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POSSIBLE MECHANISMS OF ACTION OF *Gymnodinium breve* TOXIN AT THE MAMMALIAN NEUROMUSCULAR JUNCTION

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1 The mechanism of action of a crude fraction of *Gymnodinium breve* toxin (GBTX) was investigated by intracellular recording techniques in the rat phrenic nerve diaphragm preparation.

2 GBTX (2 μg/ml) decreased the input resistance of the muscle membrane concomitantly with a depolarization of the resting membrane potential.

3 A low sodium solution reversed or prevented a GBTX-induced membrane depolarization.

4 Tetrodotoxin (TTX) antagonized a GBTX-induced increase in miniature endplate potential (m.e.p.p.) frequency and repolarized a GBTX-depolarized membrane. Pretreatment with TTX prevented GBTX effects.

5 GBTX reversibly reduced depolarizations produced by bath applied acetylcholine (ACh). The membrane depolarization was not responsible for the depression of ACh responses.

6 These findings suggest that GBTX increases m.e.p.p. frequency and depolarizes the resting membrane potential by increasing sodium permeability. The reduction of ACh-induced depolarizations suggests that GBTX may be acting at some site on the ACh receptor.

Introduction

In the preceding paper (Gallagher & Shinnick-Gallagher, 1980), it was shown that indirectly stimulated muscle was more sensitive to the blocking action of *Gymnodinium breve* toxin (GBTX) than directly stimulated muscle. At the same time, the frequency of miniature endplate potentials (m.e.p.p.s) was elevated and the postsynaptic membrane was depolarized by 10 to 15 mV. The toxin's action, both pre- and postsynaptically, may be caused by depolarization. Another explanation of the inhibition of neuromuscular transmission may be a postsynaptic block of the cholinoceptor.

Westerfield, Moore, Kim & Padilla (1977) demonstrated that tetrodotoxin (TTX), an inhibitor of transient sodium currents, prevented the GBTX-induced repetitive firing in squid giant axon. These authors suggested that the current responsible for the GBTX-induced repetitive firing flows through the sodium channel. However, the concentration of the ion itself must be altered to demonstrate an ionic mechanism of action. Sodium ion may be responsible for the GBTX effects which were observed in the rat diaphragm.

To define possible mechanisms of action of GBTX in mammalian preparations, the effect of the toxin was studied on depolarizations induced by exogenously applied acetylcholine (ACh), on the electrical characteristics of the membrane, and in the presence of either a low Na^+ solution or tetrodotoxin. The results of these studies suggested that GBTX may increase resting sodium permeability of membranes. A preliminary account of some of these experiments has been published (Shinnick-Gallagher & Gallagher, 1977).

Methods

Preparation and experimental design

All experiments were performed with isolated phrenic nerve diaphragm preparations from male Sprague-Dawley rats (200 to 300 g). The preparation and experimental recording conditions were described in the previous paper (Gallagher and Shinnick-Gallagher, 1980). Paired control and treatment values were obtained in the same cell. The data collected from a cell during the control period were compared to data collected from the same cell after treatment with the toxin. Because of the possibility of residually-acting toxin, a fresh preparation was employed for each application of the toxin.

Acetylcholine perfusion

The microelectrode was inserted at the endplate region of a muscle fibre where m.e.p.p.s of <1 ms rise

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time and <1.5 ms half fall time could be recorded. For these experiments, a small bath (0.8 ml volume) and rapid perfusion rate were used. Acetylcholine (ACh, 5×10^{-6} to 10^{-5} M) was perfused through the muscle chamber for 15 s with 15 min intervals between applications. The effect of GBTX was studied only after reproducible ACh depolarizations were attained. At the end of a 15 and 30 min treatment period, ACh was added to the GBTX-containing solution. In each cell, the magnitude of the ACh-induced depolarization was compared before and after treatment with 2 μg/ml GBTX. Since the depolarizing response of the endplate is non-linear, ACh responses were corrected for non-linear summation (Martin, 1955).

Effective membrane resistance

The method for measuring input resistance of the muscle fibre, R_0, was similar to that described by Boyd & Martin (1959). A muscle fibre was impaled with a recording electrode and a second, current passing electrode was inserted in the same cell within 50 μm of the recording electrode. Electrotonic potentials of 60 ms duration and various intensities were recorded before and after treatment with GBTX in each cell. The amplitude of the recorded electrotonic potential (mV) at the 60 ms time point was plotted against the amount of current ($\times 10^{-8}$ A) passed across the membrane. The effective membrane resistance (R_0) was determined from the slope of a regression line fitted for a minimum of squares of the deviations from the line by a computer program. Only hyperpolarizing currents were used since the current-voltage relationship is not linear with depolarizing currents greater than one-third the electrical threshold of the muscle membrane (Katz, 1948). R_m, the specific resistance, or the resistance for a unit area of membrane, was calculated from the equation: $R_m = (R_0 \cdot \pi)^2 d^3 / R_i$ (Hubbard, Llinas & Quastel, 1969), where R_i for mammalian muscle was assumed to be 125 ohm·cm at 37°C (Boyd & Martin, 1959); d, the fibre diameter, was measured directly with a microscope micrometer.

Solutions and drugs

The physiological solution and toxin preparation were described in the previous paper (Gallagher & Shinnick-Gallagher, 1980). In experiments requiring a low sodium medium, sodium or sodium chloride in the normal physiological solution was substituted with either choline chloride or sucrose and Tris buffer. The final composition of the low sodium solution was as follows (mM): choline chloride 115 or sucrose 230, Tris 4, KCl 4.6, KH_2PO_4 1.15, $CaCl_2$ 2.46, $MgSO_4$ 1.15, and glucose 8.85. The solution was bubbled vigorously with 95% O_2 and 5% CO_2 for 1 h and titrated with 1N NaOH to a pH of 7.4. The final solution contained 7.5 mM Na^+. The muscle was incubated in the low Na^+ solution for 30 min before the data collection except where indicated in the results.

Results

Acetylcholine depolarizations

A typical experiment illustrating the effect of 2 μg/ml GBTX on ACh depolarizations is depicted in Figure 1. GBTX depressed ACh depolarizations. This effect was reversible when the preparation was rinsed with normal solution for 1.5 h. The depression of ACh potentials occurred at the time when m.e.p.p. frequency was notably increased. The effect of 15 min of GBTX treatment on depolarizations induced by 10^{-5} M ACh in seven preparations is summarized in Table 1. In each cell GBTX depressed ACh-induced depolarizations and depolarized the resting membrane significantly. The amplitude of GBTX-depressed ACh depolarizations was not affected by correction for non-linear summation which takes into account alterations in resting membrane potential.

However, the depression of ACh-induced depolari-

Table 1 Effects of *Gymnodinium breve* toxin (GBTX, 2 μg/ml) on acetylcholine (ACh)-induced depolarizations

	n	*Resting membrane potential* (mV)	*ACh depolarization* (mV)	*ACh*[1] *depolarization* (mV)
Control	7	65.7 ± 3.0	5.8 ± 1.7	7.1 ± 2.3
GBTX (2 μg/ml)	7	58.1 ± 4.0*	1.5 ± 0.3*	1.6 ± 0.4*

n = number of paired experiments.
*Statistically significant difference ($P < 0.05$) between paired control and treatment values, one-tailed *t* test.
[1]Corrected for non-linear summation (Martin, 1955).

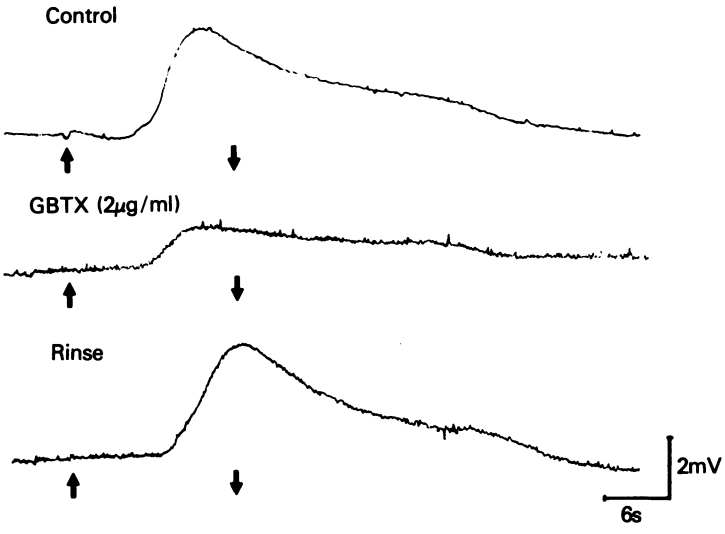

Figure 1 D.c. pen recordings of the effect of *Gymnodinium breve* toxin (GBTX) 2 μg/ml on acetylcholine (ACh) depolarizations. ACh (5×10^{-6} M) was applied between the arrows in each trace. The higher noise level in the middle and bottom trace was due to an increased miniature endplate potential (m.e.p.p.) frequency. Resting membrane potential was −66 mV, −64.5 mV, and −70 mV, respectively.

zations by GBTX could be due to a decrease in the membrane resistance of the muscle as a result of an increase in membrane permeability to some ions. The increase in membrane permeability may also account for the depolarization produced by the toxin.

Effective membrane resistance

The effect of 2 μg/ml GBTX on constant current anodal pulses at various intensities is illustrated in Figure 2a. After a 15 min treatment, GBTX decreased the anelectrotonic responses, an indication of a decreased input resistance. The effect of a GBTX-induced depolarization on the voltage-current relationship of effective membrane resistance is shown in Figure 2b. GBTX, 2 μg/ml, reduced the resting membrane potential and slightly decreased input resistance after a 15 min treatment. Control effective membrane resistance in 10 paired experiments was 1.30 ± 0.22 ($\times 10^5$) ohms. GBTX slightly but significantly decreased effective membrane resistance to 1.19 ± 0.20 ($\times 10^5$) ohms. Resistance for a unit area of membrane, R_m, was decreased from 187 ± 69 ohm cm^2 to 149 ± 45 ohm cm^2. The mean fibre diameter of these cells was 51 ± 7 μm. When constant current pulses were continuously applied to the membrane, it appeared that a decrease in input resistance occurred coincident with the membrane depolarization. GBTX depolarized the membrane from -70.5 ± 3.5 mV to -61.8 ± 3.3 mV. This decrease in the input resistance and specific membrane resistance would not be suffi-

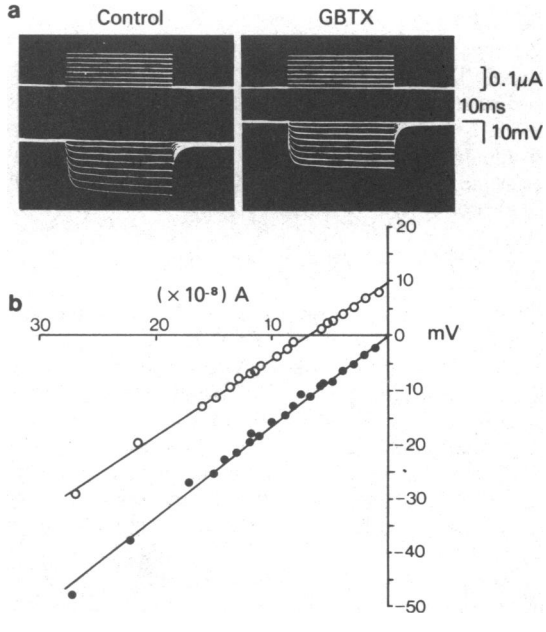

Figure 2 Effect of *Gymnodinium breve* toxin (GBTX) on input resistance. (a) Recordings of electrotonic potentials (lower traces) in control (left panel) and after 15 min of treatment with 2 μg/ml GBTX (right panel). In each panel, the top tracings are the current monitors. (b) Voltage-current plot of input resistance with respect to membrane potential. Control resting membrane potential is equal to 0 mV. Control = (●); GBTX (2 μg/ml) for 15 min = (○).

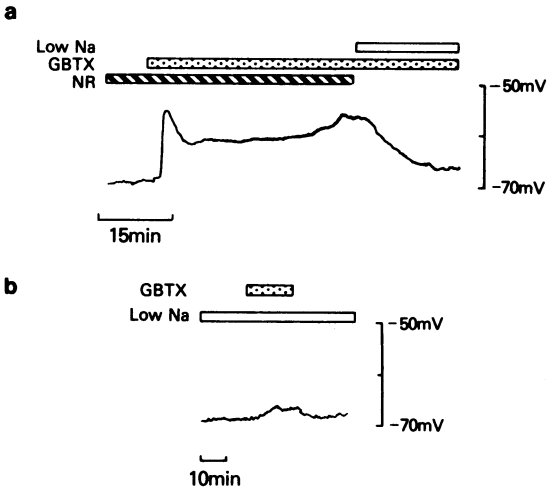

Figure 3 Effect of low sodium (choline-substitution) solution on *Gymnodinium breve* (GBTX)-induced effects on resting membrane potential in 2 different cells (a and b). Concentration of GBTX was 2 μg/ml. NR is normal Ringer.

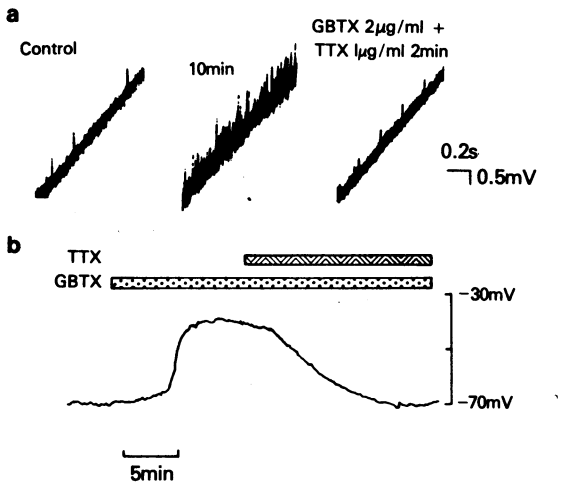

Figure 4 Action of tetrodotoxin (TTX, 1 μg/ml) on the effects of *Gymnodinium breve* toxin (GBTX, 2 μg/ml) on miniature endplate potentials (m.e.p.p.) and resting membrane potential in the same cell. (a) TTX action on GBTX-induced increase in m.e.p.p. frequency. (b) d.c. pen recording of TTX antagonism of GBTX depolarization of resting membrane potential. The 1s oscilloscope sweeps were photographed on moving film.

cient to explain the marked depression of the ACh depolarization induced by GBTX.

The decreased resistance with membrane depolarization suggested that GBTX may be increasing membrane permeability, probably to Na^+. To test this possibility, the effect of GBTX was observed in a low Na^+ solution.

Effects of GBTX in low Na^+ solutions

The effect of substitution of a low Na^+ choline solution on the action of a GBTX is shown in Figure 3a. GBTX (2 μg/ml) depolarized the resting membrane potential within 5 min. The membrane repolarized slightly but remained depolarized during the GBTX treatment. During the control period, m.e.p.p. frequency was 4.2/s; GBTX increased m.e.p.p. frequency to 275/s in 40 min of treatment. The low Na^+ solution repolarized the resting membrane potential in the continued presence of GBTX. M.e.p.p.s were abolished in a low Na^+ solution. The effects of GBTX (2 μg/ml) on resting membrane potential in low Na^+ choline solution is depicted in Figure 3b. The low Na^+ solution almost completely prevented the action of GBTX on resting membrane potential. Mean control resting membrane potential in 9 preparations in low Na^+ sucrose solution was 69.1 ± 2.1 mV; treatment with GBTX (2 μg/ml) for 15 min did not alter resting membrane potential (67.8 ± 2.1 mV) significantly.

These experiments indicated that GBTX increases mainly the resting Na^+ permeability of the muscle membrane. Because tetrodotoxin antagonized the GBTX-induced repetitive firing in squid giant axon, the effect of tetrodotixin (TTX) was tested on 2 parameters altered by GBTX in mammalian preparations, m.e.p.p. frequency and resting membrane potential.

Tetrodotoxin on GBTX effects

The effect of GBTX (2 μg/ml) in the absence and presence of TTX (1 μg/ml) is illustrated in Figure 4a and b. GBTX reduced resting membrane potential 27 mV within 3 min of application and increased m.e.p.p. frequency from 3/s to 120/s within 15 min. Addition of TTX in the continued presence of GBTX, reversed the resting membrane potential depolarization and antagonized the increased m.e.p.p. frequency within 2 min of application. The effect of TTX (1 μg/ml) on GBTX-induced alterations in m.e.p.p. amplitude and frequency and resting membrane potential in six preparations is summarized in Table 2. It is quite clear that TTX antagonized the action of GBTX on m.e.p.p. frequency and resting membrane potential of the muscle fibre. Furthermore, the membrane was hyperpolarized 4 mV beyond the control level in the presence of TTX and GBTX. TTX did not antagonize the GBTX-induced increase in m.e.p.p. amplitude

Table 2 Effect of tetrodotoxin (TTX, 1 μg/ml) on miniature endplate potentials in the presence of *Gymnodinium breve* toxin (GBTX), 2 μg/ml

	n	*m.e.p.p. amplitudes* (mV)	*m.e.p.p. frequency* (m.e.p.p.s/s)	*Resting membrane potential* (mV)
Control	6	0.94 ± 0.11	3.5 ± 0.9	65.5 ± 2.6
GBTX (2 μg/ml)	6	1.03 ± 0.12*	104.1 ± 17.9*	50.8 ± 3.8*
GBTX (2 μg/ml) + TTX (1 μg/ml)	6	1.04 ± 0.13	3.9 ± 0.9**	68.3 ± 3.3**

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

during a 15 min application. In some cells when TTX was perfused for a longer time a decrease in m.e.p.p. amplitude was observed. Also, pretreatment with TTX (1 μg/ml) prevented GBTX effects on m.e.p.p. amplitude and frequency and resting membrane potential. TTX itself hyperpolarized the membrane 4 to 5 mV.

Discussion

The principal effects of GBTX can be summarized as follows: (1) depolarization of the muscle fibre membrane, (2) an increase in m.e.p.p. frequency and blockade of endplate potential generation; and (3) biphasic effects on m.e.p.p. amplitude (Gallagher & Shinnick-Gallagher, 1980) together with the depression of ACh-induced depolarizations.

The mechanism of membrane depolarization of the muscle fibre seems to be an increase in sodium permeability, P_{Na}, since the depolarization by GBTX was accompanied by a decrease in the membrane resistance and abolished in a low Na^+ solution and by treatment with tetrodotoxin. A small decrease in effective membrane resistance (8.4%) might be insufficient to explain a depolarization of 20 mV if it is due only to a sodium permeability increase. However, there is a 20% decrease in the resistance of a unit area of membrane. Furthermore, a depolarization caused by an increase in P_{Na} of the membrane would activate the anomalous rectification of the muscle membrane. This membrane rectification should decrease the P_K, increase the input resistance, and mask the decrease in membrane resistance accompanying the P_{Na} increase (Nakajima, Iwasaki & Obata, 1962). Therefore, it seems that the depolarization of the muscle membrane may be explained by an increase in P_{Na}.

ACh-induced depolarizations were depressed to 25% of control values by GBTX. The depression cannot be explained by the depolarization of the muscle membrane since the GBTX effect on the amplitudes of the ACh depolarizations were not altered by correction for non-linear summation. A 20% decrease in the specific membrane resistance (R_m) does not seem sufficient to explain the 75% decrease in ACh depolarizations, although this factor may contribute to the depressant effect.

Tetrodotoxin antagonized the GBTX increase in m.e.p.p. frequency and depolarizations of the membrane probably through a sodium mechanism. Since the sodium channels involved in ACh responses are insensitive to TTX (Furukawa, Sasoka & Hosaya, 1959), the GBTX depression of ACh depolarizations should not be affected by TTX. It seems likely that the mechanism responsible for the GBTX-induced depression of ACh depolarizations may be different from that producing the increased m.e.p.p. frequency and depolarization of the membrane.

Perhaps, GBTX may act on the cholinoceptor and thus depress ACh depolarizations. On the other hand, m.e.p.p. amplitude was slightly increased by GBTX (Table 2; Gallagher & Shinnick-Gallagher, 1980). It would seem that the lack of a depressant effect on m.e.p.ps would not be consistent with a depressant action at the cholinoceptor. However, this paradox can be explained in part by various postsynaptic mechanisms.

Recently, a purified toxin, batrachotoxin, an alkaloid which increases resting Na^+ permeability has been shown to depress ACh depolarizations in a non-

competitive manner (Garrison, Albuquerque, Warnick, Daly & Witkop, 1978). Non-competitive blocking agents depress large ACh responses to a greater extent than smaller ones. The fact that GBTX has a greater depressant effect on large ACh responses, i.e. the depolarization with bath-applied ACh, and does not depress m.e.p.p. amplitudes, suggests that GBTX may act as a non-competitive blocking agent of ACh, perhaps by blocking channels. The lifetime of the channel opened by ACh could be shortened without affecting markedly the peak conductance as is the case with the actions of atropine (Feltz, Large & Trautmann, 1977), certain local anaesthetics (Steinbach, 1968), and alcohols (Gage, McBurney and Van Helden, 1978). In this condition, m.e.p.p. amplitude is less affected, but the ACh depolarization is significantly depressed (Dionne & Stevens, 1975; Mallart, Dreyer & Peper, 1976). Furthermore the paradox could also be explained if GBTX has a potent 'desensitizing' action on the cholinoceptor, as seen with histrionicotoxin (Albuquerque, Barnard, Chiu, Lapa, Dolly, Jansson, Daly & Witkop, 1973; Burgermeister, Catterall & Witkop, 1977). To determine the mechanism responsible for the paradoxical action of GBTX, experiments using voltage clamp techniques with purified toxin have to be carried out.

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