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EFFECTS OF *Gymnodinium breve* TOXIN ON THE SMOOTH MUSCLE PREPARATION OF GUINEA-PIG ILEUM

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1 The effects of *Gymnodinium breve* neurotoxin (GT) on smooth muscles were studied using the guinea-pig isolated ileum.

2 The toxin caused strong spasmogenic effects at 1-4 μg/ml, characterized by prolonged tonic contraction with superimposed pronounced pendular movements. Tachyphylaxis was observed upon administration of successive doses.

3 Atropine blocked the contractile response elicited by GT, whereas mepyramine and hexamethonium failed to do so. These findings tentatively suggested a cholinergic involvement at a post-ganglionic site of action.

4 In the presence of tetrodotoxin the effects of GT were abolished, excluding direct action of the toxin on the smooth muscle.

5 It is concluded that GT exerts its spasmogenic effects through stimulation of the post-ganglionic cholinergic nerve fibres.

Introduction

Blooms of toxic algae are well known, some of the most toxic among them belonging to the order of Dinoflagellates (Russell, 1965). *Gymnodinium breve*, a member of this order, has been found to be responsible for extensive toxic blooms in the seas along the southeast coast of Florida and the Gulf of Mexico (Wilson & Ray, 1956). As a result of the rapid growth of these blooms and the accumulation of toxin, massive mortality of marine organisms and fish life are observed (Gunter, Williams, Davis & Walton Smith, 1948). Although some marine organisms such as certain molluscs are apparently immune themselves to the toxic effects, the accumulated poisonous substances within them can, upon their consumption, cause severe intoxication in man (Woodcock, 1948; Ray & Aldrich, 1965; McFarren, Tanabe, Silva, Wilson, Campbell & Lewis, 1965). Extraction and purification of *Gymnodinium breve* cultures yielded two neurotoxic fractions (Martin & Chatterjee, 1969; Trieff, Spikes, Ray & Nash, 1971), both of which were further characterized by Spiegelstein, Paster & Abbott (1973) with respect to certain chemical and toxicological properties. Regarding the site and mechanism of action, Sasner, Ikawa, Thurberg & Alam (1972) attributed the main effects of *Gymnodinium breve* toxin (GT) to a synaptic block at the endplate, although they state that the specific mechanism was not clear. However, their studies using mammalian intestine, vertebrate and invertebrate heart preparations and human serum indicated an anticholinesterase-like action. Siger, Spiegelstein & Abbott (1972) found that GT caused oscillations in non-myelinated nerve fibres followed by trains of action potentials, indicating a direct effect on the nerve membrane by the toxin.

In order to obtain additional information concerning the physiological and pharmacological actions of GT, the following study, with a smooth muscle preparation, was undertaken. The importance of such a study seemed to us particularly relevant as diarrhoea was one of the major signs reported in the symptomatology of human GT intoxications (McFarren *et al.*, 1965).

Methods

Toxin

GT was extracted from axenic cultures of *Gymnodinium breve* as described by Spiegelstein *et al.* (1973). The toxin used in the present study was the semi-pure yellow powder, obtained following Step 6 of the purification procedure (as described by the above authors) and contained the two neurotoxic fractions T_1 and T_2. This partially

purified toxin was dissolved in ethanol and kept as stock solution under refrigeration (−4°C). Working solutions were prepared by appropriate dilutions of the stock solution with Tyrode of the following composition in g/litre: NaCl 8.0; KCl 0.2; $MgCl_2$ 0.1; $CaCl_2$ 0.2; NaH_2PO_4 0.05; $NaHCO_3$ 1.0 and glucose 1.0. The potency of the toxin was determined by intravenous injection into white mice. Only material possessing an LD_{50} value of 0.145-0.176 mg/kg was used.

Guinea-pig ileum

Guinea-pigs were killed by a blow on the head and bled. The ileum was removed, flushed free of food residues and placed in Tyrode solution. Segments of about 3 cm long were cut from the mid-ileum and the isolated preparation was suspended in a 5 ml organ bath containing Tyrode solution at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. Contractions were recorded on smoked kymograph paper by means of an isotonic lever with a 5-10 magnification.

Transmurally stimulated guinea-pig ileum

This preparation was essentially similar to that described by Paton (1955). The electrodes were made of teflon-coated platinum wire, stripped at the ends and placed at opposite ends of the organ bath. Monophasic square-wave pulses were applied, with a Grass S-4 stimulator.

Drugs

Acetylcholine perchlorate; nicotine sulphate; histamine hydrochloride; atropine sulphate; hexamethonium bromide; mepyramine; tetrodotoxin (No. T-6254, Sigma).

Results

Effects of Gymnodinium breve *toxin on guinea-pig ileum*

The typical responses elicited are shown in Figure 1. In general, the initial response was a tonic contraction followed in most cases by steadily increasing pendular movements. In some preparations, the spasmogenic response was delayed for a few minutes, while in others this response occurred almost immediately upon addition of the toxin. Although characteristic responses could be observed at doses lower than 1 μg/ml, due to the relatively short-lasting effect of such doses, we preferred employing higher concentrations in order to obtain a more prolonged response. Thus, in most experiments the response of the ileum to GT was recorded during a contact time of 10 min, after which GT was removed by washing it out several times. Repeated administrations of GT clearly demonstrated a diminishing effect of the same dose upon each successive addition. This tachyphylactic effect was seen with respect to both the tonic and phasic (pendular) contractions (Figure 2).

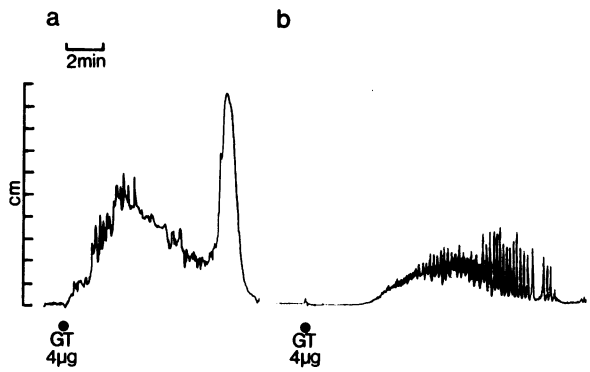

Fig. 1 Typical effects of *Gymnodinium breve* toxin (GT) in two different preparations of guinea-pig isolated ileum (a;b) suspended in Tyrode solution. The organ-bath volume was 5 ml. Same experimental conditions were used throughout study. Note tonic and phasic contractions. Doses refer to amounts per ml.

Antagonism studies

For the purpose of elucidating the mechanism underlying the contractile effect of GT, the responses in the presence of antagonists of smooth muscle stimulants were examined.

Atropine. Atropine at a concentration which suppressed the response to acetylcholine also abolished completely the effects of GT (Figure 3). This antagonistic effect was reversed after atropine had been washed out. This finding points to the possible involvement of a cholinergic mechanism in the induction of spasmogenicity by GT.

Mepyramine. As an additional antagonist, the anti-histamine drug mepyramine was used. Figure 4 shows the lack of inhibitory influence of this antagonist on GT. This result, as well as the fact of the immediate relaxation caused by atropine added at the height of the GT response (Fig. 4c) supports the assumption that the toxin interacts with the parasympathetic activity of intestinal contraction.

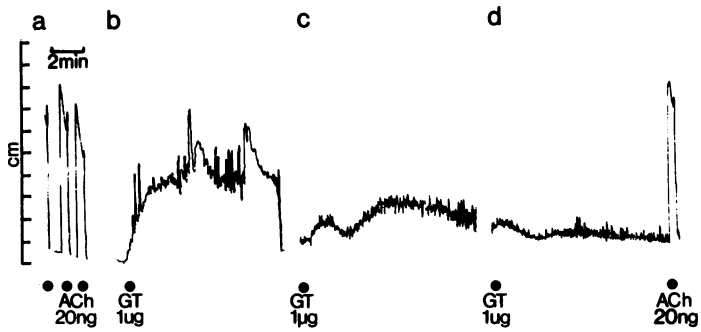

Fig. 2 Tachyphylaxis in guinea-pig ileum to successive doses of *Gymnodinium breve* toxin (GT) (b; c; d). Between records, three washings. Acetylcholine (ACh) was added as control at the start (a) and after the last dose of GT (d). Doses refer to amounts per ml.

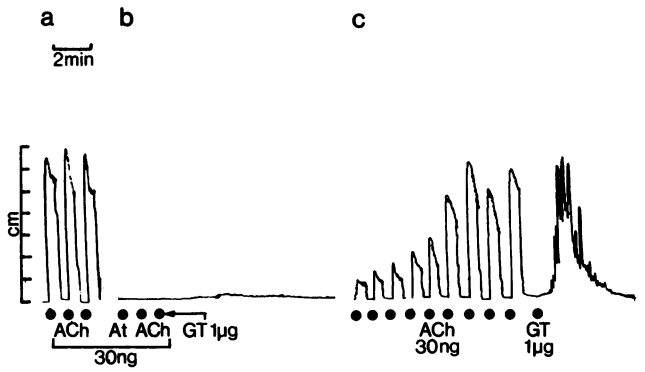

Fig. 3 Influence of atropine (At) on the action of *Gymnodinium breve* toxin (GT) in the guinea-pig ileum. (a) Control responses to acetylcholine (ACh); (b) following atropine, both responses to acetylcholine and GT were blocked; (c) gradual recovery of responses to ACh after repeated washings, followed by characteristic GT-induced contractions. Doses refer to amounts per ml.

Hexamethonium. In order to characterize further the site of action of GT, the possibility of a ganglionic stimulatory effect was examined. Figure 5 shows that responses induced by nicotine were blocked by hexamethonium while those elicited by GT were unaffected. This observation implies that the effects of GT are due to a post-ganglionic site of action.

Tetrodotoxin. Tetrodotoxin (TTX) serves as a useful pharmacological tool in the analysis of drug effects on the various post-ganglionic sites since it selectively blocks neurally elicited responses while axon-terminals and effector cells of smooth muscles remain unaffected (Kao, 1966; Ogura, Mori & Watanabe, 1966; Gershon, 1967). Figure 6 illustrates the results of a typical experiment with TTX. Following the addition of TTX, neither nicotine nor transmural stimulation elicited any contractile response (Figure 6d). Similarly, the characteristic stimulatory activity of GT was completely blocked by TTX, whereas acetylcholine-induced spasmogenicity was not susceptible to the blocking action of TTX (Figure 6e). After TTX had been washed out, the responses to nicotine and transmural stimulation remained suppressed for a time (Figure 6e). Upon complete cessation of TTX's blocking action, further addition of GT resulted in resumption of the toxin's stimulatory activity (Figure 6f).

Discussion

Among the few reported incidences of intoxication due to GT in man (McFarren *et al.*, 1965), the

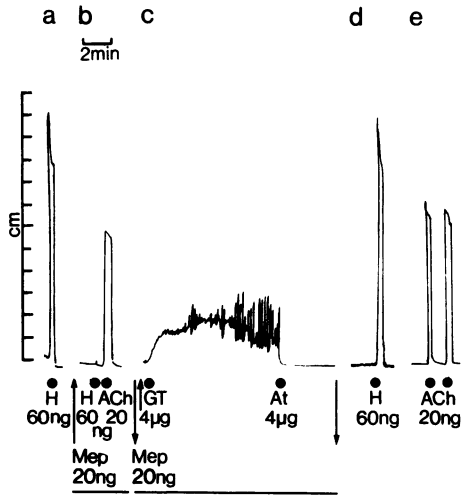

Fig. 4 Effect of mepyramine (Mep) on the response of guinea-pig ileum to *Gymnodinium breve* toxin (GT). Between records, three washings. (a) Control response to histamine (H); (b) H response blocked after mepyramine response to acetylcholine (ACh) unaffected; (c) failure of mepyramine to block GT-evoked contraction. Atropine (At) added at height of induced contraction, followed by immediate cessation of spasmogenicity; (d) restoration of response to histamine after washing; (e) control response to acetylcholine. Doses refer to amounts per ml.

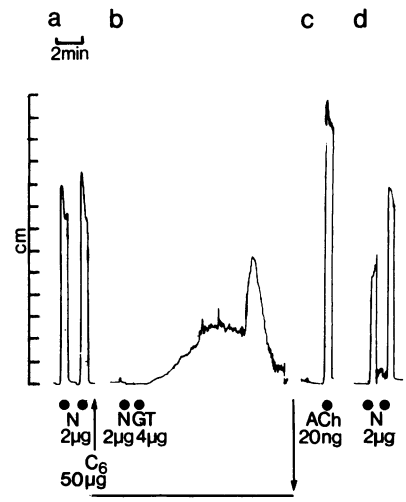

Fig. 5 Effect of hexamethonium (C_6) on the response of guinea-pig ileum to *Gymnodinium breve* toxin (GT). (a) Control response to nicotine (N); (b) hexamethonium blocked the response to nicotine but had no effect on the contractile action of GT; (c) control response to acetylcholine; (d) recovery of the response to nicotine after repeated washings. Doses refer to amounts per ml.

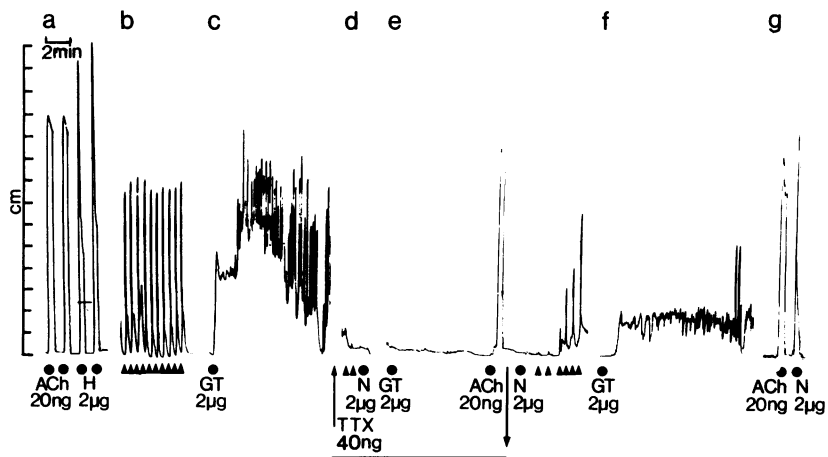

Fig. 6 Effects of *Gymnodinium breve* toxin (GT) on the transmurally stimulated (▲) and tetrodotoxin (TTX)-treated guinea-pig ileum. (a) Control response to acetylcholine (ACh) and nicotine (N); (b) control responses to transmural stimulation (every 30 s, 10 V, 500 ms duration); (c) effect of GT; (d) block of contractions due to transmural stimulation and nicotine; (e) failure of GT to evoke contraction after TTX, response to acetylcholine unchanged. Gradual recovery of the response to stimulation after washing of TTX (arrow downward); (f) effect of GT following recovery from TTX; (g) control responses to acetylcholine and nicotine. Doses refer to amounts per ml.

occurrence of diarrhoea implies a possible effect of the toxin on gastrointestinal motility. Our results corroborated this assumption, as shown by the pronounced spasmogenicity elicited by GT when applied to the guinea-pig isolated ileum. In comparison with other known intestinal smooth muscle stimulants, the nature of its activity appeared to be more complex, judging by the characteristic pattern of prolonged tonic and superimposed phasic contraction. Several sites of action of the toxin might be considered, such as a direct effect on the smooth muscle fibres or stimulation of one of the components associated with the neural network innervating the gut.

Initially, the failure of the preparation to respond to GT in the presence of atropine merely suggested involvement of a cholinergic mechanism. Subsequent experiments with TTX, which in itself does not affect the electrical or mechanical activity of smooth muscle fibres (Kao, 1966) but which abolished GT-induced contraction, excluded the possibility of a direct action on the muscle fibres, including the cholinergic receptors which retain their function under these conditions (Gershon, 1967). Siger *et al.* (1972) and Paster (1972) reached similar conclusions about the action of GT on striated muscle. However, Paster demonstrated that when the Ca concentration was reduced, the muscle membrane became sensitive to GT as shown by the spontaneous contractions of fibres in an asynchronous sequence.

The neural network in the gut wall contains elements of both the sympathetic and parasympathetic system in the pre- and post-ganglionic fibres of the autonomic plexuses (Schofield, 1968). The fact that treatment with hexamethonium failed to block the typical contraction induced by GT, pointed to a post-ganglionic site of action.

In accounting for the pharmacological action exerted by a drug on the post-ganglionic neurone, one has to consider the nerve cell body, its processes and the axon terminals. In this connection, TTX provides a delicate and selective experimental tool. Thus, TTX blocks action potentials by prevention of sodium conductance in nerve and striated muscle, but fails to affect spontaneous miniature endplate potentials or bring about local depolarization of the nerve terminal (Elmquist & Feldman, 1965; Katz & Miledi, 1966). TTX has no effect on the spontaneous or evoked electrical activity of smooth muscle (Toida & Osa, 1965; Kao, 1966).

As mentioned before, following treatment of the ileum preparation with TTX (40 ng/ml), no spasmogenic responses were obtained by applying GT. Since it can be assumed that in the presence of TTX the nerve terminals continue to function normally, it is evident that GT cannot have caused spontaneous release of transmitter by a direct action on the post-ganglionic axon terminals. This observation led us to conclude that in smooth muscle preparations, the toxin must act directly on the post-ganglionic cholinergic non-myelinated nerve fibres with the resultant release of acetylcholine to initiate the events of contraction. Support for this proposed site of action is given by the studies of Siger *et al.* (1972). These workers found that in the frog isolated sartorious nerve-muscle preparation, GT caused fasciculations which could be blocked by curare. Further analysis of intracellular recordings at the neuromuscular junction implied that the effects of GT are apparently caused by subthreshold oscillations in the nerve. This effect has been shown in the squid giant axon where the toxin brought about oscillations followed by trains of action potentials. Since these phenomena resembled the effects obtained following the reduction of calcium concentration, the authors suggested that the toxin is an antagonist of the normal function of calcium in the nerve membrane. Additional evidence for this mode of action has recently been presented by Paster (1972).

An important additional finding of the present study is the typical tachyphylaxis of the smooth muscle to repeated administrations of GT. An explanation for the mechanism underlying tachyphylaxis in this case could be sought in the blocking effect of action potentials in the nerve fibres by the toxin. This seems to us a most likely postulate when considering the findings of several investigators (Abbott & Paster, 1970; Siger *et al.*, 1972; Spiegelstein & Siger, 1972) that GT in high doses blocks nerve action potentials. Thus, repeated application of GT might create a situation in which more and more of the toxin is accumulated, ultimately leading to partial or complete nerve block. Unpublished observations from this laboratory showed that nerve block, due to GT in frog sciaticus, could not be reversed even by repeated washings.

Besides the results reported here, the only other available data in the literature on effects of GT on smooth muscle are in the study of Sasner *et al.* (1972). Using the mouse gut, these authors attributed the spasmogenic effect of GT in that preparation to an anti-cholinesterase-like activity of the toxin. Our results do not support this conclusion, since, if this were the case, we should have observed a considerable increase in height of contractions induced by GT following treatment of the ileum with TTX, which has no effect on the spontaneous release of acetylcholine from nerve terminals. Such an increase in contractility was shown by Gershon (1967) when the anti-

cholinesterase drug physostigmine was added to a TTX-poisoned guinea-pig ileum preparation. Additional experimental proof that GT did not possess anti-cholinesterase activity has been presented by Siger *et al.* (1972).

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