Zinc antagonizes the effect of botulinum type A toxin at the mouse neuromuscular junction

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Summary. Zn^{2+} (10–100 μM) elevated the frequency of miniature end-plate potentials (MEPPs) in the mouse diaphragm. The effect did not depend on external Ca^{2+}. Botulinum type A toxin (BTX_A, 50 ng/ml) abolished MEPPs almost completely within 30 min. Zn^{2+} (100 μM) restored MEPPs and increased their frequency after they had been abolished by BTX_A in Ca^{2+}-free solutions. The antagonistic effect of Zn^{2+} in the Ca^{2+}-free solution was reduced by exposing the diaphragm to the toxin in the Ca^{2+}-free solutions containing high K^+. Thus, the action of BTX_A is probably enhanced by depolarization of the motor nerve terminals.

Key words. Botulinum type A toxin; neuromuscular junction; zinc antagonism; depolarization dependency; mouse.

Botulinum neurotoxin (BTX), produced by *Clostridium botulinum*, blocks transmitter release from motor nerve terminals [1]. Several models have been proposed for studying the mechanism of action of BTX, but the precise mechanism of action is not yet known. Proposed models include alteration of the vesicular membrane to impede refilling with transmitter [2] and blockade of depolarization-induced influx of Ca^{2+} into the nerve terminals [3–5]. It has also been suggested that BTX interferes with the release of transmitter after entry of Ca^{2+} into the nerve terminals [6–8]. If this suggestion is true, BTX should reduce the effects of agents or procedures enhancing transmitter release independently from Ca^{2+}. There are some substances that increase transmitter release from motor nerve terminals poisoned with BTX. Such substances include guanidine [9], Ca^{2+}-ionophore [6], tetraethylammonium [10, 11], 4-aminopyridine [9, 12] and 3,4-diaminopyridine [12]. Their effects are dependent on external Ca^{2+} and they enhance transmembranous movement of this ion. The effect of BTX is antagonized also by beta-bungarotoxin in the presence of external Ca^{2+} [13]. We do not know, however, whether the effect of BTX can be antagonized by any agent or procedure whose effects are independent of external Ca^{2+} except black widow spider venom [7]. In a search for such agents or procedures, we have found that hypoxia markedly facilitates transmitter release from nerve terminals in a Ca^{2+}-free solution [14] and also antagonizes the effect of BTX in a Ca^{2+}-free solution [15]. Divalent and trivalent cations such as Zn^{2+}, Hg^{2+}, Pb^{2+}, La^{3+}, Cd^{2+}, Co^{2+}, and Mn^{2+} are effective ions in promoting increases in asynchronous release of transmitter [10]. Recently we found that Zn^{2+} can restore transmitter release that has been blocked by type A BTX (BTX_A) in Ca^{2+}-free solution. Since the effect of Zn^{2+} was independent of external Ca^{2+}, this observation made it possible to investigate the blocking effect of BTX_A on an exocytotic process of the transmitter release. We examined in detail the effects of Zn^{2+} and its efficacy in antagonizing the effect of BTX_A under different conditions.

Methods. Hemidiaphragms were isolated from male mice of the *ddy* strain (12–14 weeks old) and soaked in a solution of the following composition (mM): NaCl, 136; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 15; glucose, 11. Ca^{2+}-free solutions containing KCl at different concentrations (10 and 15 mM) were prepared isosmotically by replacing NaCl with KCl. The solution was bubbled with 95% O_2 and 5% CO_2. Transmembrane potentials of the end-plate regions were recorded by conventional intracellular techniques. MEPPs of 0.1 mV or larger amplitude were counted with a computer (Nihon Kohden, DAB-1100) for successive 1-min periods after exposure of the preparations to a given solution; the mean frequency of the MEPPs (s^{-1}) was calculated from these data. The plots of the frequency of MEPPs in the figures 1 and 2 are the values obtained with the same end-plate throughout the observation period. BTX_A was added to the solution (20 ml) in the bath to give a final concentration of 50 ng/ml (specific activity, 63.3 mouse LD_{50}/ng).

Results. Figure 1 shows the blocking effect of BTX_A on the frequency of MEPPs in Ca^{2+}-free depolarizing solution. MEPPs were measured in a Ca^{2+}-free, 15 mM KCl bathing solution and a mean value of the frequency of MEPPs was obtained ($1.88 \pm 0.23\ s^{-1}$, mean ± SEM of N = 38 end-plates of 10 preparations). Addition of Ca^{2+} (2 mM) to the solution markedly elevated the frequency of MEPPs in the absence of the toxin ($77.8 \pm 8.2\ s^{-1}$, N = 14 end-plates of 5 muscles, left panel). BTX_A (50 ng/ml) lowered the frequency of MEPPs in the Ca^{2+}-free, 15 mM KCl bathing solution almost to zero within about 30 min (right panel). The 2 mM Ca^{2+} added to 15 mM KCl bathing solution 35 min after the addition of BTX_A caused only a small, transient increase in the frequency of MEPPs. A peak value of the frequency of MEPPs after the addition of the 2 mM Ca^{2+} was $0.22 \pm 0.09\ s^{-1}$ (N = 5 muscles). Thus, the blocking effect of BTX_A is complete within 35 min of exposure.

Zn^{2+} transiently elevated the frequency of MEPPs in a standard bathing solution (fig. 2A, left). Such a stimulatory effect of Zn^{2+} could be detected in Ca^{2+}-free, depolarizing solutions (fig. 2), hence it must be independent of external Ca^{2+}. The effect of 100 μM Zn^{2+} was high; the maximum value of the frequency of MEPPs varied from 80 to 120 s^{-1} independently of external Ca^{2+}. A latent period was required for manifestation of the effect.

Zn^{2+} was examined for its effect on the frequency of MEPPs in preparations poisoned with BTX_A in bathing solutions

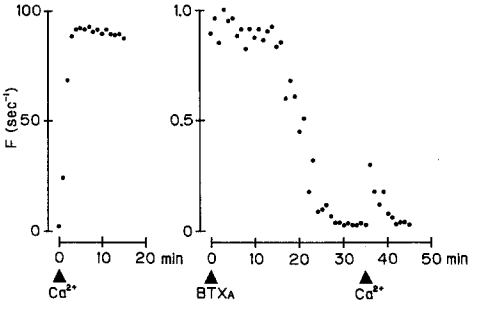

Figure 1. Blockade by BTX_A of MEPPs in a Ca^{2+}-free, 15 mM KCl solution, and of a stimulatory effect of Ca^{2+} on the frequency of MEPPs. In the left panel, a preparation was incubated in Ca^{2+}-free, 15 mM KCl solution for about 60 min and calcium chloride (2 mM) was added to the solution at the time indicated by the triangle marked Ca^{2+}. In the right panel, a preparation was incubated in Ca^{2+}-free, 15 mM KCl solution for 30 min and BTX_A (50 ng/ml) was added to the solution at the time shown by the triangle marked BTX_A. Calcium chloride (2 mM) was added at the time shown by the triangle marked Ca^{2+} 35 min after the addition of BTX_A. Typical results from 5 sets of similar observations are presented in both panels.

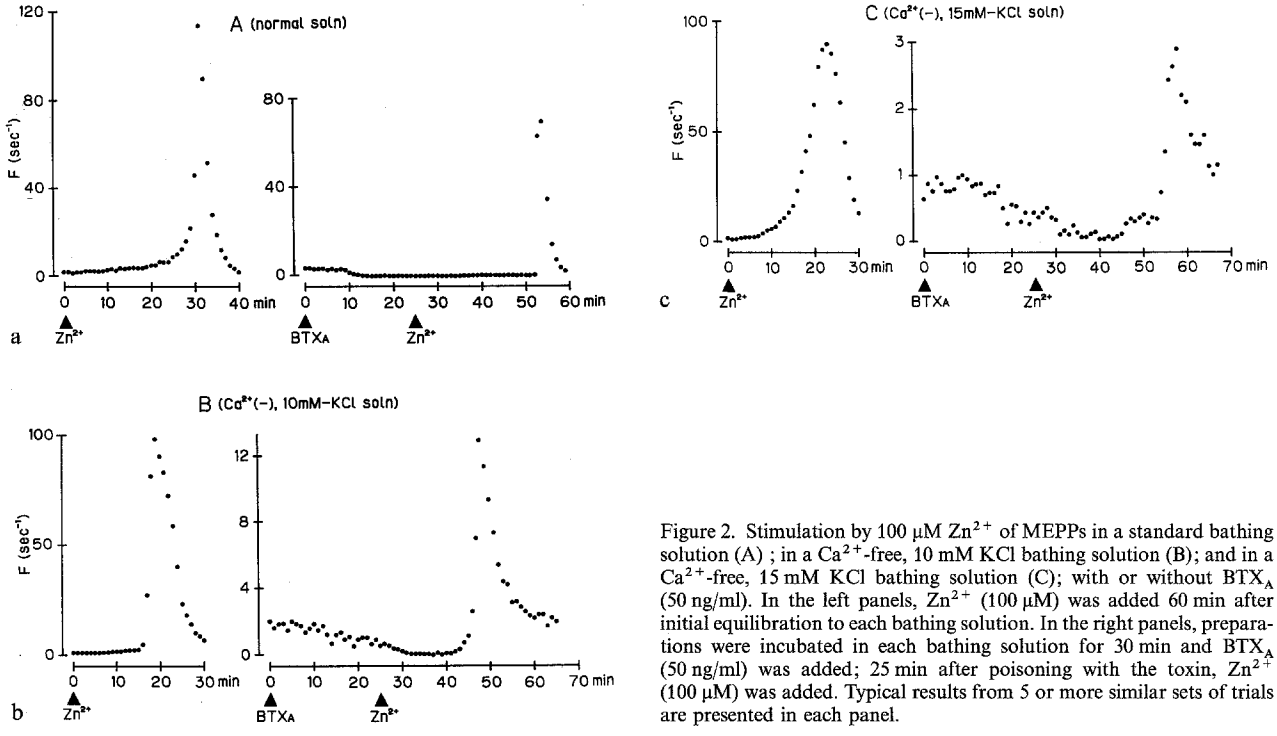

Figure 2. Stimulation by 100 μM Zn^{2+} of MEPPs in a standard bathing solution (A); in a Ca^{2+}-free, 10 mM KCl bathing solution (B); and in a Ca^{2+}-free, 15 mM KCl bathing solution (C); with or without BTX_A (50 ng/ml). In the left panels, Zn^{2+} (100 μM) was added 60 min after initial equilibration to each bathing solution. In the right panels, preparations were incubated in each bathing solution for 30 min and BTX_A (50 ng/ml) was added; 25 min after poisoning with the toxin, Zn^{2+} (100 μM) was added. Typical results from 5 or more similar sets of trials are presented in each panel.

with standard compositions, Ca^{2+}-free, 10 mM KCl, or Ca^{2+}-free, 15 mM KCl (figs 2 and 3). The MEPPs which were abolished completely by the action of 50 ng/ml BTX_A were restored, and their frequency was transiently elevated by adding Zn^{2+} (100 μM) in the presence and absence of external Ca^{2+}. The maximum effect of Zn^{2+} in those solutions was significantly reduced in preparations poisoned with BTX_A (fig. 3). The antagonistic effect declined with increase in the concentration of KCl in the medium.

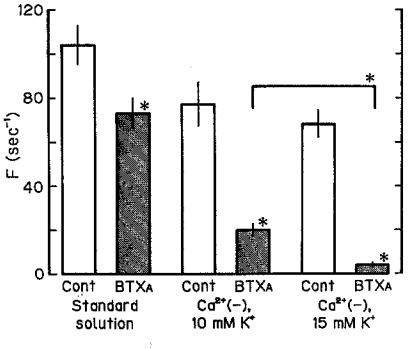

Figure 3. Stimulatory effect of Zn^{2+} on MEPP frequency in preparations poisoned with BTX_A in bathing solutions with different compositions as standard solution. Ca^{2+}-free, 10 mM KCl solution ($Ca^{2+}(-)$, 10 mM K^+), and Ca^{2+}-free, 15 mM KCl solution ($Ca^{2+}(-)$, 15 mM K^+). In control experiments (Cont), Zn^{2+} (100 μM) was added to each solution 60 min after initial equilibration of preparations. BTX_A (50 ng/ml) was added 30 min after *starting the equilibrium* in those bathing solutions (BTX_A). Zn^{2+} (100 μM) was added 25 min after adding BTX_A. Presented are MEPP frequency at peak effect produced by Zn^{2+}. F (s^{-1}); frequency of MEPPs. Vertical bars show SEM of 5 or 6 experiments. *$p < 0.05$ by Student's t-tests.

Discussion. Zn^{2+} augmented transmitter release independently of external Ca^{2+}. Theoretically, inside the nerve terminal, the effect of Zn^{2+} might be brought about: a) by directly activating exocytosis, as Ca^{2+} does, b) by screening internal negative fixed charges, and c) by interfering with internal Ca-buffering mechanisms or energy metabolism to increase intraterminal Ca^{2+} [10]. The effect of Zn^{2+} was similar to those of Cd^{2+} and 2,3-bisphosphoglycerate (Nishimura, unpublished results). These agents have been reported to inhibit inositol 1,4,5-triphosphate 5-phosphatase, thereby increasing intracellular Ca^{2+} by releasing Ca^{2+} from a non-mitochondrial storage site [16]. Thus, it is suggested that Zn^{2+} may augment the transmitter release by a release of Ca^{2+} stored intracellularly.

Present experiments indicate that BTX_A blocks spontaneous transmitter release at the motor nerve terminals in Ca^{2+}-free solution. This result supports the hypothesis that the toxin affects a step in the process of transmitter release occurring after entry of Ca^{2+} [6, 7, 16]. Zn^{2+} restored, but less potently, the spontaneous transmitter release after the blockade by BTX_A in the presence and absence of external Ca^{2+}. Thus, these results imply that BTX_A may act on the release of Ca^{2+} ions stored intracellularly, perhaps by altering their availability, and/or on a exocytotic process of transmitter release. The antagonistic effect of Zn^{2+} declined in high K^+ solutions containing no Ca^{2+}. The higher the level of external KCl produced the higher the blocking effect of BTX_A. The stimulatory effect of black widow spider venom has been shown to be independent of prior treatment with BTX_A in Trowel's T-8 medium [7]. It is not clear from the report, however, whether the experiment was performed in a depolarizing solution. Elevation of the concentration of KCl depolarizes excitable membranes. Depolarization of the nerve terminal membrane opens Ca^{2+} channels [17]. Depolarization would also be an exocytotic event independent of external Ca^{2+} in such a sequence leading to transmitter release in the nerve terminal membrane. The event must be essential for the action of BTX_A and may involve a certain alteration of

the conformation of membrane constituents to an extent that is directly related to transmitter release.

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Light and temperature affect retinyl ester hydrolase activity and visual pigment composition

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Summary. Dim light, in combination with high water temperature, resulted in a significant increase in the retinyl ester hydrolase activity in the goldfish retina. This rise in enzyme activity may relate to a selective increase in the availability of retinal chromophores thereby favoring the formation of rhodopsin under these light and temperature conditions.
Key words. Goldfish; rhodopsin and porphyropsin; retinyl ester hydrolase; light and temperature treatments.

Visual pigments are light sensitive molecules located in the photoreceptor cells (rods and cones) of the eye, serving the function of photo-transduction[1]. These photopigments are derived from the conjugation of opsin (a protein) to either retinal or 3,4-didehydroretinal[2]. Because retinal and 3,4-didehydroretinal based pigments exhibit different absorption properties[3], an alteration of visual pigment composition (i.e. from retinal based pigments to 3,4-didehydroretinal based pigments or vice versa) will result in a change in the visual sensitivity, thereby improving the animal's adaptation to its (new) environment[4]. This change in the visual pigment composition occurs seasonally in certain species and in others, during spawning migrations[2, 5–7]. Although laboratory light and temperature treatments have been successful in the (artificial) induction of visual pigment changes in some animals[8–10], the mechanism underlying this visual pigment change is not understood. In the present communication, experimental evidence is presented to show that light and temperature exert a profound influence on the ocular retinyl ester hydrolase activity (which governs the release of retinol from retinyl esters in the eye) as well as on visual pigment composition. Assuming that the activity of retinyl ester hydrolase is specific for retinyl palmitate, an increase in this enzyme activity should selectively increase the amount of retinal (in comparison to 3,4-didehydroretinal) available for rhodopsin (over porphyropsin) formation. These results, therefore suggest that the hydrolase enzyme may hold a control mechanism through which light and temperature exert their influence on the visual pigment composition.

Goldfish were used in these experiments because they possess both rhodopsin and porphyropsin in their retinas[13]. The composition of these visual pigments is known to change in response to an artificial alteration of their light and temperature environments[9, 15]. Visual pigment analyses were performed by standard procedures[9, 10]. Retinyl ester hydrolase (REH) activity was assayed by a method using HPLC (high performance liquid chromatography) and described in detail previously[11, 12]. Retinyl palmitate was used as the substrate of the REH. The enzyme activity was linear to 3 mg protein. The distribution of REH activity in goldfish is shown in table 1. The highest REH activity was found in retinal homogenates, followed by homogenates prepared from the retinal pigment epithelium (RPE/choroid). These values are similar to those from the bovine retina (72.5 pmol retinol/mg/h, n = 19)[11] and RPE/choroid (100.8 pmol retinol/mg/h, n = 10, unpublished observation). Both brain and liver tissues had significantly lower REH activity than those in ocular tissues (table 1). The unusually low REH activity found in the liver (i.e. compared to 130 pmol retinol/mg/h in the bovine liver[11], and 571 pmol retinol/mg/h in the rat[12]) may be attributable to the low level of vitamin A stored in the goldfish hepatic tissue[15].

The effect of light and temperature on the REH activity in ocular tissues is shown in table 2. The REH activities in the retina and RPE/choroid of fish in group 1 (held under room

Table 1. Distribution of REH activity in the goldfish

Tissue type	Specific enzyme activity (pmol retinol/mg/h)
Retina	114.3 ± 18.9 (n = 12)
RPE/choroid	105.1 ± 10.4 (n = 8)
Brain	41.7 ± 5.2 (n = 4)
Liver	13.4 ± 3.3 (n = 4)

Fish were dark adapted 2–5 h before decapitation and eye removal. Two retinas of a fish were dissected from their underlying retinal pigment epithelium (RPE) and homogenized in 0.5 ml Tris-maleate buffer (0.05 M, pH = 8). The RPE/choroid were also peeled from the eye cup and homogenized in 0.5 ml of Tris-maleate. The liver and the brain were dissected from the fish and homogenized in 1 ml each of Tris-maleate. The specific activities of REH (mean ± SD, n = number of samples analyzed) are given. The animals possessed in excess of 90% porphyropsin in their retinas. Boiling (by adding the tissue homogenate in test tube placed in 100 °C for 30 min) completely abolished the REH activity in the retina and the RPE/choroid.