit iris dilator muscle.

Conclusions: These data indicated that botulinum A toxin may inhibit not only the acetylcholine release in the cholinergic nerve terminals, but also substance P release from the trigeminal nerve terminals of the rabbit iris sphincter muscle. However, the neurotoxin has little effect on the adrenergic nerve terminals of the rabbit iris dilator muscle. Furthermore, the botulinum A toxin binding to the pigment melanin appears to influence the response quantitatively in the two types of irides. **Jpn J Ophthalmol 2000;44:106–109**

Key Words: Albino rabbit, botulinum toxin type A, electrical field stimulation, pigmented rabbit, substance P.

Introduction

Botulinum toxin type A (botulinum A toxin) is a derivative of one of eight exotoxins produced by *Clostridium botulinum.* Its crystalline form is a high-molecular-weight protein consisting of two subunits that dissociate in solution. In 1973, Scott et al. found that botulinum A toxin in low doses was very effective in weakening an overactive extraocular muscle without serious side effects.[1] After successful clinical trials, the use of purified botulinum A toxin for the treatment of blepharospasm was approved in Japan. Systemic side effects, in particular those affecting the autonomic nervous system, including the pupillary response, have recently been investigated.

When injected into muscle tissue, the neurotoxin acts at the level of the neuromuscular junction.[2,3] Botulinum A toxin binds rapidly and firmly to receptor sites on the motor nerve terminal and enters the intracellular compartment by a process occurring in the synaptic vesicles.[2] At this point, the neurotoxin prevents calcium ion transport and specifically blocks acetylcholine (Ach) release.[4] The mechanisms of action of botulinum A toxin in weakening the muscle contraction at the motor nerve endings are well-recognized, but little is known about the presynaptic effects of botulinum A toxin on autonomic and other nonadrenergic, noncholinergic nerve terminals. Therefore, the present study was initiated to

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investigate the effects on twitch contractions evoked by electrical field stimulation (EFS) of the albino and pigmented rabbit iris sphincter muscle in comparison with the effects of botulinum A toxin on the dilator muscle with the use of isometric tension recording methods.

Materials and Methods

General

All experiments were performed according to the Guide for Care and Use of Laboratory Animals (Department of Health, Education and Welfare publication, NIH 80-23).

Ten male albino and 5 pigmented rabbits, weighing 2–3 kg, were sacrificed with an overdose of intravenous pentobarbital sodium (60 mg/kg; Abbott, North Chicago, IL, USA). The eyes were immediately enucleated and placed in oxygenated Krebs solution of the following composition (mM): NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7. A ring-shaped iris sphincter specimen (2–3 mm) and a dilator muscle specimen (1–2 mm wide, 3–4 mm long) were prepared under a microscope.[5]

Electrical Field Stimulation Experiment

The specimen was mounted vertically in a 1.5 mL organ bath and connected to an isometric tension transducer (EF-601G; Nihon Koden, Tokyo) with initial loads of 100 mg for the iris sphincter and 50 mg for the iris dilator muscle. The organ bath was perfused continuously (0.3 mL/s) with oxygenated Krebs solution that was warmed to 37°C. The test drugs (see chemicals below) were dissolved in the Krebs solution in a reservoir. Transmural EFS was applied through a pair of silver-plated electrodes placed in the organ bath. These electrodes were separated by 5 mm and were placed so that the current pulses would pass transversely across each tissue. Stimulation with 70 pulses of 1.0 milliseconds in duration and 100 V in strength at 20 Hz was applied to the tissues. Electrical field stimulation was usually applied at intervals of 5 minutes for the iris sphincter and 3 minutes for the iris dilator. To obtain constant twitch contractions, the experiment was started after the muscle tone had reached a steady level.

Statistical Analysis

Data are expressed as percentages of the control contraction that took place without treatment and are presented as mean ± standard error of the means (SEM). The Student *t*-test was used for statistical evaluation of the difference of means and $P < .05$ was considered to be statistically significant. Statistical analyses were performed using StatView 4.0 (Abacus Concepts, Berkeley, CA, USA) for a Macintosh personal computer.

Chemicals

The following drugs were used: botulinum A toxin, (Wako Pure Chemical Industries, Osaka), substance P (Peptide Institute, Osaka), carbachol and tetrodotoxin (Sigma Chemical, St. Louis, MO, USA). All other compounds used were of reagent grade.

Results

The specimens of the rabbit iris sphincter and dilator muscles, mounted in an organ bath, gradually relaxed to a steady tone during a 60-minute equilibration period. The muscle tone then remained constant for several hours. Spontaneous mechanical responses did not occur at any time during the procedures. Electrical field stimulation caused twitch contractions of the iris sphincter and dilator muscle preparations.

The twitch contraction was blocked by pretreatment with tetrodotoxin (0.1 μmol/L), indicating that the mechanical response was neurogenic in origin (data not shown).

In the iris sphincter muscle, EFS produced contractions with fast and slow components (Figure 1A). The fast component was inhibited by atropine

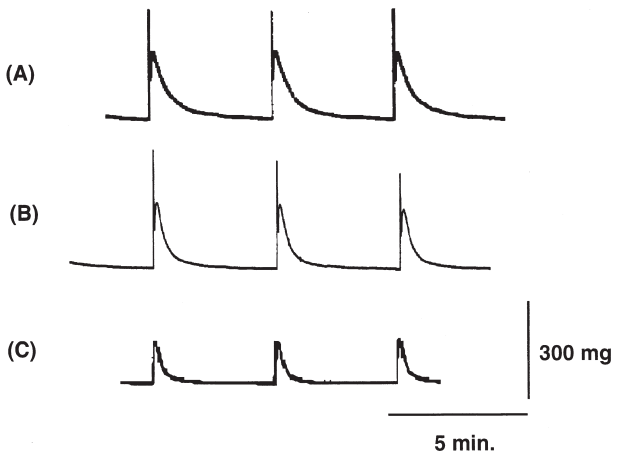

Figure 1. Typical tracings showing electrical field stimulation-induced twitch contractions in albino rabbit iris sphincter muscle. After control tracing (**A**), preparation was equilibrated with botulinum A toxin (150 nmol/L) for 100 minutes (**B**) and 200 minutes (**C**).

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Table 1. Comparison of Inhibitory Effects of Botulinum A Toxin (15 and 150 nmol/L) in Albino and Pigmented Rabbit Iris Sphincter and Dilator Muscles*

Concentration of Botulinum A Toxin	Type of Rabbit	Iris Sphincter		Iris Dilator
		Fast Component	Slow Component	
15 nmol/L	Albino	62.0 ± 2.1% (n = 4/3)	44.2 ± 4.0% (n = 4/3)	
150 nmol/L	Albino	0%† (n = 12/7)	62.1 ± 7.2% (n = 14/7)	104.2 ± 2.7% (n = 6/5)
150 nmol/L	Pigmented	36.0 ± 22% (n = 4/3)	75.0 ± 25% (n = 4/3)	

*Results are expressed as percentages of control contraction that took place without treatment. Preparation was equilibrated with botulinum A toxin for 300 minutes. n = number of observations/number of animals.

†Statistically significant difference from value of fast component in pigmented rabbit iris sphincter.

(1 μmol/L), while the slow component was little affected (data not shown). Figures 1B and 1C provide typical records of the inhibitory patterns of the iris sphincter after the addition of botulinum A toxin (150 nmol/L). The fast component of contraction was completely abolished by the botulinum A toxin (150 nmol/L) within 3 hours of the start of incubation (Figure 1C). By that time, the amplitude of the slow component was also significantly decreased.

Botulinum A toxin, as shown in Table 1 in detail, inhibited the fast component of the twitch contractions in the iris sphincter muscle dose-dependently.

The inhibitory effect of botulinum A toxin on the fast component in the albino iris sphincter was significantly greater than that of pigmented rabbit (Table 1). Furthermore, pretreatment with botulinum A toxin (150 nmol/L) did not affect the contractions induced by carbachol or substance-P in the albino rabbit iris sphincter (Figure 2).

Botulinum A toxin (150 nmol/L) did not affect the EFS-induced contraction in the albino rabbit iris dilator muscle (Table 1, Figure 3).

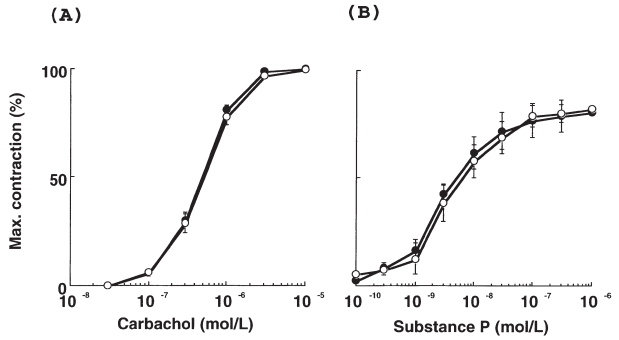

Figure 2. Dose-response curves for (**A**) carbachol and (**B**) substance P for albino rabbit iris sphincter, in the presence (open circles) and absence (closed circles) of botulinum A toxin (150 nmol/L). Each point represents the mean of 3 to 7 experiments with bar indicating SEM.

Discussion

Botulinum A toxin is an effective therapy for a wide variety of muscle hyperactive states and focal dystonia. Systemic side effects, especially those related to the autonomic nervous system, have recently been investigated. It was reported that retrobulbar injection of botulinum A toxin causes mydriasis in rabbits[6] and rats.[7] A rise in intraocular pressure resulting from mydriasis after botulinum A toxin injection was also reported.[8] These effects may be explained by local diffusion of botulinum A toxin to the ciliary ganglion, because the toxin molecule appears to be too large to penetrate the cornea and sclera.[9]

In the rabbit iris sphincter muscle, EFS produced biphasic contractions with fast and slow components. The fast component was thought to be cholinergic, while the slow one was considered to be mediated by substance P or a related peptide.[10,11] In the present study, botulinum A toxin inhibited the fast cholin-

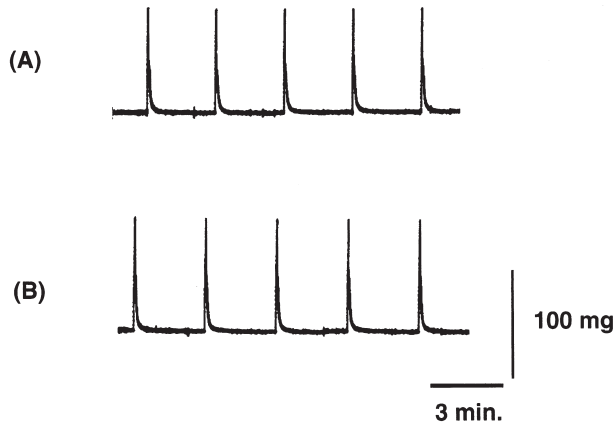

Figure 3. Tracings showing EFS-induced twitch contractions in albino rabbit iris dilator muscle (**A**). Preparation was equilibrated with botulinum A toxin (150 nmol/L) for 200 minutes (**B**).

ergic component of the twitch contraction in a dose-dependent manner. Furthermore, pretreatment with botulinum A toxin (150 nmol/L) did not affect the contractile activity of exogenously applied carbachol. These results suggest that botulinum A toxin inhibits Ach release from the parasympathetic nerve terminals of the rabbit iris sphincter.

The mechanisms of action of botulinum A toxin in weakening the muscle contraction at the motor nerve endings are well recognized,[2–4] but little is known about the other nonadrenergic, noncholinergic nerve terminals. Montecucco et al[12] demonstrated that botulinum toxins bind to an as yet unidentified presynaptic component of the neuromuscular junction. Botulinum A toxin also significantly inhibited the slow substance P-ergic response to exogenously applied substance P without affecting contractile activity (Figures 1 and 2, and Table 1). Therefore, our present results appear to indicate that botulinum A toxin also binds to the trigeminal nerve endings in the rabbit iris sphincter[13] and inhibits substance P release. In general, sensory nerves have been thought to have a bipolar structure and stimulation of a nerve is said to cause the release of substance P from both central and peripheral terminals.[13] Prostaglandins are known to enhance the substance P-ergic response induced by EFS in the rabbit iris sphincter;[14] on the other hand, endothelins inhibit this response.[15]

It is well-known that some drugs, such as atropine[16] and prazosin,[17] bind to melanin pigment in the iris smooth muscle. In the present study, the inhibitory effects of botulinum A toxin on cholinergic and substance P-ergic neurotransmission are more marked in the albino rabbit than in the pigmented rabbit, indicating that the botulinum A toxin binding to the melanin pigment may influence the quantitative aspects of the response in the two types of iris.

People who are systemically poisoned with botulinum toxin characteristically have fixed, moderately dilated pupils resulting from paralysis of the sympathetic and parasympathetic nervous system.[18] However, in the present study, the toxin appeared not to affect the contraction induced by EFS in the albino rabbit iris dilator. This suggests that botulinum A toxin has little effect on adrenergic neurotransmission in this tissue.

Conclusions

A pharmacological analysis of the sphincter and dilator contraction indicated that botulinum A toxin may inhibit not only Ach release from the cholinergic nerve terminals, but also substance P release from the trigeminal nerve terminals of the rabbit iris sphincter muscle.

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