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Establishment of alternative potency test for botulinum toxin type A using compound muscle action potential (CMAP) in rats

Yasushi Torii [a, b, *], Yoshitaka Goto [a], Shinji Nakahira [a], Akihiro Ginnaga [a]

[a] The Chemo-Sero- Therapeutic Research Institute (KAKETSUKEN), 1-6-1 Okubo, Kita-ku, Kumamoto-shi, Kumamoto 860-8568, Japan
[b] Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

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ABSTRACT

The biological activity of botulinum toxin type A has been evaluated using the mouse intraperitoneal (ip) LD_{50} test. This method requires a large number of mice to precisely determine toxin activity, and, as such, poses problems with regard to animal welfare. We previously developed a compound muscle action potential (CMAP) assay using rats as an alternative method to the mouse ip LD_{50} test. In this study, to evaluate this quantitative method of measuring toxin activity using CMAP, we assessed the parameters necessary for quantitative tests according to ICH Q2 (R1).

This assay could be used to evaluate the activity of the toxin, even when inactive toxin was mixed with the sample. To reduce the number of animals needed, this assay was set to measure two samples per animal. Linearity was detected over a range of 0.1–12.8 U/mL, and the measurement range was set at 0.4–6.4 U/mL. The results for accuracy and precision showed low variability. The body weight was selected as a variable factor, but it showed no effect on the CMAP amplitude.

In this study, potency tests using the rat CMAP assay of botulinum toxin type A demonstrated that it met the criteria for a quantitative analysis method.

1. Introduction

Clostridium botulinum is classified into 8 types, A–H, based on the immunological characteristics of the botulinum toxin that they produce (Schiavo et al., 2000; Barash and Arnon, 2014). All types of toxin act on the neuromuscular junction and inhibit the release of acetylcholine from the presynaptic membrane, inducing muscle relaxation, and serious cases result in death from dyspnea (Sakagichi, 1983; Jahn and Niemann, 1994). Type A toxins are protein complexes, called progenitor toxins, containing a 150-kDa neurotoxin (NTX) and nontoxic components. Type A progenitor toxins are classified by their molecular weight into three forms: LL toxin, 900 kDa; L toxin, 500 kDa; and M toxin, 300 kDa (Sugii and Sakaguchi, 1975). The L and LL toxins of the nontoxic component have hemagglutinin (HA), which shows active hemagglutination, and the M toxin has no HA (Montecucco et al., 1996). NTX consists of an approximately 50-kDa light chain and 100-kDa heavy chain connected by a disulfide bond. The C-terminal of the heavy chain binds to synaptic vesicle protein 2 and gangliosides, which are the receptors, the N-terminal of the heavy chain can translocate light chains from the inside of the synaptic vesicle to the cytosol of the nerve terminal, and the light chain is known to cleave SNAP-25 (Arnon et al., 2001).

* Corresponding author. Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan. Tel.: +81 6 6879 3301; fax: +81 6 6879 3309.
E-mail addresses: torii@bact.med.osaka-u.ac.jp, chabo@pop17.odn.ne.jp (Y. Torii).

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The standard method to determine the biological activities of botulinum toxin products has been the lethal dose 50% (LD_{50}) test, involving intraperitoneal (ip) injections of mice, defining one mouse ip LD_{50} = 1 unit (U) (Pearce et al., 1994). This method is applicable for up to 2 U/mL of the toxin. It is an assessment of lethal activity, and the results vary widely due to several factors, such as differences in individual and strain-specific sensitivity to toxins, measurers' techniques, and between institutes, etc. To establish an international standard for botulinum toxin, the mouse ip LD_{50} assay was investigated in several laboratories. However, large variations between institutes were reported (Sesardic et al., 2003). Due to these differences, many mice are required to ensure sufficient accuracy levels for the quality control of preparations. Additionally, this method, which is lethal for mice, poses a problem with regard to animal welfare. Many alternative methods have been reported (Straughan, 2006; Hallis et al., 1996; Wictome et al., 1999; Yoneda et al., 2005; Bigalke et al., 2001; Rasetti-Escargueil et al., 2011; Aoki, 2001; Takahashi et al., 1990; Sesardic et al., 1996).

In a previous report, we proposed the compound muscle action potential (CMAP) assay using rats as an alternative method to the mouse ip LD_{50} assay (Sakamoto et al., 2009). CMAP is the microcurrent generated by the contraction of muscle fibers. The microcurrent is amplified and recorded. Botulinum toxin suppresses neurotransmission by affecting nerve endings. Stimuli from neurons are not transmitted to the muscles due to the botulinum toxin, and the CMAP amplitude decreases depending on the toxin dosage. Using this rat CMAP assay, a linear relation was observed at 0.01–30 U/head (0.1–300 U/mL) of type A toxin one day after toxin application, and the time course of the pharmacological effects of the toxins could be measured.

In this study, we investigated an optimal method for determining the biological activity of botulinum toxin using the rat CMAP assay. In our previous report, we administered one sample per rat at 8 weeks of age. To reduce the number of animals and respond to concerns over animal welfare, we investigated altering the test method involving the administration of two samples per animal into the left and right gastrocnemius muscles using rats at 5 weeks of age. We have been developing botulinum toxin products. The rat CMAP assay will be used as a quantitative method to measure the biological activities of botulinum toxin products in place of the mouse ip LD_{50} method. We evaluated the parameters necessary for quantitative tests (specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, and robustness) as outlined by the International Conference on Harmonization (ICH) Q2 (R1) (ICH Harmonised Tripartite Guideline, 2005).

2. Materials and methods

2.1. Purification of toxin

Botulinum type A neurotoxins (150 kDa, NTX) were prepared using a modified version of a previously reported method (Sakaguchi et al., 1981). *C. botulinum* type A strain Chiba-H, belonging to subtype A2, was cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate in a fermenter. The organisms were removed from culture fluid by filtration. M toxin was purified from the culture fluid by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration. M toxin was adsorbed onto a DEAE Sepharose column equilibrated with 10 mM phosphate buffer, and eluted with a 0–0.3 M NaCl gradient buffer to separate NTX and non-toxic components. The NTX was stored at −70 °C until use.

2.2. Experimental animals

ICR/CD-1 mice (4 weeks of age, female, about 20 g, Charles River Laboratories Japan, Yokohama, Japan) were used for the assay of toxic activity, and S/D rats (5 weeks of age, female, 110–140 g, Charles River Laboratories Japan, Yokohama, Japan) were used for the rat CMAP test. Animals were maintained under controlled light/dark conditions and had free access to food and water. This study was performed in accordance with the guidelines concerning experimental animals established by the Japanese Pharmacological Society, and was approved by the Animal Ethics Committee of our institute.

2.3. Toxic activity assay (mouse ip LD_{50} test)

The toxic activities of NTX were determined employing the mouse ip LD_{50} test (Pearce et al., 1994). The mouse ip LD_{50} was determined using a series of 7 doses with a dilution interval of 1.25, and 20 mice per dose. The observation period was the first 96 h after administration, and the LD_{50} was calculated using the probit method. One mouse ip LD_{50} was defined as 1 unit (U).

2.4. Rat CMAP assay

2.4.1. Examination of conditions for CMAP measurement

Rats were anesthetized by the intraperitoneal administration of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan), and the left and right hind legs were shaved. CMAP was measured using a modified version of a previously reported method (Sakamoto et al., 2009). CMAP was measured using Nicolet Viking Quest (Viasys Healthcare, Tokyo, Japan). The electrode employed was an alligator clip lead wire (Viasys Healthcare, Tokyo, Japan). Rats were anesthetized and placed in a prone position. Ten animals were used for each examination.

The optimal position for the recording electrode was investigated in the following manner. The stimulating electrode was placed on the root of the spinal cord, the anode recording electrode was positioned on the hind gastrocnemius tendon, and the earth electrode on the tail root. The belly of the hind gastrocnemius muscle was set as the starting point, and measurement was performed with the cathode recording electrode positioned at 4 mm proximal, 4 mm distal, and 8 mm distal. Electric stimulation was loaded at 25 mA for 0.2 msec.

Examinations of the stimulating electrode position and stimulation intensity were performed as follows. The cathode recording electrode was positioned on the belly of