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PRODUCTION OF A PURIFIED MARINE NEUROTOXIN AND DEMONSTRATION OF ITS
BINDING AFFINITY TO ION CHANNEL RECEPTORS

Ciguatera is a tropical fish-borne disease in which both a lipid soluble (ciguatera) and water soluble (maitotoxin) toxin have been implicated. The determination of toxin structure, cell binding characteristics, and sensitive assays are all dependent on an increased supply of toxins. In order to increase our production of toxin we developed a mass culture technique using a Gambierdiscus toxicus dinoflagellate strain obtained from Fort-de-France Bay near the Caribbean island of Martinique. The clones were maintained at

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27° under an illumination of $30-40 \text{ uE} \cdot \text{M}^{-2} \cdot \text{S}^{-1}$ and a 16:8 hours light:dark cycle without aeration. Cells are grown in an enriched seawater medium (K-medium) with seawater collected from Vero Beach, Florida or Charleston, South Carolina. The vitamin mixtures and enrichments for the media are prepared in concentrated stocks and sterilized. *G. toxicus* clones were harvested by filtration through 12 μm polycarbonate membranes and inoculated for mass culture into 12 liter glass carboy microcarrier flasks. The cells were kept in suspension by a combination of magnetic stirring and aeration. We currently employ solvent partitioning, pre-column filtering and HPLC chromatography to prepare a semi-purified extract of maitotoxin, the water soluble toxin produced by *Gambierdiscus toxicus*. Whole cells are sonicated, extracted in hot methanol and subjected to ether/water and water/butanol solvent partitioning. The aqueous extract is then filtered through a C18 filter. The toxin is separated from additional contaminants using a semi-prep C18 HPLC column (Alltech Econosil 22 mm x 250 mm) using 70% methanol as the mobile phase (injection 2 ml; flow rate 10 ml/min). The toxic 'peak', eluting at 1.5 relative to phenol, has a toxicity of 4 $\mu\text{g}/\text{kg}$ mouse and represents an overall increase in toxicity from the whole cell stage of 140 fold (toxicity is reported in mouse units, i.e., the dose that kills 50% of mice in 48 hours). The incorporation of this extraction method has resulted in a more efficient preparation of semi-purified toxin. The mode of action of many of the marine neurotoxins such as ciguatoxin and brevetoxin have been reported to involve a common binding site on sodium channels. An in vitro binding study using a rat brain synaptosome preparation demonstrated that brevetoxin binds with a high affinity and specificity. The synaptosomes showed a saturable binding of brevetoxin with increasing concentrations of toxin from 2-20 nM. Scatchard analysis of "total binding" resulted in a line with a slope of $-.3027$ representing an apparent dissociation constant (K_d) of 3.30 nM with a binding maximum of 14.61 pmoles brevetoxin bound/mg synaptosome protein. In a subsequent study, the effect of maitotoxin on brevetoxin binding was determined. Incubation of synaptosomes with .1-20 μM maitotoxin resulted in brevetoxin binding of 97-114% of control. The effect of ciguatoxin on brevetoxin binding to synaptosome receptors was also determined. Incubation of synaptosomes with 2-100 nM ciguatoxin resulted in a reduction in the binding of brevetoxin as the concentration of ciguatoxin was increased. At 20 nM ciguatoxin, brevetoxin binding was reduced to 62% of the control and at 100 nM ciguatoxin the brevetoxin was reduced to only 12% of the control. With the use of a rapid centrifugation technique to separate synaptosome bound toxin from free toxin following in vitro binding we have demonstrated that one of the ciguatera implicated toxins, maitotoxin, does not displace brevetoxin from its unique receptor and therefore must produce its toxic effects with a mode of action different from that of brevetoxin. But, our results demonstrate that ciguatoxin does affect the binding of brevetoxin to synaptosomes. Therefore, our results lend further support to the conclusion that maitotoxin and ciguatoxin possess different pharmacological modes of action.

PRODUCTION OF A PURIFIED MARINE NEUROTOXIN AND DEMONSTRATION OF ITS BINDING
AFFINITY TO ION CHANNEL RECEPTORS

FINAL REPORT

JAMES E. BALTHROP, JOHN A. BABINCHAK, PENNY B. TRAVIS
TERESA L. HERRING AND PAM Y. BROWN-EYO

JUNE 10, 1989

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"Production of a Purified Marine Neurotoxin and Demonstration of Its Binding Affinity to Ion Channel Receptors"

**James E. Balthrop, John A. Babinchak, Penny B. Travis
Teresa L. Herring and Pam Y. Brown-Eyo**

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PRODUCTION OF A PURIFIED MARINE NEUROTOXIN AND DEMONSTRATION OF ITS
BINDING AFFINITY TO ION CHANNEL RECEPTORS

James E. Balthrop, John A. Babinchak, Penny B. Travis,
Teresa L. Herring and Pam Y. Brown-Eyo

Ciguatera is a tropical fish-borne disease in which both a lipid soluble (ciguatoxin) and water soluble (maitotoxin) toxin have been implicated. The determination of toxin structure, cell binding characteristics, and sensitive assays are all dependent on an increased supply of toxins. In order to increase our production of toxin we developed a mass culture technique using a *Gambierdiscus toxicus* dinoflagellate strain obtained from Fort-de-France Bay near the Caribbean island of Martinique. The clones were maintained at 27° under an illumination of 30-40 $\mu\text{E}\cdot\text{M}^{-2}\cdot\text{S}^{-1}$ and a 16:8 hours light:dark cycle without aeration. Cells are grown in an enriched seawater medium (K-medium) with seawater collected from Vero Beach, Florida or Charleston, South Carolina. The vitamin mixtures and enrichments for the media are prepared in concentrated stocks and sterilized. *G. toxicus* clones were harvested by filtration through 12 μm polycarbonate membranes and inoculated for mass culture into 12 liter glass carboy microcarrier flasks. The cells were kept in suspension by a combination of magnetic stirring and aeration. We currently employ solvent partitioning, pre-column filtering and HPLC chromatography to prepare a semi-purified extract of maitotoxin, the water soluble toxin produced by *Gambierdiscus toxicus*. Whole cells are sonicated, extracted in hot methanol and subjected to ether/water and water/butanol solvent partitioning. The aqueous extract is then filtered through a C18 filter. The toxin is separated from additional contaminants using a semi-prep C18 HPLC column (Alltech Econosil 22 mm x 250 mm) using 70% methanol as the mobile phase (injection 2 ml; flow rate 10 ml/min). The toxic 'peak', eluting at 1.5 relative to phenol, has a toxicity of 4 $\mu\text{g}/\text{kg}$ mouse and represents an overall increase in toxicity from the whole cell stage of 140 fold (toxicity is reported in mouse units, i.e., the dose that kills 50% of mice in 48 hours). The incorporation of this extraction method has resulted in a more efficient preparation of semi-purified toxin. The mode of action of many of the marine neurotoxins such as ciguatoxin and brevetoxin have been reported to involve a common binding site on sodium channels. An in vitro binding study using a rat brain synaptosome preparation demonstrated that brevetoxin binds with a high affinity and specificity. The synaptosomes showed a saturable binding of brevetoxin with increasing concentrations of toxin from 2-20 nM. Scatchard analysis of "total binding" resulted in a line with a slope of -.3027 representing an apparent dissociation constant (K_d) of 3.30 nM with a binding maximum of 14.61 pmoles brevetoxin bound/mg synaptosome protein. In a subsequent study, the effect of maitotoxin on brevetoxin binding was determined. Incubation of synaptosomes with .1-20 μM maitotoxin resulted in brevetoxin binding of 97-114% of control. The effect of ciguatoxin on brevetoxin binding to synaptosome receptors was also determined. Incubation of synaptosomes with 2-100 nM ciguatoxin resulted in a reduction in the binding of brevetoxin as the concentration of ciguatoxin was increased. At 20 nM ciguatoxin, brevetoxin binding was reduced to 65% of the control and at 100 nM ciguatoxin the brevetoxin was reduced to only 12% of the control. With the use of a rapid centrifugation technique to separate synaptosome bound toxin from free toxin following in vitro binding we have demonstrated that one of the ciguatera implicated toxins, maitotoxin, does not displace brevetoxin from its unique receptor and therefore must produce its toxic effects with a mode of action different from that of brevetoxin. But, our results demonstrate that ciguatoxin does affect the binding of brevetoxin to synaptosomes. Therefore, our results lend further support to the conclusion that maitotoxin and ciguatoxin possess different pharmacological modes of action.

A RAPID AND EFFICIENT METHOD OF MAITOTOXIN EXTRACTION AND SEMI-PURIFICATION

P. Travis, T. Herring and J. Balthrop

National Marine Fisheries Service

, PO Box 12607

Charleston, SC 29412

FASEB Journal 3(4):A1189, 1989

We are currently employing solvent partitioning, pre-column filtering and HPLC chromatography to prepare a semi-purified extract of maitotoxin, the water soluble toxin produced by Gambierdiscus toxicus. Whole cells from a strain isolated from Martinique are sonicated, extracted in hot methanol and subjected to ether/water and water/butanol solvent partitioning. The aqueous extract is then filtered through a C18 filter. The filtering procedure recovers approximately 95% of the toxin and removes approximately 23% of the total mass. The toxin is then separated from additional contaminants using a semi-prep C18 HPLC column (Alltech Econosil 22 mm x 250 mm) using 70% methanol as the mobile phase (injection 2 ml; flow rate 10 ml/min). The toxic 'peak', eluting at 1.5 relative to phenol, has a toxicity of 4 ug/kg mouse and represents an overall increase in toxicity from the whole cell stage of 140 fold (toxicity is reported in mouse units, i.e., the dose that kills 50% of mice in 48 hours). The incorporation of this extraction method has resulted in a more efficient preparation of semi-purified toxin.

Mass Culturing Gambierdiscus toxicus in Microcarrier Spinner Flasks

Tenth Southeastern Phycological Colloquy, 14-16 October, 1988

Dolphin Beach Resort, St. Petersburg Beach, FL

John A. Babinchak and Pamela Brown-Eyo

NMFS, PO Box 12607, Charleston SC 29412

ABSTRACT

Microcarrier spinner flasks were applied as a new technique for mass culture of the epiphytic dinoflagellate, Gambierdiscus toxicus. The gentle agitation of the flask design maintained the dinoflagellate in suspension and with aeration, stabilized the pH of the medium during the final growth phase. Cell yield of Martinique G. toxicus clone, MQ2, in mass culture (4532 $\pm$ 526 cells/ml, N44) was equivalent to the yield obtained in one liter culture using 2.8 liter Fernbach flasks (4735 $\pm$ 1105 cells/ml, N26). Culturing facilities were established at the NMFS Charleston Laboratory to handle 24 culture flasks. Maintaining a continuous schedule of alternate weekly harvests and inoculations of 12 flasks provided for the harvest of 288 liters of culture or 1.3×10^9 cells (dry w . 66 g) every 28 days. Determined by mouse bioassay, G. toxicus clone, MQ2, had a whole cell toxicity (LD50) of 223 cells/MU and a crude methanol extract toxicity (LD50) of 310 cells/MU. The capacity for toxin production of the mass culture facility is > 4 million mouse units (crude methanol extract) every 28 days.

Annual Report, FY88

MARINE BIOTOXIN PROGRAM

DINOFLAGELLATE TOXIN PRODUCTION

John A. Babinchak

Introduction

Ciguatera is a tropical fish-borne disease in which a lipid-soluble toxin (ciguatoxin) and water-soluble toxin (maitotoxin) are known to cause the disease (8). Obtaining sufficient quantities of these toxins from toxic fish is a tedious process (13) which has not yielded sufficient quantities for detailed analyses. The marine dinoflagellate, Gambierdiscus toxicus (1) has been implicated as the producer of ciguateric toxins and readily produces maitotoxin, however, only limited quantities of ciguatoxin (11, 12, 4, 8, 9). Production of purified maitotoxin for use in defining its structure and development of assay techniques for detection of ciguatoxin and maitotoxin in fishery products are goals of the Marine Biotoxin Program at the Charleston Laboratory. An adequate supply of dinoflagellate produced maitotoxin is a prerequisite for developing this information. The goal for FY88 in G. toxicus production was to develop a mass culturing system which could consistently achieve maximum cell yield of G. toxicus per unit volume of culture medium.

Statement of Work Performed

Stock Cultures

Three clonal strains of Gambierdiscus toxicus were maintained as stock cultures for toxin production, a Hawaiian strain, T39 (9) and MQ1 and MQ2, clones isolated from samples collected 24 April 1986, in Fort-de-France Bay near the Caribbean island of Martinique. Isolation procedure was as previously described (2). The clones were maintained at 27°C under an illumination of 30-40 $\mu\text{E}\cdot\text{M}^{-2}\cdot\text{S}^{-1}$ and a 16:8 hours light:dark cycle without aeration. Illumination was provided by a mixture of Cool White (North American Philips Lighting Corp., Bloomfield, NJ) and Vita-Lite (Duro-Test Corp., North Bergen,

NJ) fluorescent bulbs. K medium (7), an enriched seawater medium, was used in all culturing. Seawater was collected either from a saltwater well in Vero Beach, FL, or 10 miles off Charleston, SC, filtered through 0.45 μ m cartridge filters into polycarbonate carboys and refrigerated in the dark until used to prepare media. The vitamin mixtures and enrichments for K medium were prepared in concentrated stocks, filter-sterilized and autoclaved respectively, stored frozen, and added aseptically to autoclaved seawater. G. toxicus clones were harvested by filtration through 12 μ m polycarbonate membranes, washed with sterile seawater and inoculated at 200-300 cells/ml into 2.8 liter Fernbach flasks containing 1 liter of medium. Stock cultures of T39, MQ1 and MQ2 were harvested for transfers at 14 days and for toxin extraction at 18 to 27 days. In FY88, a total of 451.5 liters of mixed stock culture was harvested yielding 2.0×10^9 cells (dry wt., 102 g) for toxin extraction.

Mass Culture

The Martinique G. toxicus clone, MQ2, was selected for mass culture since it consistently reached cell densities > 4000 cells/ml and had a whole cell toxicity (Time-to-death LD50, 47 cells/MU) comparable to the highest toxicity reported for cultured G. toxicus (3).

Microcarrier spinner flasks (Bellco Glass Inc., Vineland, NJ) were selected as mass culture vessels. The 8 liter model was the largest that could be accommodated upright in our sterilizer and was of a design (wide mouth), and weight that could be easily handled and cleaned. The maximal working volume of these flasks for dinoflagellate culture was 12 liters. Magnetic stirring units (Bellco), designed for gentle agitation, maintained a stirring speed of 20 RPM, sufficient to prevent cell sedimentation during growth without causing cell damage, and also provide adequate exchange of gases in the culture medium.

Shelving units (Figure 1) were designed to accommodate six culture vessels and stirrers and provide illumination from above and behind at $30\mu\text{E}\cdot\text{M}^{-2}\cdot\text{S}^{-1}$ and a 16:8 hour light:dark cycle. As with stock cultures, illumination was provided by a mixture of Cool White and Vita-Lite fluorescent bulbs. Two shelving units were installed in each of two walkin environmental rooms (large walkin (LWI) 1800 cu. ft.; small walkin (SWI) 600 cu. ft.) maintained at 27°C , providing space for culturing 24 vessels at one time. Vessels were inoculated at a rate of 500 washed cells/ml. A schedule of alternate weekly harvests and inoculations of 10 flasks provided for the harvest of 240 liters every 28 days. Recent receipt of additional culture vessels will allow use of all 24 stirring units, increasing production to 288 liters per 28 days in FY89.

The only major modification to the original design of the culture system was the manner of providing aeration to the cultures. Aeration supplies the carbon needed for growth and also stabilizes the pH in seawater by keeping it from getting too high too fast through the interaction of the carbonate system with pH (6). Initially, air supplied by Whisper 800 aquarium air pumps (Willinger Bros. Inc., Englewood, NJ) and filtered through AQ microfibre disposable filter tubes, gas efficiency (at 0.1 micron) of 99.9999+%, (Balston Filter Products, Lexington, MA) was bubbled into the culture vessels through one ml glass pipettes. Using this aeration system, cell yields were 30% less than produced in 2.8 liter Fernbach flasks and pH values ≥ 9.6 (Table 1). Plastic air diffusers, "discard-a-stone", (Lee's Aquarium Products, San Marcos, CA) were attached to the pipette tips, resulting in a stabilization of the culture medium pH and an increase in cell yields (35% SWI; 18% LWI; Table 1). The average cellular yield differed between the two environmental rooms with the smaller

walkin producing cell densities equivalent to those produced by one liter culture in 2.8 liter Fernbach flasks (4735 ± 1105 cell ml^{-1} , N26; 3).

In FY88, a total of 1923 liters of G. toxicus clone, MQ2, was harvested from microcarrier flasks yielding 7.4×10^9 cells (dry wt., 376 g) for toxin extraction.

Harvesting Procedure

A system for harvesting the microcarrier spinner flasks was established which required minimal handling of the vessels. After 21 days of incubation, the stirring and aeration apparatus were removed, the flask swirled and a 10 ml sample taken for determining cell counts. The cells were allowed to settle and the supernatant removed with a peristaltic pump and filtered through a 142 mm stainless filter holder (30 or 41 micron nylon or polyester membrane; Figure 2). The concentrated mass of settled cells from all the vessels were then combined and collected on a 30 or 41 micron membrane in a 90 mm glass filter holder which produced a cake of wet cells (Figure 3). The cells were refrigerated in 80% methanol until extracted for toxin.

Cell Counts

Cell counts on individual microcarrier flasks were determined at harvest using natural chlorophyll autofluorescence and direct epifluorescence microscopy. Duplicate 0.5 ml volumes of each microcarrier sample were collected on 25 mm black polycarbonate membranes (5 micron) and observed at 320x with a video display. A Leitz Dialux 20 microscope was used, equipped with a 150W xenon lamp, fluorescence vertical illuminator, KG1 heat filter, BG23 blue filter and a Leitz I2 filter block. Twelve fields or a minimum of 400 cells per sample

were counted. Cell densities for harvested cells resuspended in 1% Tween 80 were determined with five replicated counts using Palmer-Maloney chambers. Cell densities for pooled stock cultures at harvest were determined counting 20 fields each of duplicate samples using Palmer-Maloney chambers.

Algal Extract

Bomber (5) determined in tube culture that K medium with a reduced concentration of Na_2EDTA ($1 \times 10^{-4.5}\text{M}$) and added aqueous rhodophyte extract (50 mg/liter), "Super K medium", supported the highest yield $> 1 \text{ g l}^{-1}$ and growth rate $0.85 \text{ division day}^{-1}$ of Martinique strain MQ1. A study was initiated to determine what effect these cultural parameters would have on final cell densities of clone MQ2 grown in microcarrier flasks. The macroalgal extract was prepared by homogenizing Heterosiphonia gibbesi in distilled H_2O (1:2, weight:volume), autoclaving, filtering through 12 micron polycarbonate membranes and reautoclaving. G. toxicus strains may take up to six transfers to acclimate to new cultural parameters (Bomber, pers. commun.), however the amount of macroalgal extract needed to accomplish this in mass culture was not available. Extraction of 160 g of H. gibbesi produced 1995 mg of extract, enough to statistically test "Super K medium" in tube culture, but only enough for 3.3 microcarrier flasks. Cell yields of MQ2 in microcarrier flasks using "Super K medium" were less than for K medium (Table 2). The study was terminated because of the prohibitively large quantities of the rhodophyte extract required to sustain mass culture even if acclimated culture demonstrated increased cell yields.

Toxicity of MQ2

Cells for whole cell bioassay were harvested by filtration (12 micron polycarbonate membranes) and resuspended in distilled water containing 1% Tween 80. Toxicities of whole cells (LD50) were determined by ultrasonic disintegrating (2 30-s bursts at 0°C) the cell suspensions and injecting 0.2-0.3 ml of appropriate cell concentrations intraperitoneally into female ICR mice weighing approximately 20 g. Whole cell toxicities for MQ2 from mass culture was 0.57 mg/kg mice or 223 cells/MU. Toxicity of a crude methanol extract of these same cells was 0.16 mg/kg mice or 310 cells/MU. Toxicity recovery in a crude methanol extract ranged from 75 to 95% of whole cell values (pers. commun., Jim Balthrop).

Summary

Microcarrier spinner flasks were applied as a new technique for mass culture of the epiphytic dinoflagellate, Gambierdiscus toxicus. The gentle agitation of the flask design maintained the dinoflagellate in suspension without any detrimental effect on its growth rate, and with aeration, stabilized the pH of the medium during the final growth phase. Cell yield of Martinique G. toxicus clone, MQ2, in mass culture (4532 ± 526 cells/ml, N44) was equivalent to the yield obtained in one liter culture using 2.8 l Fernbach flasks (4735 ± 1105 cell/ml, N26). Culturing facilities were established at the NMFS Charleston Laboratory to handle 24 culture vessels and using a continuous schedule of alternate weekly harvests and inoculations of 12 flasks, provided for the harvest of 288 liters of culture, or 1.3×10^9 cells (dry wt. 66 g) every 28 days. Using mouse bioassay, G. toxicus clone, MQ2, had a whole cell toxicity (LD50) of 223 cells/MU and a crude methanol extract toxicity (LD50) of 310 cells/MU. The capacity for toxin production of the mass culture facility is > 4 million mouse units (crude methanol extract) every 28 days. Total G. toxicus production for FY88 was 9.4×10^9 cells (dry wt., 478 g) representing 30 million mouse units (crude methanol extract).

Table 1. Mean cell densities of G. toxicus clone, MQ2, mass cultured in microcarrier spinner flasks.

Incubator	Aeration	Cells/ml	pH ^a
SWI	nonairstone	3357 $\pm$ 488 (N25)	9.9 (N6)
	airstone	4532 $\pm$ 526 (N44)	8.9 (N44)
LWI	nonairstone	3296 $\pm$ 515 (N27)	9.6 (N17)
	airstone	3886 $\pm$ 473 (N45)	8.8 (N45)

^a K medium at harvest

Table 2. Mean cell densities of G. toxicus clone, MQ2, grown in microcarrier spinner flasks.

Incubator	Medium	Cells/ml
SWI	Super K	4024 $\pm$ 756 (N3)
	K	4784 $\pm$ 523 (N7)
LWI	Super K	3506 $\pm$ 414 (N3)
	K	4272 $\pm$ 570 (N7)

Figure 1. Mass culturing setup for microcarrier flasks.

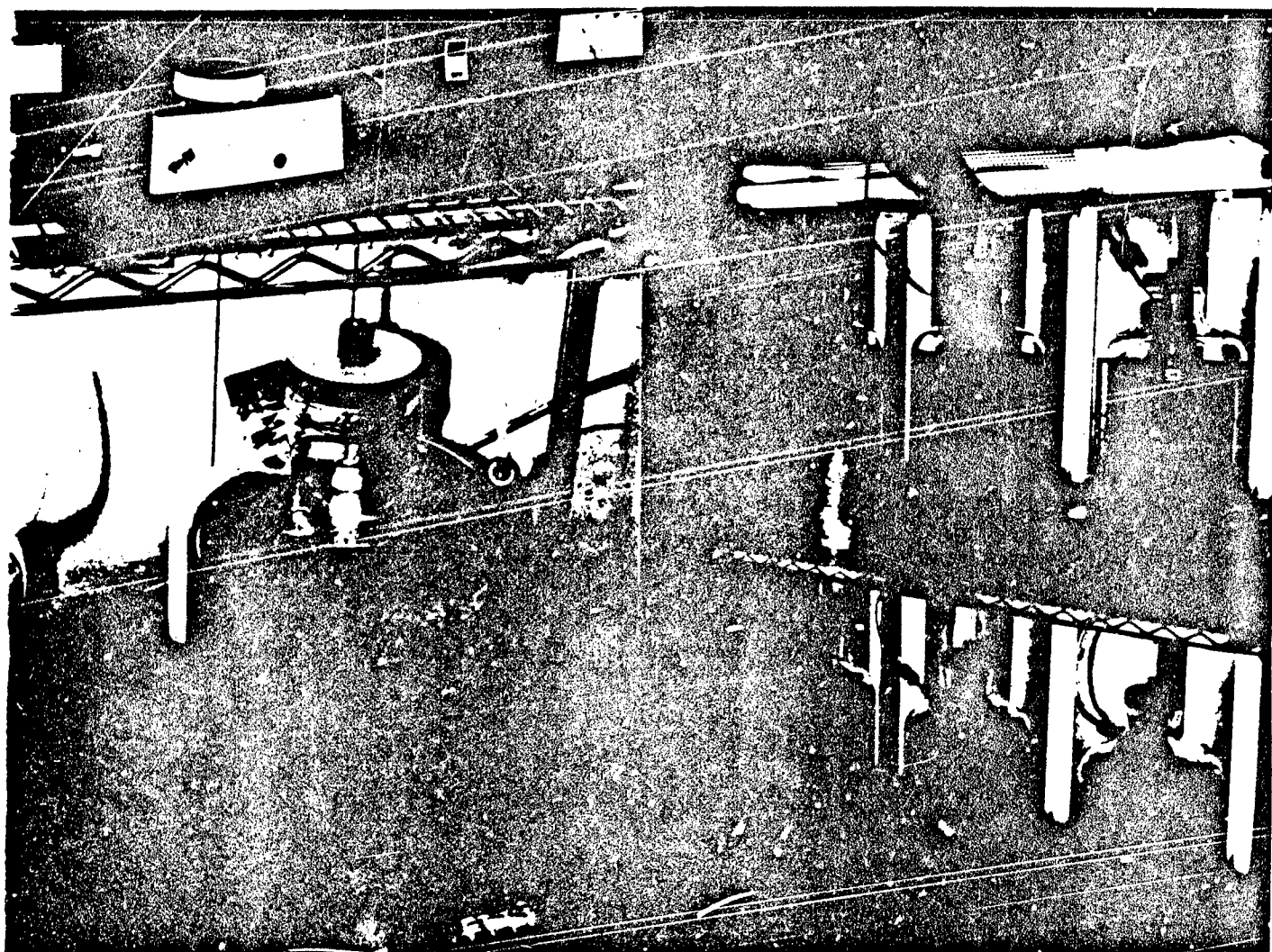


Figure 2. Harvesting equipment for microcarrier flasks.

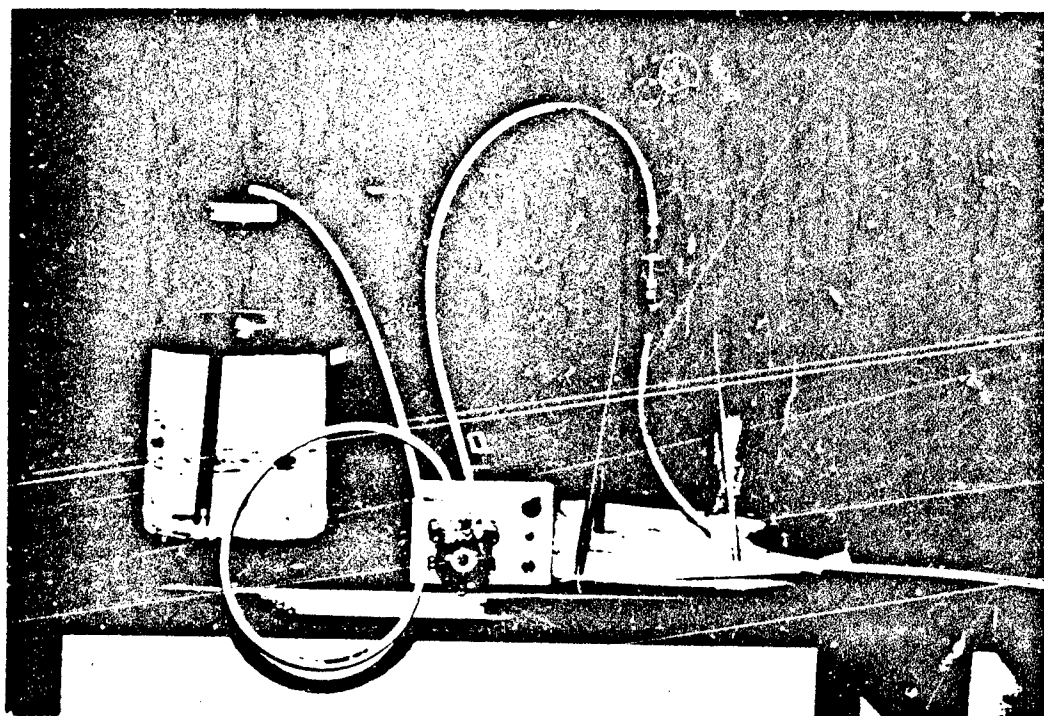
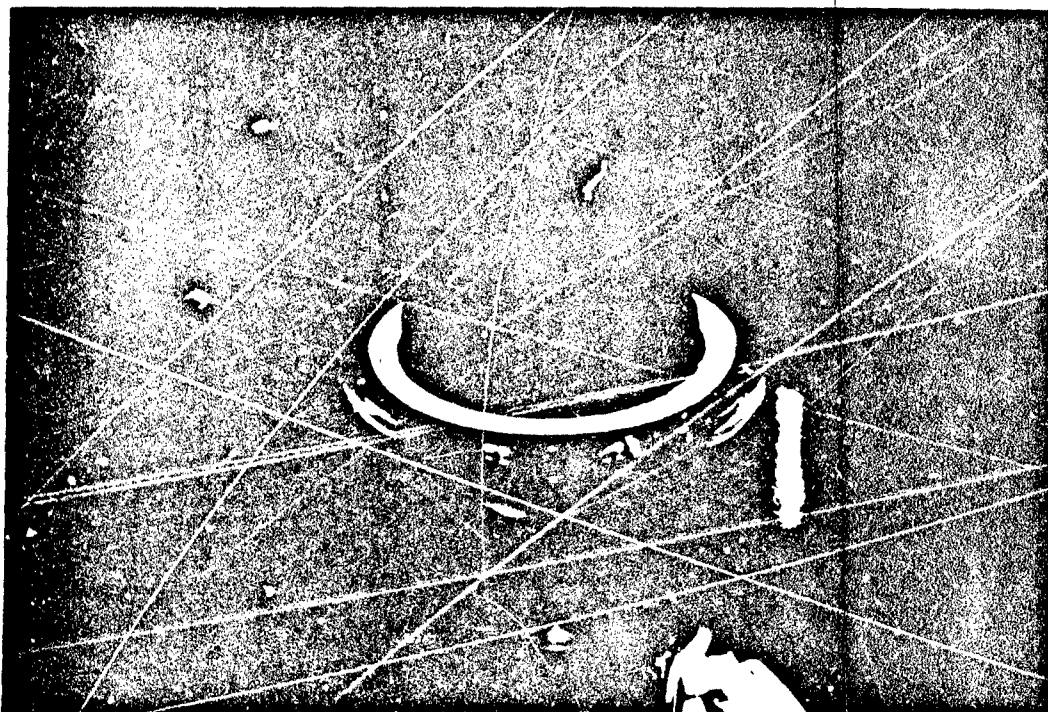


Figure 3. Cell cake of harvested G. toxicus.



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THE INTERACTION OF BREVETOXIN, CIGUATOXIN, AND MAITOTOXIN
WITH RAT BRAIN SYNAPTOSOMES

TERESA L. HERRING AND JAMES E. BALTHROP

SOUTHEAST FISHERIES CENTER
NATIONAL MARINE FISHERIES SERVICE
CHARLESTON, SOUTH CAROLINA 29412

INTRODUCTION

Several potent marine neurotoxins such as maitotoxin, ciguatoxin, saxitoxin, brevetoxin and tetrodotoxin are known to threaten human health (Table 1). Ciguatoxin poisoning results from eating predaceous subtropic or tropic finfish, such as grouper, red snapper, barracuda, sea bass and amberjack. These fish accumulate the toxin from their diet (Nukina et al., 1984) by feeding on smaller fish that have in turn fed on marine plankton carrying the toxin.

Table 1. Marine Neurotoxins

Toxin	Source	LD50/Kg mice *
Palytoxin	Palythoa mammosa	50-100 ng
Maitotoxin	Gambierdiscus toxicus	170 ng
Ciguatoxin	Gambierdiscus toxicus	450 ng
Tetrodotoxin	Tapes semidecussata	8-20 ug
Brevetoxin	Ptychodiscus brevis	250 ug

* Kaul and Daftari, 1986

Ciguatoxin and maitotoxin are the principal toxins responsible for ciguatera poisoning; the dinoflagellate, *Gambierdiscus toxicus*, is the primary producer of ciguatoxin and maitotoxin (Yasumoto et al., 1977). Since the cultured dinoflagellate has been shown to produce mostly maitotoxin and only trace amounts of ciguatoxin, an assay for purified ciguatoxin has been limited. However, culturing efforts in our laboratory have produced a viable dinoflagellate strain from Martinique from which maitotoxin and ciguatoxin have been isolated.

The mode of action of many of the marine neurotoxins involves binding to cell membrane ion receptors (Table 2). Saxitoxin and tetrodotoxin bind to a common receptor site (site 1) affecting sodium channels (Davio et al., 1984; Krueger et al., 1979). Ciguatoxin and brevetoxin have also been reported to act at a common binding site (site 5) on sodium channels (Lombet et al., 1987). Maitotoxin is reported to be a calcium channel activator. Evidence for specific maitotoxin-receptor binding is based on evidence such as calcium flux (Freedman et al., 1984, Takahashi et al., 1982) and calcium dependent contraction of smooth muscle (Ohizumi et al., 1983; Ohizumi and Yasumoto 1983 a,b; and Takahashi et al., 1983).

Table 2. Toxins and Sodium Channels *

Receptor Site	Ligand	Physiological Effect
1	Tetrodotoxin	Inhibit ion conductance
	Saxitoxin	
	Conotoxins	
2	Batrachotoxin	Persistent activation
	Veratrum alkaloids	
	Grayanotoxins	
3	α -scorpion toxins	Inhibit inactivation
	sea anemone toxins	
4	β -scorpion toxins	Shift activation
5	Brevetoxins	Shift activation and
	Ciguatoxin	Inhibit inactivation

* Baden 1989

The objectives of this study include:

- 1) demonstration of dinoflagellate toxin binding to synaptosome ion channels
- 2) investigation of the effects of maitotoxin on the binding of brevetoxin to synaptosome ion receptors
- 3) investigation of the interaction of ciguatoxin and brevetoxin with synaptosome ion receptors.

MATERIALS & METHODS

Cell Culture

A mass culture technique using a *G. toxicus* dinoflagellate strain obtained from Fort-de-France Bay near the Caribbean island of Martinique was used to produce ciguatoxin and maitotoxin. The clones were maintained at 27° under an illumination of 30-40 $\mu\text{E} \cdot \text{M}^{-2} \cdot \text{S}^{-1}$ and a 16:8 hours light:dark cycle without aeration. Cells are grown in an enriched seawater medium (K-medium) with seawater collected from Vero Beach, FL or Charleston, SC. The vitamin mixtures and enrichments for the media are prepared in concentrated stocks and sterilized. *G. toxicus* clones are harvested by filtration through 12 μM polycarbonate membrane filters and inoculated for mass culture into 12 liter glass carboy microcarrier flasks (Bellco). The cells are kept in suspension by a combination of magnetic stirring and aeration.

Toxin Extraction and Purification

We employed solvent partitioning, pre-column filtering, and HPLC chromatography to prepare a semi-purified extract of maitotoxin, the water soluble toxin produced by *G. toxicus*. Whole cells are sonicated, extracted in methanol, and subjected to ether/water and water/butanol solvent partitioning. The aqueous extract is then filtered through a C18 cartridge. The filtering procedure recovers approximately 95% of the toxin and removes approximately 23% of the total mass. The toxin is then separated from additional contaminants using a semi-preparative C18 HPLC column (Alltech Econosil 22 mm x 250 mm) with 70% methanol as the mobile phase (Figure 1).

Figure 1. Maitotoxin Preparation

Harvest Mass Cultured *G. toxicus*
Sonicate Cells
Extract in 80% Methanol
Filter With .2 Micron PTFE
Partition Between Ether/Water
Partition Between Butanol/Water
Prefilter With C18 Cartridge
HPLC C18 Reverse Phase 70% Methanol

The ciguatoxin, a lipid soluble compound, was isolated by extracting the *G. toxicus* cells with 80% methanol, followed by liquid-liquid solvent partitioning using ether/water and silicic acid column chromatography using chloroform/methanol (9:1) as the eluant (Figure 2).

Figure 2. Ciguatoxin Preparation

Harvest Mass Cultured *G. toxicus*

Scnicate Cells

Extract in 80% Methanol

Filter With .2 Micron PTFE

Partition Between Ether/Water

Silicic Acid Chromatography 9:1 Chloroform:Methanol

Synaptosome Preparation

Synaptosomes were prepared on the day of each experiment from a single rat brain (Harlan Sprague Dawley, Outbred rat HSD:(SD)BR, 300-400 g) using the technique described by Dodd et al. (1981) for the isolation of ion channel receptors (Figure 3). Cerebral cortices were homogenized in 0.32 M sucrose and centrifuged at low speed (2400 x g) in a Sorvall model RC5B centrifuge and SM 24 rotor. The supernatant was layered directly onto 1.2 M sucrose and

centrifuged at high speed (230,000 x g) in a Sorvall model RC70 ultracentrifuge and TH641 rotor. The interface was removed, layered onto 0.8 M sucrose and again centrifuged at high speed. The resulting pellet contained the synaptosomes with active ion binding sites (Figure 3). The protein content of the synaptosome preparation was determined using the Lowry protein assay with bovine serum albumin as a standard. A correction in absorbance was made for the HEPES present in the preparation.

Figure 3. Synaptosome Preparation

Homogenize Cerebral Cortices in 10 Volumes of 0.32 M Sucrose

Centrifuge at 2400 x g, 10 min

Layer Supernatant on 1.2 M Sucrose

Centrifuge at 230,000 x g, 15 min

Remove Interface and Dilute With .32 M Sucrose

Layer Diluted Interface on 0.8 M Sucrose

Centrifuge at 230,000 x g, 15 min

Suspend Synaptosome Pellet in Binding Medium

Binding Studies

Experiment 1

A 100 μ l aliquot of the synaptosome preparation containing 97 μ g total protein was incubated with increasing concentrations (2-20 nM) of [3 H]-Brevetoxin (PbTx-3), [42- 3 H(N)] (New England Nuclear). The binding medium (Poli et al., 1986) consisted of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/ml BSA and 0.02 % Emulphor-EL 620 (GAF) a non-ionic detergent.

Triplicate binding assays were performed in 1.5 ml Eppendorff microfuge tubes for each brevetoxin concentration (1 ml total volume, 1 hour, 4°). The binding reaction was stopped and the bound and free brevetoxin were separated by centrifuging for 2 minutes at 15,000 x g in an Eppendorff microfuge. Supernatant solutions were aspirated and the pellets were rinsed with 2 ml of a rinse medium consisting of 5 mM HEPES (pH 7.4), 163 mM choline chloride, 1.8 mM calcium chloride, 0.8 mM magnesium sulfate, and 1 mg/ml BSA. A 500 μ l aliquot of the supernatant (free brevetoxin) from each tube was transferred to liquid scintillation vials containing 15 ml of Econofluor (NEN) liquid scintillation cocktail. The pellets (bound brevetoxin) were transferred to liquid scintillation vials containing 15 ml of Econofluor. The [3 H] Brevetoxin was counted in a LKB 1211 scintillation counter.

Experiment 2

A 100 μ l aliquot of the synaptosome preparation containing 93 μ g total protein and 10 nM [3 H] Brevetoxin was incubated in triplicate with increasing concentrations of maitotoxin (0, .1, 1, 2, 10, and 20 μ M) assuming a molecular weight of 3425 and a toxicity of .13 μ g/kg as reported by Yasumoto et al. (1988). The incubation medium and procedures to isolate the bound and free brevetoxin were the same as in experiment 1.

Experiment 3

A 100 μ l aliquot of the synaptosome preparation containing 98 μ g total protein and 10 nM [3 H] Brevetoxin was incubated in triplicate with increasing concentrations of ciguatoxin (0, 2, 10, 20, 100 nM) assuming a molecular weight of 1112 and a toxicity of .45 μ g/kg as reported by Scheuer et al. (1967). The incubation medium and procedures to isolate the bound and free brevetoxin were the same as in experiment 1.

RESULTS AND DISCUSSION

Maitotoxin and ciguatoxin are the primary toxins involved in ciguatera toxicity. Many such neurotoxins are known to act specifically on various kinds of ion channels and neurotransmitter receptors (Catterall, 1980). The demonstration that maitotoxin and structurally known polyether compounds such as brevetoxins and okadaic acid can inhibit ciguatoxin antibody binding in toxic fish samples suggests that both maitotoxin and brevetoxin are closely related to ciguatoxin (Hokama et al., 1984).

An in vitro binding study, Experiment 1, using a rat brain synaptosome preparation demonstrated that brevetoxin binds with a high affinity and specificity. The synaptosomes showed a saturable binding of brevetoxin with increasing concentrations of toxin from 2-20 nM (Figure 4). Scatchard analysis of "total binding" resulted in a line with a slope of -0.3027 representing an apparent dissociation constant (K_d) of 3.30 nM with a binding maximum of 14.61 pmoles brevetoxin bound/mg synaptosome protein (Figure 5).

Poli et al. (1986) has reported from 7-12% for nonspecific binding at 4°. The microcentrifuge techniques used to separate bound and free toxin results in less nonspecific binding than would be obtained with a filtration assay. Correcting for "nonspecific binding", resulted in a line with a slope of -0.2988 and an apparent dissociation constant of 3.35 nM with a binding maximum of 13.25 pmoles toxin/mg of synaptosome protein.

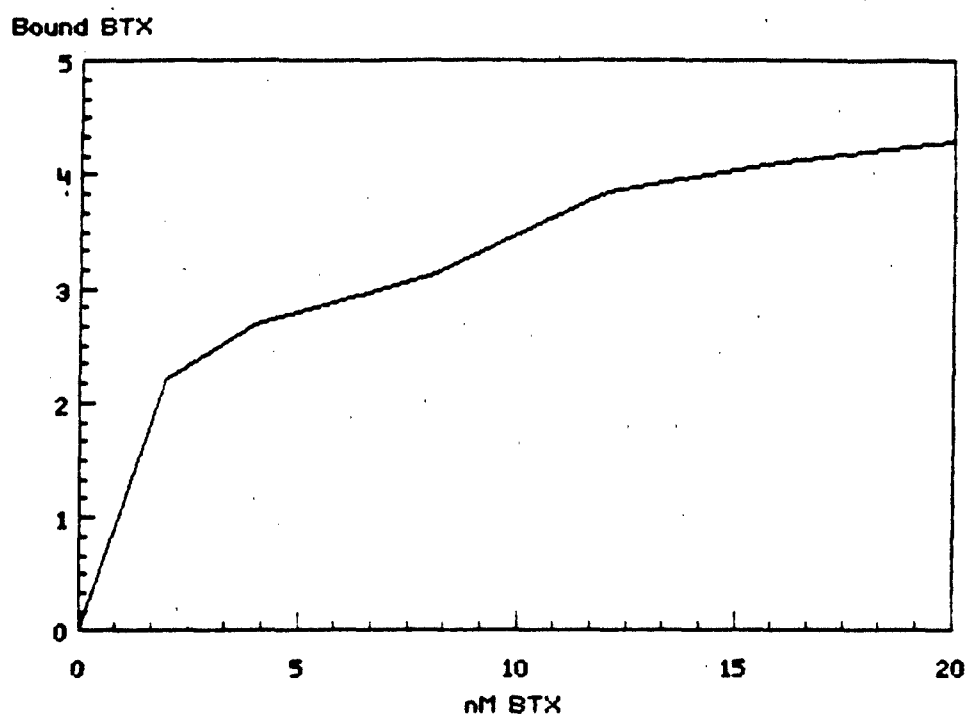


Figure 4. The Binding of Brevetoxin to Rat Brain Synaptosomes. Bound BTX represents tritiated brevetoxin $\text{dpm} \times 10^4$. Triplicate tubes containing 97 μg of rat brain synaptosomes were incubated for 1 hour at 4° with 2-20 nM brevetoxin.

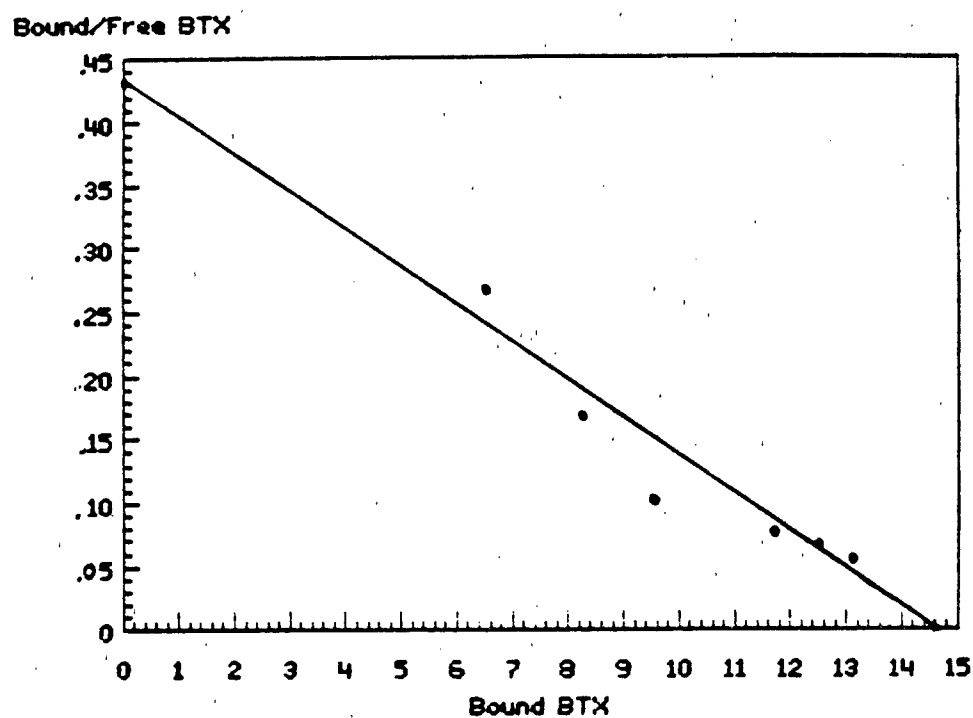


Figure 5. Scatchard Analysis of Total Binding. Bound BTX represents pmoles brevetoxin/mg protein. Rat brain synaptosomes (97ug total protein) were incubated in triplicate with 2-20 nM tritiated brevetoxin.

The calculated dissociation constant of 3.30-3.35 nM for brevetoxin and rat brain synaptosomes is in good agreement with the K_d of 2.9nM reported by Poli et al. (1986). Furthermore, it is also similar to those values reported by Wu et al. (1985) and Huang et al. (1984) for squid axons (1.7nM) and by Baden et al. (1984) for rat phrenic nerve hemi-diaphragm (5nM). Other marine toxins such as tetrodotoxin and saxitoxin have shown similar affinities (K_d) for synaptosomes (1.7-2.3 nM).

In a subsequent study, Experiment 2, the effect of maitotoxin on brevetoxin binding was determined. Incubation of synaptosomes with 0.1-20 μ M maitotoxin resulted in brevetoxin binding of 97-114% of control (Figure 6). These results indicate maitotoxin was unable to displace brevetoxin from synaptosomes. Brevetoxin has been shown to bind to a different sodium receptor site (site 5) than other toxins (Poli et al., 1986). Therefore, it appears that maitotoxin does not bind to the same unique brevetoxin receptor site.

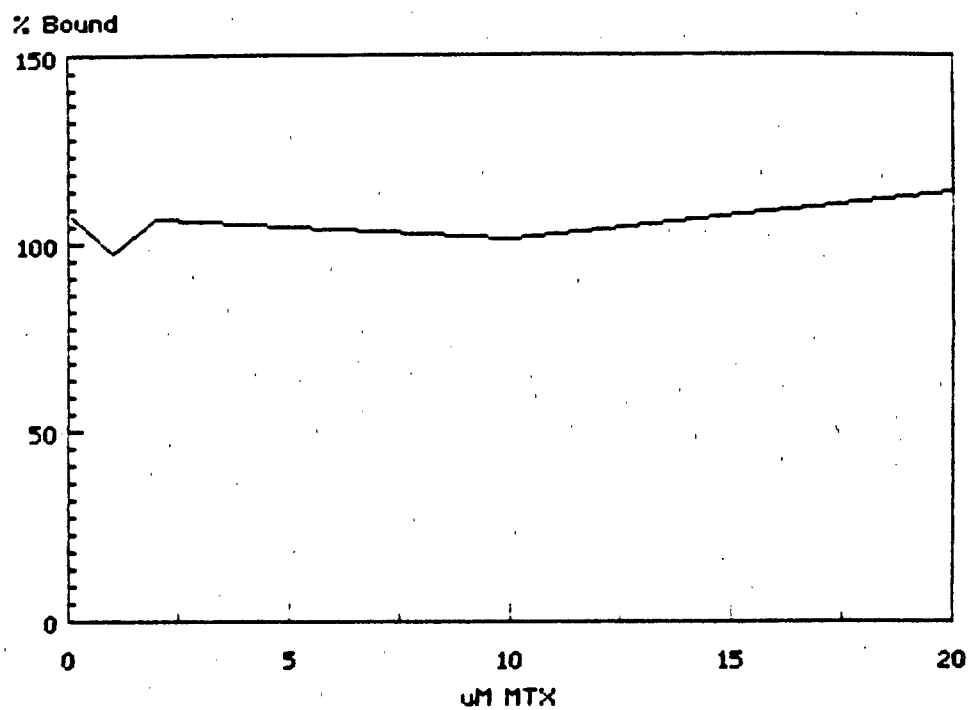


Figure 6. Effect of Maitotoxin(MTX) on Brevetoxin Binding to Synaptosomes. A synaptosome preparation containing 93 ug total protein and 10 nM brevetoxin was incubated in triplicate with .1 to 20 uM maitotoxin.

However, ciguatoxin has been reported to share a common and unique receptor site on the sodium channel with brevetoxin (Lombet et al., 1987). Furthermore, the cross-reactivity of brevetoxin antibodies to ciguatoxin implies a close structural and perhaps pharmacological relationship between brevetoxin and ciguatera toxins. The effect of ciguatoxin on brevetoxin binding to synaptosome receptors was determined in Experiment 3. Incubation of synaptosomes with 2-100 nM ciguatoxin resulted in a reduction in the binding of brevetoxin as the concentration of ciguatoxin was increased (Figure 7). At 20 nM ciguatoxin, brevetoxin binding was reduced to 65% of the control and at 100 nM ciguatoxin the brevetoxin was reduced to only 12% of the control.

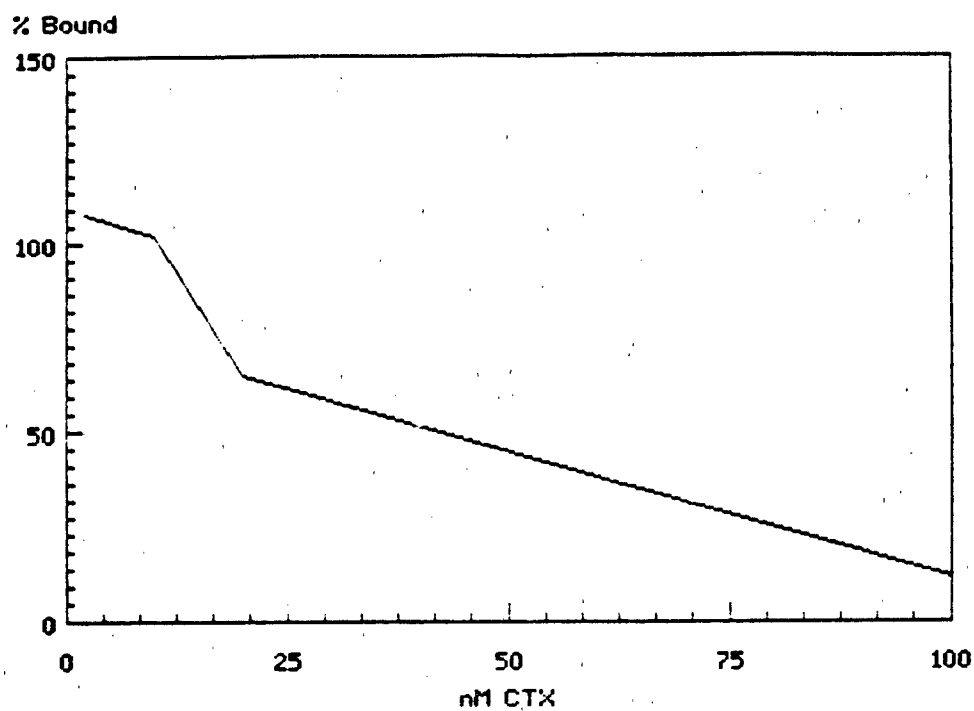


Figure 7. Effect of Ciguatoxin (CTX) on the Binding of Brevetoxin to Synaptosomes. Ciguatoxin (2-100 nM) was incubated in triplicate with 98 ug of rat brain synaptosome protein and 10 nM tritiated brevetoxin.

With the use of a rapid centrifugation technique to separate synaptosome bound toxin from free toxin following in vitro binding we have demonstrated that one of the ciguatera implicated toxins, maitotoxin, does not displace brevetoxin from its unique receptor and therefore must produce its toxic effects with a mode of action different from that of brevetoxin. But, our results demonstrate that ciguatoxin does affect the binding of brevetoxin to synaptosomes. Therefore, our results lend further support to the conclusion that maitotoxin and ciguatoxin possess different pharmacological modes of action.

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