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Biological Activity of the Functional Epitope of Ciguatoxin Fragment AB on the Neuroblastoma Sodium Channel in Tissue Culture

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It is well established that the targeted receptor for ciguatoxin (CTX) in mammalian tissues is the sodium channel, affecting the influx of sodium into cells and altering the action potential and function of the cell. Since the syntheses of fragments of CTX has become available, our focus has been on the receptor functions of the west sphere AB and east sphere JKLM fragments using the neuroblastoma cell assay, guinea pig atrium assay, and the membrane immunobead assay (MIA). The data presented here suggest that the west sphere AB of the ciguatoxin molecule is the active portion and is responsible for the activation of the sodium channels. J. Clin. Lab. Anal. 20:126–132, 2006. © 2006 Wiley-Liss, Inc.

Key words: ciguatoxin; ciguatera; marine toxins; sodium channel

INTRODUCTION

It is well established that ciguatoxin (CTX), a marine neurotoxin, acts on the sodium channel of various cells and increases action potential by influx of sodium into the cells via the sodium channel (1). This has been demonstrated in guinea pig atrial tissue in a pharmacological assay in vitro (2) and by the effect of CTX on mice neuroblastoma (NB) cells in tissue culture by measuring the dehydrogenase activity of the NB cells (3). Through analyses with ciguatoxin synthetic fragments of action on the sodium channel using the guinea pig atrium and neuroblastoma cell assays, evidence is presented to show that the major epitope in CTX associated with the activation of the sodium channel is the west sphere, AB fragment. Experiments with the AB fragment (shown in Fig. 1a) of CTX (shown in Fig. 1b), and sodium channel receptor proteins extracted from epithelial membrane of pig intestine, which acts as an inhibitor to the west sphere of CTX, the AB portion of CTX is shown to be associated with the activation of the sodium channel. Conversely, the east sphere of CTX, fragment JKLM (Fig. 1c), showed no effects in the activation of the sodium channel in guinea pig atrial tissue and NB cells in tissue culture. Conclusions from the data reported in this study are that the active site of CTX on the sodium channel is the west sphere of the molecule—the AB region.

MATERIALS AND METHODS

Ciguatoxin Extraction

The organic solvent extraction and partial purification of CTX from fish flesh and viscera were performed as previously reported (4). The partial purification steps include separation in silica gel chromatography and further partitioning into four major fractions. Fraction 1 was partitioned in 100% chloroform; fraction 2 in 10% methanol/90% chloroform; fraction 3 in 50% methanol/50% chloroform; and fraction 4 in 100%

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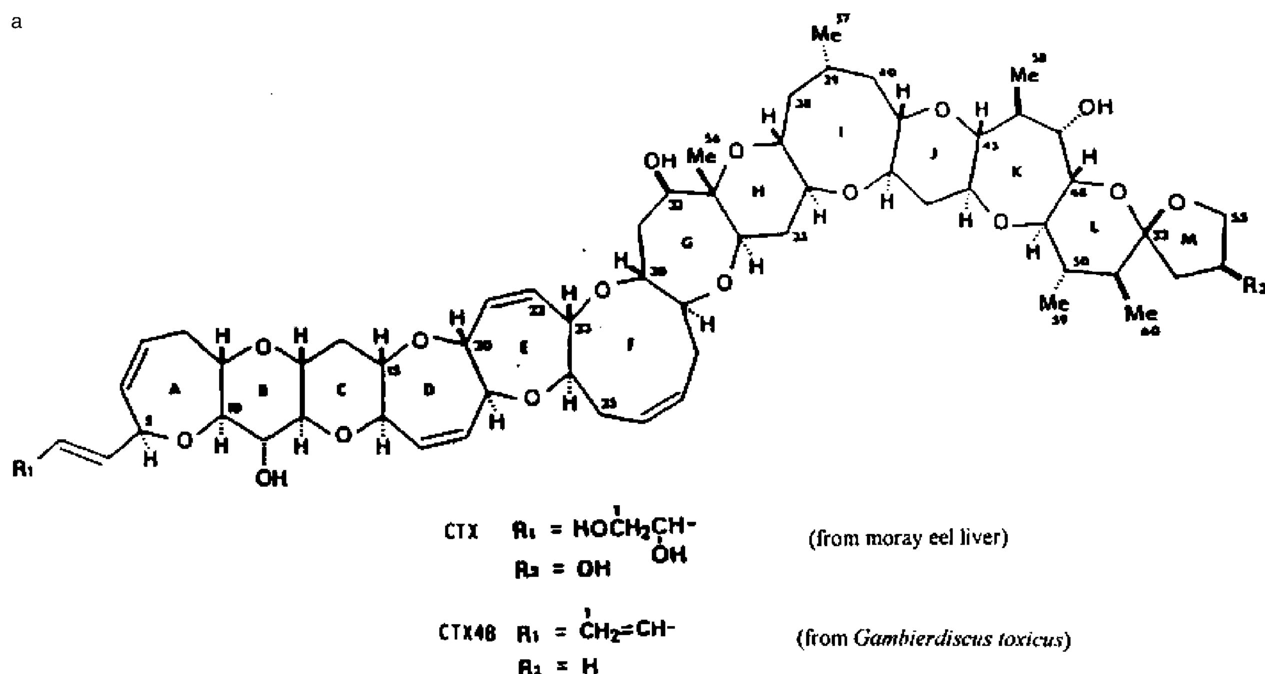
a



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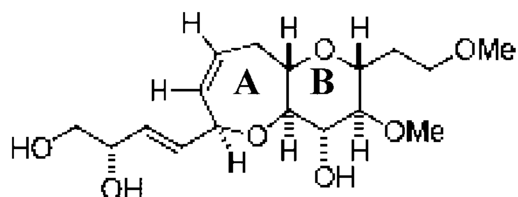
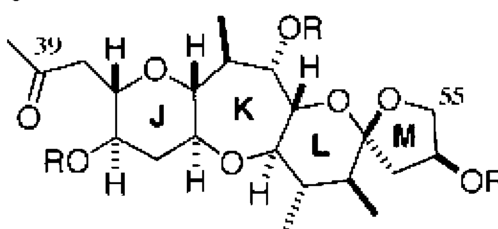


AB-ring methyl ether

c



JKLM-rings

Fig. 1. Ciguatoxin molecule (**a**) showing the AB fragment (**b**) and the JKLM region (**c**).

methanol. A mixture of fractions 2 and 3 was further purified chromatographically on the C18 solid phase column and eluted with ethyl acetate and hexane (5). Both fractions were active in the membrane immunobead assay (MIA) test and examined in the NB assay. The MIA immunological inhibition test of CTX fragments with partially purified CTX extracted from fish clinically implicated in ciguatera outbreaks by the Hawaii Department of Health (DOH-CTX) demonstrated that the monoclonal antibody to CTX (MAb-CTX) reacted with the JKLM fragment but not with the AB fragment (6,7).

Guinea Pig Atrial Assay for Sodium Channel Activity

Inotropic effects of partially purified DOH-CTX and CTX synthetic fragments AB and JKLM were determined by the method developed by Miyahara et al. (1) and Hokama and Miyahara (2). Atrial tissue dissected from adult male Hartley guinea pigs was suspended in a physiobath containing 25 mL Krebs bicarbonate solution, pH 7.4 maintained at a temperature of 30°C and aerated with 95% air and 5% CO_2. After 15 to 30 min and after an equilibrium of the heartbeat had

been established, partially purified DOH-CTX (10 mg/bath), pure maitotoxin (MTX) (50 ng/bath), palytoxin (PTX) (32 ng/bath), and brevetoxin (pbTx-3; Isomer of Brevetoxin) (1 μg/bath) were added to the physiobath to obtain inotropic response curves. At 10 min after the initial inotropic response, solutions of various inhibitors were added to the physiobath using separate segments of atrium for each toxin. These in included 0.15 μL of the following at 1×10^{-7} M: tetradotoxin (TTX), verapamil, propanol, and phentolamine. The physiobath was rinsed with Krebs solution to complete the assay.

Neuroblastoma Cell Assay

The NB cell assay was modified after the method outlined by Manger et al. (3) and preparation of toxins at various concentrations was performed as previously described (8). Mouse neuroblastoma cells (neuro-2a, CCL131, ATCC) were grown in RPMI 1640 supplemented with 10% Gibco fetal bovine serum (Invitrogen, Carlsbad, CA) 2 mM glutamine, 1 mM sodium pyruvate, 50 μg/mL streptomycin, and 50 units/mL penicillin. Cultures were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere.

NB cells from stock culture were seeded in complete media into 96-well Costar microtiter plates (Cole-Parmer, Vernon Hills, IL) at a concentration of 5×10^5 cells/ml in 100 μL volumes per well and maintained for 24 hr at 37°C. After 24 hr, 10 μL each of 10 mM of ouabain, 1 mM of veratridine, and dilutions of purified toxins, synthetic fragments, or toxic extracts from ciguateric fish were added to replicate wells. Control wells of ouabain/veratridine and untreated controls received added incomplete RPMI media to make up for volume

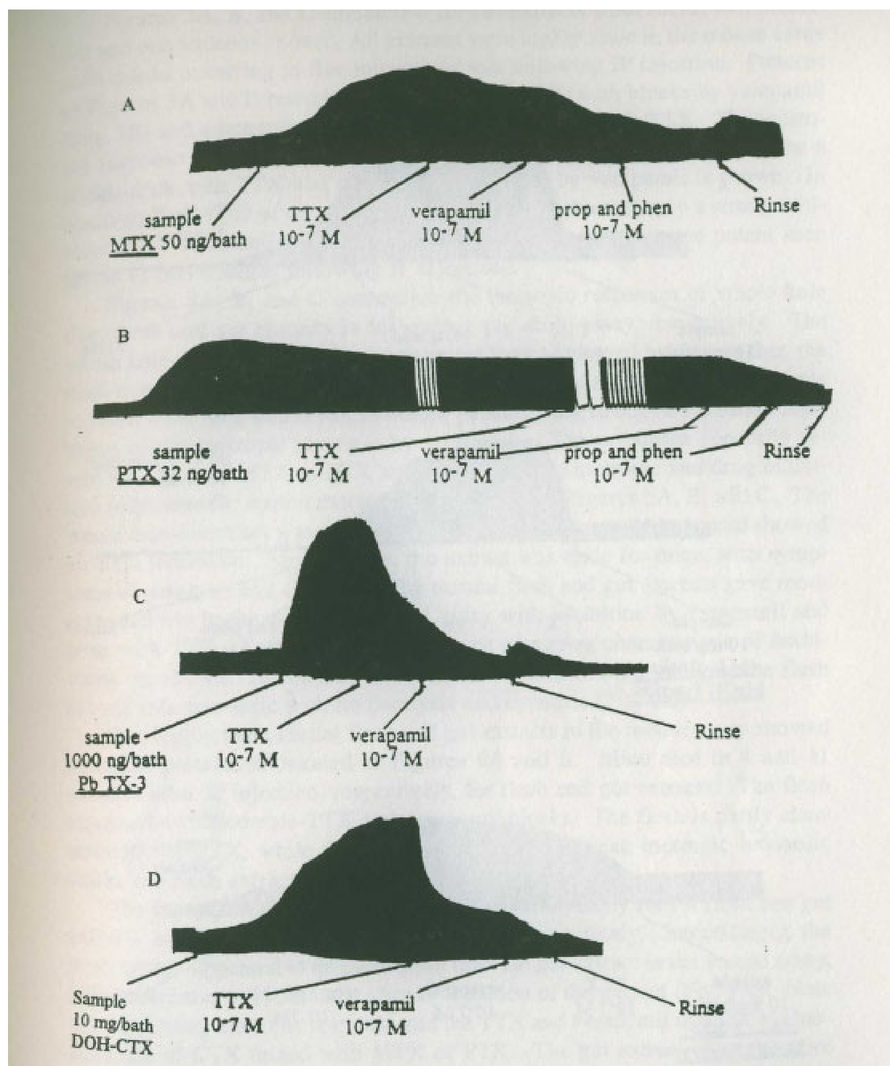


Fig. 2. Typical inotropic curves for the guinea pig atrial assay are shown for maitotoxin (MTX) at 50 ng (**A**), palytoxin (PTX) at 32 ng (**B**), brevetoxin –3 (BTX) at 1 μg (**C**) , and crude partially purified DOH-ciguatoxin (CTX) at 10 mg (**D**).

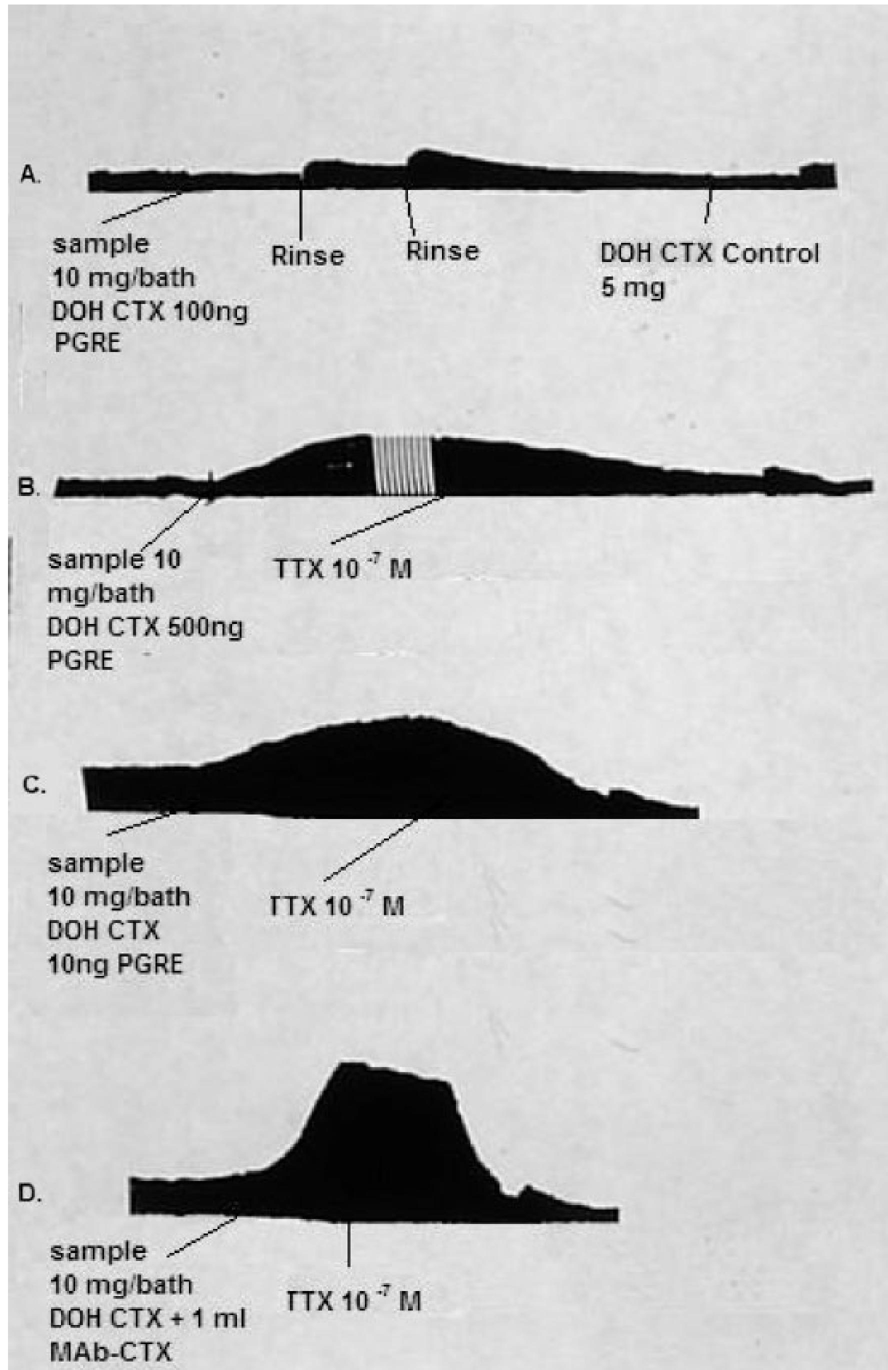


Fig. 3. Inhibition of the inotropic response (the guinea pig atrium assay) by pig gut extract containing soluble sodium channel receptors. (**A**) Standard DOH CTX with 100 ng of PGRE (Pig Gut Receptor Extract) show almost complete inhibition. (**B**) Half of the concentration of PGRE (50 ng) shows partial inhibition, but complete inhibition upon addition of TTX. (**C**) A tenth of the initial concentration of PGRE (10 ng) shows less inhibition, but complete inhibition when TTX added. (**D**) Control with MA6-CTX and no PGRE shows slight inhibition, but complete inhibition upon addition of TTX.

differences. The plate was then incubated at 37°C for 16–20 hr for the detection of sodium channel inhibition. End-point assessment with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoleum), was carried out as previously described (3,8,9). The color end-point was read at a wavelength of 495 nm with a Titertek microplate reader (BioTek, Winooski, VT). Reagents were obtained from Sigma Chemical (St. Louis, MO)

unless otherwise indicated. Each well was carried out in quadruplicate and the mean and standard deviation (SD) were recorded, as shown in Figures 4, 5 and 6.

Extraction of Sodium Channel Receptor From Pig Gut Epithelial Cell Layer

Fresh, cleaned pig gut was obtained from a commercial food market. It was dissected longitudinally to expose the epithelial layer. The latter layer was scraped with a scalpel and the free tissue layer was soaked in distilled water and mixed with a magnetic stirrer for 1 hr at room temperature. The suspension was centrifuged at 2,500 rpm for 30 min. The clear supernatant containing the soluble sodium channel receptor was decanted and used previously in the MIA procedure to detect CTX (10). In addition, three concentrations of the pig gut extract containing the sodium channel receptor, 10 μg/protein, 50 μg/protein, and 100 μg/protein, respectively, were added to the physiobath containing the guinea pig atrial tissues suspended in Krebs bicarbonate solution for inotropic analysis.

RESULTS

Guinea Pig Atrial Assay

Typical inotropic curves for the guinea pig atrial assay are shown in Fig. 2A–D for four marine toxins: MTX at 50 ng, PTX at 32 ng, pbtx-3 at 1 μg, and crude partially purified DOH-CTX at 10 mg. MTX reactivity in Fig. 2A shows a sloping ascending curve with slight block with 0.15 μL of 10^{-7} M TTX, moderate block with 10^{-7} M verapamil (0.15 μL), and slight block with a 10^{-7} M propanol and phentolamine mixture (0.15 μL); the mixture serves as an adrenergic blocker. The pure PTX response shows a moderated ascending slope with a long descending curve, showing inhibition with 10^{-7} M verapamil (0.15 μL) and no inhibition with 10^{-7} M TTX (0.15 μL) and the 10^{-7} M adrenergic blocker mixture. Brevetoxin shows a strong rapid ascending curve with a strong block with 10^{-7} M TTX (0.15 μL) but none with 10^{-7} M verapamil (0.15 μl), while the crude DOH-CTX shows an initial moderate ascending slope that was strongly inhibited by 10^{-7} M TTX (0.15 μL) and was moderately blocked by verapamil. Figure 2C and D show characteristic patterns of sodium channel potentiation action drugs readily inhibited by

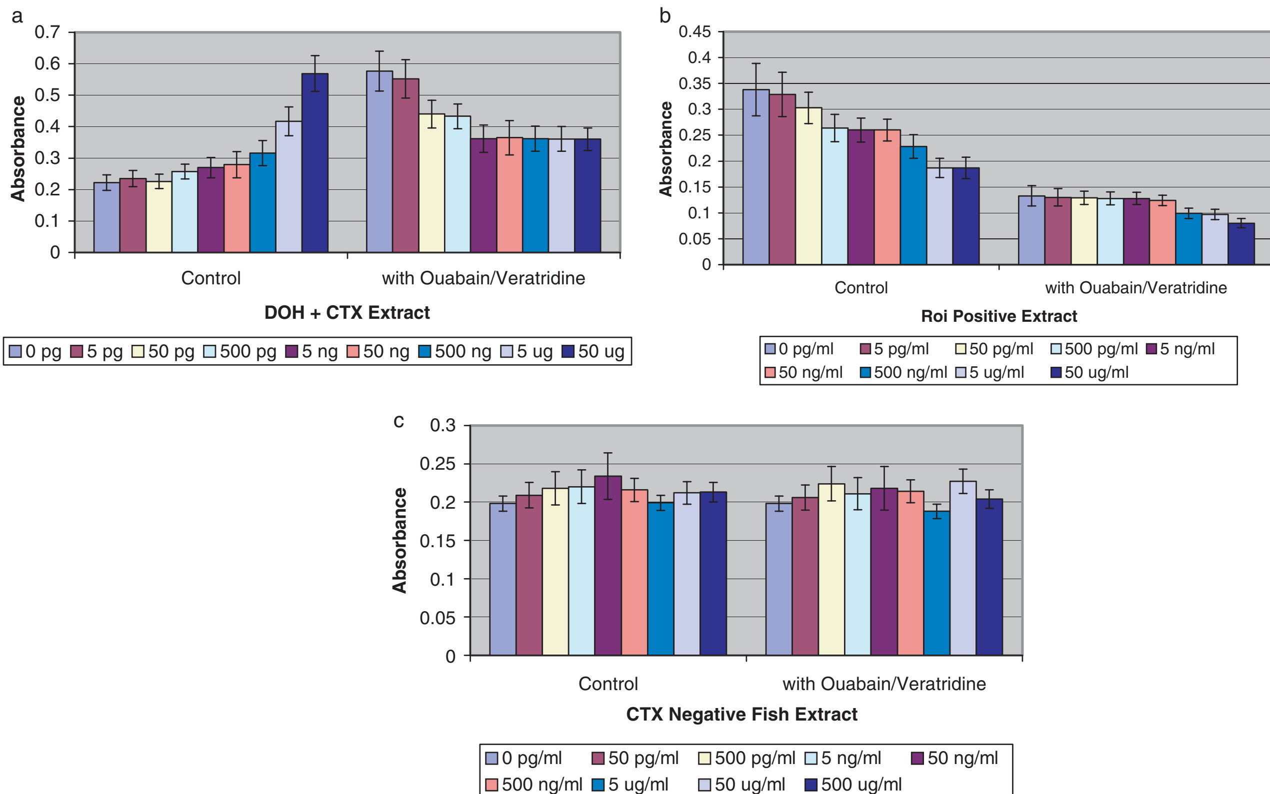


Fig. 4. Neuroblastoma cell assay of the effect of partially purified DOH-CTX (**a**), Roi (Hawaiian name for cephalopholic argus, a.k.a. Blue spotted grouper) positive extract (**b**), and a CTX negative fish extract (**c**) on the sodium channel.

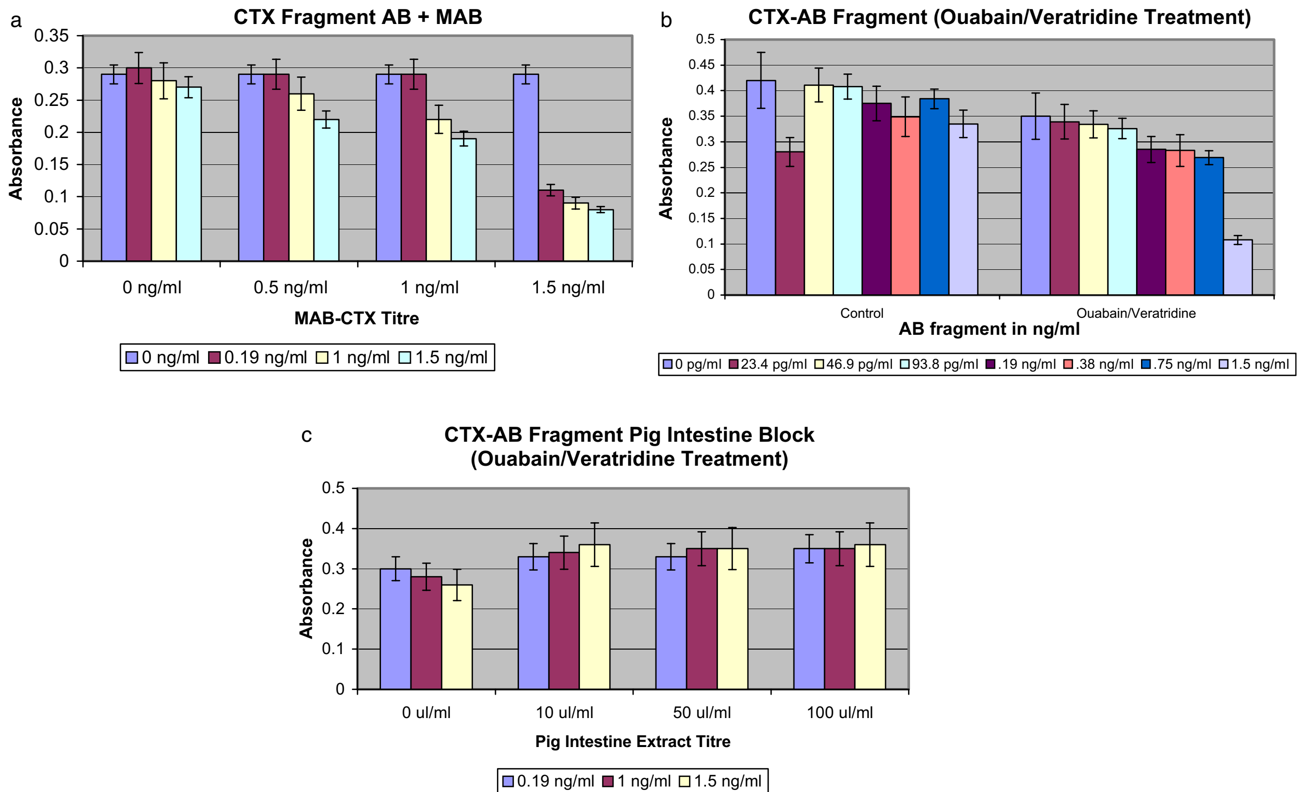


Fig. 5. The effect of CTX west sphere fragment AB on the sodium channel using the neuroblastoma cell assay. Fragment AB alone (**a**), fragment AB+Mab-CTX at different concentrations, (**a**) CTX-AB fragment (control) showing no inhibition of AB fragment (**b**), and fragment AB with pig gut receptor extract titer (pig intestine block)+ouabain/veratridine treatment showing complete inhibition by Pig Gut extract (**c**).

TTX or paralytic shellfish poisoning (PSP) implicated marine toxins acting on domain II of sodium channels.

MTX and PTX have greater in toxicity and tend to be destructive in the lysis of erythrocyte and muscle tissues, causing severe tissue destruction, as suggested by electron micrograph pathological studies (11).

Using the partially purified DOH-CTX, sodium channel activity was analyzed using competitive inhibition with the guinea pig atrial assay in the presence and absence of MAb-CTX and pig gut sodium channel receptors. Studies have shown that the JKLM ring of the CTX molecule represents the specific region that reacts immunologically with the MAb-CTX, confirmed by the lack of an immunological reaction with the AB ring fragments, which show no reaction with MAb-CTX using the MIA test (10).

Figure 3 shows the inhibition of the inotropic response of the guinea pig atrium assay by pig gut extract containing soluble pig gut sodium channel receptors extract (PGSCRE). MAb-CTX in high concentrations (1 mL) added to the guinea pig atrium assay showed no inhibition of the inotropic effect (Fig. 6), while three graded concentrations of the pig epithelial extracts (10, 50, and 100 ng) added to a constant dose of DOH CTX at 10 mg showed a dose response inhibitor of the inotropic effect. This is presented in Fig. 3A–D. The TTX effect also appeared to be nullified by the pig gut sodium channel epithelial extract (PGSCRE).

Neuroblastoma cell assay

The effects of DOH-CTX fish extract on the NB cells are shown in Fig. 4a (bar graph with mean $\pm$ SD). The control, various concentrations of the extract, and the NB cells show a stimulation effect in the cell proliferation at 50 μg. However, in the presence of ouabain and veratridine with various concentrations of DOH-CTX, a typical dose–response characteristic of suppression of NB cell proliferation is shown, leveling off at the 5 ng/mL dose and greater. Figure 4b shows an NB cell assay for an extract of a specific positive CTX fish extract showing the typical CTX effect. For comparison, typical NB assay results using CTX-negative mullet fish tissue extract are shown in Fig. 4c.

Figure 5a shows the inhibition of the NB cell proliferation in the presence of ouabain and veratridine when treated with varying concentrations of the synthetic AB fragment of CTX, with inhibition showing

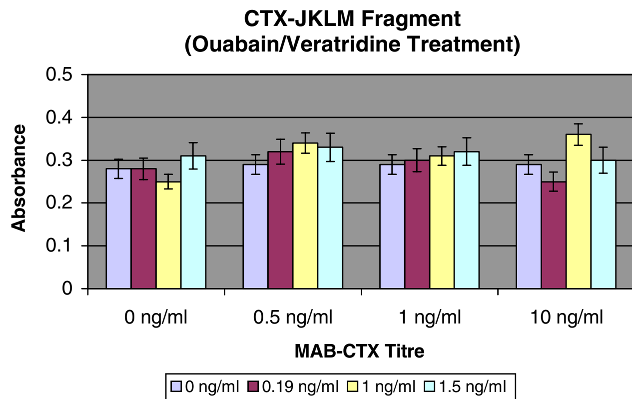


Fig. 6. The effect of CTX east sphere fragment JKLM on the sodium channel using the neuroblastoma cell assay and Mab-CTX+ ouabain/veratridine treatment.

greatest effects at the 1.5 ng/mL dose. The addition of MAb-CTX at 10 ng/mL did not neutralize the action of the CTX AB fragment suppression as shown in Fig. 5b. A total of 100 μg/well of pig extract added to three concentrations of synthetic CTX AB fragment (west sphere) completely antagonized or neutralized the effect of fragment AB suppression of the NB cells, as shown in Fig. 5c when compared to Fig. 5a. Figure 6 demonstrates that the addition of CTX synthetic JKLM fragment at concentration of 0, 0.19, 1.0, and 1.5 ng per well in the presence of ouabain and veratridine had no effect on the NB cells. Similarly, the addition of pig gut extract or MAb-CTX shows no effect (data not shown).

DISCUSSION

Data presented in the guinea pig atrial and NB cell assays strongly support the contention that ciguatoxin acts via the AB rings of the west sphere on sodium channels of the NB cells. Presumably, the action of the west sphere of CTX acts on the sodium channels of nerve, cardiac, and muscle cells via the sodium channels of the cells. It is interesting to note that the MAb-CTX, which binds to the east sphere of CTX (JKLM rings), shows no inhibition of the sodium channel effect, though it is useful in detection of whole ciguatoxin by the MIA procedure from ciguateric fish tissues (6). We concluded that the west sphere AB rings are associated with the important sodium channel activation and that the east sphere JKLM rings are the antigenic epitope for the MAb-CTX used in the MIA test for identifying ciguateric fish. Availability of polyvalent chicken antibody to the west sphere (ABCD rings) will make it possible to reexamine the NB cell analysis in future sodium channel studies.

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