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EFFECT OF *Gymnodinium breve* TOXIN IN THE RAT PHRENIC NERVE DIAPHRAGM PREPARATION

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1 The effects of a crude fraction of *Gymnodinium breve* toxin (GBTX) were studied on the rat phrenic nerve diaphragm preparation.

2 Indirectly stimulated muscle contractions were more sensitive to blockade by increasing concentrations of GBTX (0.25 μg/ml to 10 μg/ml) than direct muscle contractions.

3 GBTX increased miniature endplate potential (m.e.p.p.) frequency and depolarized the resting membrane potential of the muscle fibres at the endplate and in non-synaptic regions of muscle fibre.

4 A concentration-dependent biphasic effect on m.e.p.p. amplitude was evident. At lower concentrations m.e.p.p. amplitudes were depressed whereas at high concentrations they were increased.

5 GBTX blocked endplate potentials (e.p.ps) in concentrations (0.5 μg/ml to 2 μg/ml) that did not block m.e.p.ps.

6 Muscle fibre action potentials recorded from cells held at control membrane potential by hyperpolarizing current were not altered by toxin treatment. No repetitive e.p.ps, or muscle action potentials were observed.

7 These data suggest that GBTX may block indirectly stimulated muscle contractions and e.p.ps by depolarizing the nerve terminal in a manner similar to the observed depolarization of the muscle fibre. The depolarization of the nerve terminal may be sufficient to inhibit transmitter release.

Introduction

Red tides caused by the dinoflagellate, *Gymnodinium breve*, have been reported to produce massive numbers of fish deaths and shellfish toxicity in humans (Eldred, Steininger & Williams, 1964; McFarren, Tanabe, Sliva, Campbell & Lewis, 1965; Cummins, Jones & Stevens, 1971). Persons on beaches contaminated by *G. breve* experience severe eye irritation, respiratory problems and tingling sensations around the mouth, face and extremeties (Music, Howell & Brumback, 1973). These reports suggested that there was a potential for human toxicity from a *G. breve* red tide. Various methods have been used to purify the toxin synthesized by *G. breve*. In nerve and muscle preparations, experiments with *G. breve* toxin (GBTX) purified by different techniques yielded conflicting results (Sasner, Ikawa, Thurberg & Alam, 1972; Abbott, Siger & Spiegelstein, 1975; Westerfield, Moore, Kim & Padilla, 1977). In the only study with crude toxin, Sasner (1965) reported that GBTX depolarized both nerve and muscle membranes. There is no criterion to detect which toxin factors have been altered by purification techniques since the effect of crude GBTX has not been studied in detail. In addition, GBTX effects should be studied on mammalian neuromuscular preparations because of the potential sensitivity of humans to *G. breve* red tides and because at the neuromuscular junction toxin effects on nerve, muscle and synapse can be assessed.

This study was designed to define in detail the effects of crude *Gymnodinium breve* at a mammalian neuromuscular junction. It was hoped that the results of this investigation with crude toxin would serve as a basis of comparison for further studies with purified toxin fractions. A preliminary account of this study has been published (Shinnick-Gallagher & Gallagher, 1977).

Methods

Preparation

We used a total of 62 preparations. Experiments were carried out *in vitro* with isolated right and left hemidiaphragms from male Sprague-Dawley rats (200 to 300 g). The muscles were removed under pentobarbi-

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tone (60 mg/kg) anaesthesia and pinned in a muscle bath. The diaphragm muscle was superfused with Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2. The solution consisted of (mM): NaCl 115, KCl 4.6, KH_2PO_4 1.15, $NaHCO_3$ 24.1, $CaCl_2$ 2.46, $MgSO_4$ 1.15 and glucose 8.85. The phrenic nerve was placed over platinum electrodes in a mineral pool and when necessary was stimulated at a frequency of 0.1 Hz. Muscle contraction was prevented by superfusion with (+)-tubocurarine (1.45×10^{-6} M) or treatment with ethylene glycol (Sevik & Narahashi, 1972). The preparations were maintained at 31 to 33°C.

In muscle twitch experiments a wedge of hemidiaphragm was suspended vertically in a 100 ml organ bath. Initially, the preparation was allowed to stabilize in the bath for 30 min. For directly stimulated muscle contractions, supramaximal square wave pulses of 1 ms duration were applied through one pair of electrodes placed directly on the muscle. The muscle was stimulated indirectly by alternate pulses of supramaximal intensity and 50 μs duration which were applied to the nerve with a second pair of electrodes. The frequency for alternating direct and indirect stimuli was 0.1 pulse/s. Muscle contractions were measured with a Grass force transducer and continuously recorded on a Grass polygraph.

Intracellular recording

Conventional intracellular recording techniques were used as described previously (Fatt & Katz, 1951; Shinnick-Gallagher & Jacobs, 1976) to record from muscle fibres in rat hemidiaphragm preparations. Standard 3 M KCl-filled glass capillary electrodes of 3 to 10 MΩ resistance were used for intracellular recording of membrane potentials. Only those cells having miniature endplate potentials (m.e.p.ps) with rise times of <1 ms were considered acceptable for study. M.e.p.ps were either counted visually from films of oscilloscope tracings or tabulated by a counter attached to a Mentor neuronal spike analyzer. Cells were maintained for a 15 min control period before application of toxin. Control and treatment values were obtained in the same fibre and a paired analysis was performed using Student's t test; statistical significance was determined as $P < 0.05$. All values were expressed as mean ± s.e. mean.

Drugs and stock solutions

The crude toxin was extracted from *G. breve* organisms by the method of Spikes, Ray, Aldrich & Nash (1968). Extracted toxin was kept in the dark at 4°C; before use, an aliquot of the toxin (10 mg) was dissolved in a small quantity of ether. A drop of Tween 80 was added together with 1 ml of deionized distilled H_2O and the mixture was agitated. The ether was evaporated leaving an emulsion of crude toxin in H_2O. The final volume was adjusted to 5 ml with deionized distilled H_2O.

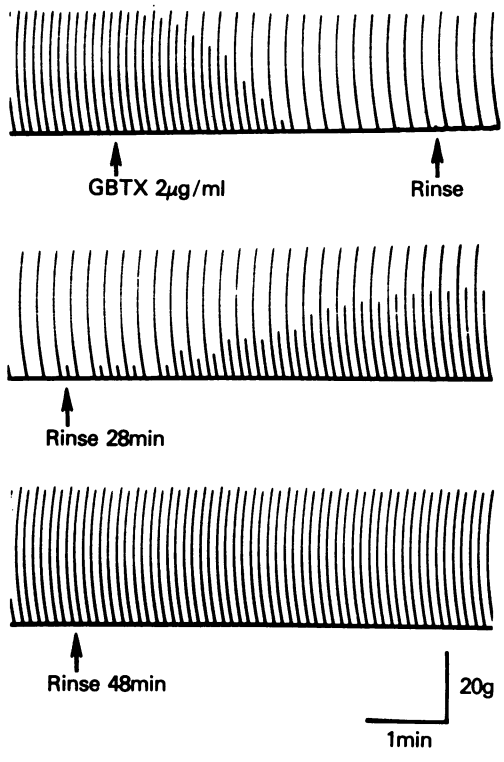

Figure 1 Effect of *Gymnodinium breve* toxin (GBTX) on muscle contractions. GBTX (2 μg/ml) blocks contractions of rat phrenic nerve diaphragm elicited indirectly but does not block contractions to direct electrical stimulation; frequency of stimulation was 0.1 Hz. Records are continuous.

The crude toxin was obtained from Dr Sammy Ray, Moody College of Marine Sciences, Texas A & M University, Galveston, Texas. Other drugs used were (+)-tubocurarine chloride (Squibb) and ethylene glycol (Sigma). All stock solutions were refrigerated until just before use.

Results

Muscle twitch experiments

Application of GBTX (2 μg/ml) selectively blocked indirectly stimulated muscle contraction in 2 min but had no effect on directly stimulated contractions (Figure 1). However, at 10 μg/ml, GBTX depressed direct muscle contractions to 50% of control values. The time required to produce complete inhibition of

indirectly stimulated muscle contractions depended on the concentration of the toxin. At 0.25 μg/ml, GBTX blocked the contractions in 30 min whereas 1 μg/ml GBTX required only 6 min. Inhibition of the indirect response was reversible; however, additional applications of GBTX to the same preparation enhanced the effect of the toxin. A fresh preparation was used in each experiment to eliminate possible residually acting toxin. Addition of the suspending agent, Tween 80, in the dilution used in these studies had no effect on direct and indirectly stimulated muscle. The fact that indirect muscle contraction was more sensitive to GBTX action than direct muscle contraction suggested that GBTX affected transmission through the synapse. For this reason the effect of GBTX was studied further by recording membrane potential changes at the neuromuscular junction.

Effects on resting membrane potential

The resting membrane of the muscle fibre was depolarized 3 to 30 mV within 3 min of GBTX application (Table 1). The depolarization was usually sustained during application of GBTX; occasionally, the membrane would repolarize to some extent in the continued presence of the toxin. Depolarization occurred at both subsynaptic and non-synaptic regions of the muscle fibre and was readily reversed upon rinsing the preparation.

Effects on miniature endplate potentials

The effect of various concentrations of GBTX on spontaneous m.e.p.ps is shown in Table 1. Effects on m.e.p.p. frequency and amplitude developed within 15 min. The values in the presence of toxin were obtained after 15 min of treatment with the respective concentrations. A biphasic effect on m.e.p.p. amplitude was observed with GBTX treatment. At lower concentrations of GBTX (0.25 μg/ml to 0.5 μg/ml), m.e.p.p. amplitude was significantly decreased. At 1 μg/ml GBTX, the m.e.p.p. amplitude was unchanged, and, at 2 μg/ml, significantly increased in spite of muscle membrane depolarization.

GBTX increased m.e.p.p. frequency. At concentrations, 0.25 μg/ml to 1 μg/ml, m.e.p.p. frequency was augmented to about three times control. The high concentration, 2 μg/ml, of GBTX increased m.e.p.p. frequency over thirty fold. The onset of the m.e.p.p. frequency increase was variable and may be related to the position and accessibility of the nerve terminal to the action of the toxin. The increased frequency was maintained as long as 2 to 3 h during application of the toxin. M.e.p.p. frequency remained elevated for 30 min to 2 h after removal of the toxin solution. The increases in m.e.p.p. frequency and alterations in m.e.p.p. amplitude were accompanied by a significant muscle membrane depolarization.

M.e.p.p. frequency was increased in every cell recorded; furthermore, in 6 of 65 cells, a very marked response to GBTX occurred (Figure 2). In those cells, m.e.p.p. frequency was dramatically increased to about 500 times control. The increase in frequency was progressive until m.e.p.ps were superimposed. With continued toxin application, it appeared that spontaneous release had ceased. After the preparation was rinsed with normal solution m.e.p.ps reappeared.

These experiments on m.e.p.ps indicated that GBTX had a presynaptic action, which resulted in an increased frequency of m.e.p.ps. GBTX effects were studied on endplate potentials to define other possible sites of action.

Table 1 Effect of various concentrations of *Gymnodinium breve* toxin (GBTX) on miniature endplate potentials (m.e.p.ps)

	n	*m.e.p.p. amplitude* (mV)	*m.e.p.p. frequency* (m.e.p.ps/s)	*Resting membrane potential* (mV)
Control	6	0.67 ± 0.08	2.5 ± 0.5	73.6 ± 2.7
0.25 μg/ml GBTX		0.57 ± 0.08*	7.7 ± 2.4*	62.8 ± 4.6*
Control	4	0.83 ± 0.08	3.0 ± 0.6	71.5 ± 5.2
0.5 μg/ml GBTX		0.66 ± 0.10*	15.7 ± 4.2*	61.2 ± 4.5*
Control	5	0.76 ± 0.07	3.5 ± 0.4	68.4 ± 4.1
1 μg/ml GBTX		0.74 ± 0.07	10.4 ± 2.3*	55.4 ± 2.6*
Control	6	0.95 ± 0.12	4.1 ± 0.8	64.6 ± 2.8
2 μg/ml GBTX		1.10 ± 0.11*	142.0 ± 46.0*	51.6 ± 3.7*

n = number of paired experiments.
* Statistically significant difference ($P < 0.05$) between paired control and treatment values, one-tailed *t* test.

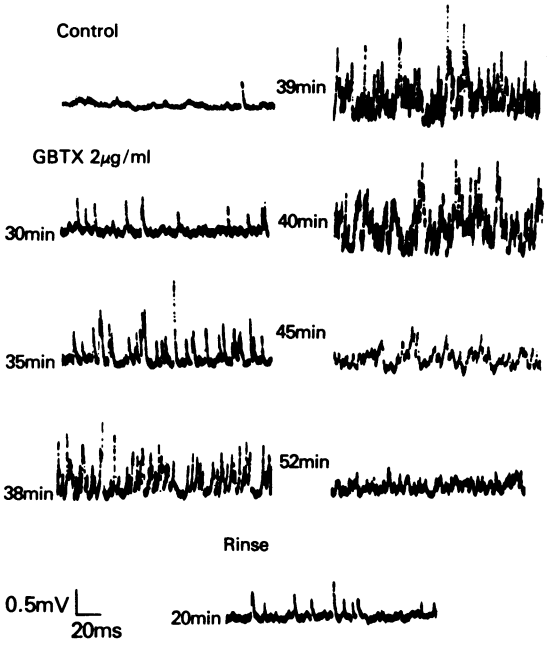

Figure 2 Effect of *Gymnodinium breve* toxin (GBTX) 2 μg/ml on miniature endplate potentials (m.e.p.ps). Control m.e.p.p. frequency was 2/s; after a 30 min GBTX application, frequency was 30/s; at 35 min, 143/s; at 38 min, 850/s; at 39 min, 1200/s; at 40 min, 1050/s; at 45 min, m.e.p.ps could not be distinguished from noise and at 52 min, it appeared that spontaneous release had ceased; after a 20 min rinse with normal solution, frequency was 15/s. Amplitude of m.e.p.ps increased from 0.45 ± 0.1 mV to 0.63 ± 0.2 mV.

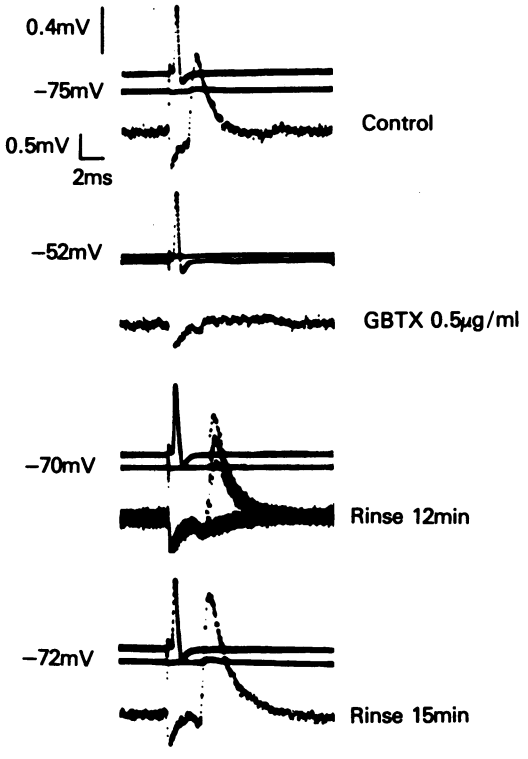

Figure 3 Effect of *Gymnodinium breve* toxin (GBTX) on endplate potentials, antidromic compound nerve action potentials, and resting membrane potential. Except in the second panel, the upper trace is the antidromic compound nerve action potentials, the middle trace is the resting membrane potential and the lower trace is the endplate potential. Top panel is control; second panel depicts complete block of the endplate potential with a 15 min application of GBTX (0.5 μg/ml); third panel shows the 12 min rinse with normal solution and the bottom panel, the 15 min rinse with normal solution.

Effects on endplate potentials

The effect of GBTX (0.5 μg/ml) in a preparation in which the endplate potential had been reduced in size with (+)-tubocurarine is depicted in Figure 3. GBTX (0.5 μg/ml to 2 μg/ml) completely blocked endplate potentials; the higher the concentration, the shorter the time required to produce a complete block. At 0.25 μg/ml, endplate potentials were only slightly depressed after 30 min of exposure to GBTX. Depolarization of the resting membrane potential occurred immediately upon addition of the toxin to the perfusion media while block of endplate potentials was observed a few minutes later. The disappearance of the endplate potential was abrupt and not decremental. The action was reversed upon rinsing with normal solution. Endplate potentials reappeared suddenly at almost control amplitude. The resting membrane potential repolarized before the reappearance of endplate potentials. Repetitive endplate potentials were not observed in any preparation.

Effects on action potentials of muscle fibres

Action potentials were elicited from a muscle fibre with a second, current-passing electrode which was placed in the same fibre within 100 μm of the recording electrode. The membrane potential was maintained at the control level by passing a continuous anodal current across the muscle membrane. The effect of 2 μg/ml GBTX on directly stimulated action potentials of a muscle fibre in an ethylene glycol-treated muscle is shown in Figure 4. When the membrane potential was held at −70 mV, GBTX treatment for 10 min did not affect the amplitude of the action potential. When the anodal current was shut off, the action potential was depressed. The actual

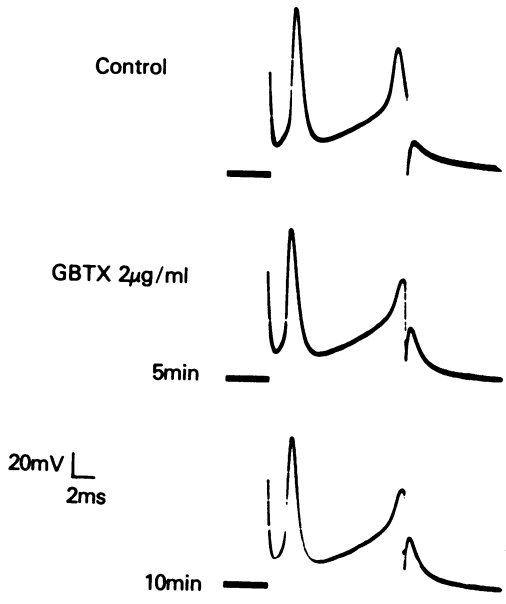

Figure 4 Effect of *Gymnodinium breve* toxin (GBTX) 2 μg/ml on an action potential recorded intracellularly from a muscle fibre in an ethylene glycol blocked preparation. The membrane potential was held at −70 mV.

membrane potential after 10 min of treatment was −52 mV. These results suggested that GBTX probably does not affect action potential generation and that the depression of muscle fibre action potentials was due to the depolarization. No repetitive muscle action potentials were observed.

Discussion

The present study shows that GBTX: (1) has a greater effect on indirect muscle contractions than direct contractions, (2) depolarizes the membrane potential of muscle, (3) increases m.e.p.p. frequency, (4) has a biphasic effect on m.e.p.p. amplitudes, (5) blocks endplate potential, (6) does not produce repetitive endplate potentials or muscle action potentials, and (7) occasionally causes cessation of spontaneous release. These effects demonstrate that *Gymnodinium breve* toxin acts both pre- and postsynaptically at the rat phrenic nerve diaphragm preparation.

GBTX depolarized the muscle membrane, a postsynaptic action. Sasner (1965) also found that crude GBTX depolarized all membranes, in general. Purified fractions of GBTX, tested by Sasner *et al.* (1972) and Abbott *et al.* (1975), did not depolarize frog sartorius muscle or squid giant axon. More recently, Westerfield *et al.* (1977) showed that their purified GBTX fraction did depolarize the squid giant axon. These differing results may be due to the various purification methods which could yield dissimilar fractions of GBTX.

The presynaptic action of GBTX is manifested by an increase in m.e.p.p. frequency. It is highly possible that the membrane of nerve terminals would have been depolarized by GBTX as observed at the muscle membrane and other cell membranes (Sasner, 1965). A GBTX-induced depolarization of the nerve terminal membrane may be responsible for the increased m.e.p.p. frequency. The fact that tetrodotoxin reverses a GBTX-induced increase in m.e.p.p. frequency supports this hypothesis (Shinnick-Gallagher, 1980). In contrast, Abbott *et al.* (1975) report that their purified GBTX fraction does not alter m.e.p.p. frequency in frog sartorius muscle nor does it depolarize the muscle membrane. The inconsistent results may be due to purification procedures or to the use of different preparations.

In some cells spontaneous release on m.e.p.ps appeared to cease. The dramatic increase in frequency and cessation of spontaneous release in some cells may be related to the extent of the depolarization of the presynaptic terminal, such that a greater depolarization may have caused a depression of spontaneous transmitter release rather than enhancement.

GBTX effects on m.e.p.p. amplitude do not appear to be caused by the depolarization of the resting muscle membrane. The effects were biphasic; at lower concentrations (0.25 μg/ml to 0.5 μg/ml), GBTX was depressant whereas at a high concentration (2.0 μg/ml) it was facilitatory. At an intermediate concentration, 1 μg/ml, GBTX did not change m.e.p.p. amplitudes. Spiegelstein & Siger (1972) reported that purified GBTX had no effect on m.e.p.p. amplitudes in frog sartorius at concentrations of 1 to 2 μg/ml. Similarly, Abbott *et al.* (1975) stated that purified GBTX did not alter m.e.p.p. amplitude in frog preparations. The lack of effect of GBTX in these two studies may be due to (1) use of a different species, the frog, or (2) the fact that the concentration of toxin employed may have been on a part of the GBTX dose-response curve that exhibited no effect, or (3) the purified toxins may have lost their actions.

GBTX produces a sudden blockade of endplate potentials at concentrations which do not block m.e.p.ps. Depolarization of nerve terminals decreases the amplitude of the nerve terminal action potential and subsequently blocks endplate potentials (Hubbard & Willis, 1968). In fact, a certain small critical depolarization of the presynaptic membrane may inhibit invasion of the action potential into the nerve terminal (Krnjević & Miledi, 1958). Since the muscle membrane is depolarized, the block of the endplate may be due to a similar presynaptic effect, namely that the nerve terminal is depolarized. The increased

m.e.p.p. frequency supports this hypothesis. The depolarization of the nerve terminal which depresses transmitter release may be responsible for the greater sensitivity of indirectly stimulated muscle contractions to blockade by GBTX.

Nerve action potentials are blocked by cumulative depolarizing effects of repetitive firing in squid (Westerfield *et al.*, 1977). However, in the present studies with the rat phrenic nerve diaphragms, no repetitive endplate potentials or muscle action potentials were recorded although the resting membrane was depolarized. Hyperpolarization restores squid axon action potentials (Westerfield *et al.*, 1977) as it does action potentials of muscle fibres in the rat. The membrane of the squid giant axon also depolarizes before the onset of repetitive firing (Westerfield *et al.*, 1977). In these studies membrane depolarization appears to be a common entity observed with crude and purified toxin in both the rat diaphragm preparation and squid giant axon.

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