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Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel

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Binding studies indicate that ciguatoxin and brevetoxin allosterically enhance in a very similar way the binding of [^{3}H]batrachotoxinin A 20-α-benzoate to the neuronal Na^+ channel protein. Moreovr ciguatoxin competitively inhibits the binding of [^{3}H]brevetoxin-3 to rat brain membranes. The affinity of ciguatoxin for the Na^+ channel is at least 20–50-times higher than that of brevetoxin. These results indicate that ciguatoxin and brevetoxins act at the same binding site on the sodium channel.

Ciguatoxin; Na channel, Ciguatera; Brevetoxin; Receptor; Pyrethroid

1. INTRODUCTION

Ciguatoxin is the principal toxin involved in ciguatera. Ciguatera is a type of ichthyosarcotoxism caused by the ingestion of toxic individuals of many species of fishes associated with coral reefs. Ciguatera syndromes typically include both gastrointestinal and neurological symptoms [1]. Ciguatoxin is biosynthesized by the benthic dinoflagellate *Gambierdiscus toxicus* which adheres to dead coral surfaces and bottom-associated algae [2]. CgTx, one of the most toxic compounds known, has a molecular mass of 1112 Da and is of polycyclic ethereal nature [3] as also reported for PbTxs that are produced by the dinoflagellate *Ptychodiscus brevis* [4]. PbTxs cause massive fish fatality, neurotoxic shellfish poisoning and contact irritation of the human skin [5]. The aerosol of the toxin induces in man such symptoms as non-productive cough, shortness of breath, tearing of the eyes, a burning sensation of the conjunctiva, rhinorrhea and sneezing and may cause wheezing in persons predisposed to asthma [6]. While the exact structure of PbTxs is well known [4], the structure of CgTx is still unknown. The independent analysis of the mode of action of these two types of marine toxins led to the conclusion that both CgTx and PbTxs act on the voltage-dependent Na^+ channel [7–11]. This paper shows that CgTx and PbTxs act at the same binding site on the Na^+ channel protein.

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Abbreviations: As_2, *Anemonia sulcata* toxin 2; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20-α-benzoate; CgTx, ciguatoxin; PbTx, brevetoxin (*Ptychodiscus brevis* toxin); TTX, tetrodotoxin

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]BTX-B and NaB^3H_4 were obtained from Dupont de Nemours-NEN (Paris, France). TTX was from Sankyo (Tokyo, Japan) and veratridine from Sigma. The pyrethroid RU 39568 was kindly provided by Dr M. Roche (Procida-Roussel-Uclaf, Marseilles, France). As_2 was purified according to Schweitz et al. [12]. CgTx was prepared as described [7]. [^{3}H]PbTx-3 was prepared from PbTx-2 and purified as described [13,14]. The labeled toxin had a specific radioactivity of 0.65 Ci/mmol.

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2.2. *Binding assays*

Binding of [^{3}H]BTX-B was carried out at 22°C using rat brain membranes as described [15]. Binding was measured in the absence or presence of 40 μM As_2. The effects of increasing concentrations of PbTx-2 or CgTx on [^{3}H]BTX-B binding were measured by the rapid filtration method on GF/C glass fiber filters. The non-specific binding component was measured in the presence of 100 μM veratridine.

Binding of [^{3}H]PbTx-3 was measured using the same rapid filtration technique and the same buffer as for [^{3}H]BTX-B binding. A large decrease of non-specific binding to the filters was obtained by pre-treating them with 0.1% polyethyleneimine and by using polyethylene tubes with low protein adsorption capacity. In inhibition experiments of the binding of 25 nM [^{3}H]PbTx-3 by increasing concentrations of PbTx-2 or CgTx the incubation time was of 2 h at 4°C. Final measurements were made by filtration of two aliquots of 200 μl of an incubation medium (500 μl) containing 130 mM *N*-methylglucamine, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mg/ml BSA, 50 mM Hepes-Tris buffer, pH 7.4, and 300–400 μg of rat brain membranes. Radioactivity was measured using Biofluor (NEN) on a Packard Tricarb 3350 spectrophotometer. K_i values for CgTx and PbTx-2 could be obtained using the relationship $K_i = K_{0.5}/1 + [L/K_d]$ where $K_{0.5}$ was the inhibitor concentration that decreased the specific binding of [^{3}H]PbTx-3 by 50%, L was the concentration of [^{3}H]PbTx-3 used in the experiment, and K_d was the equilibrium dissociation constant of [^{3}H]PbTx-3 obtained with the same material [14]. In direct binding experiments increasing concentrations of [^{3}H]PbTx-3 were used to saturate the binding sites of the toxin in the same conditions of incubation as above in the presence or absence of 0.2 ng/ml of CgTx. Dissociation kinetics of the [^{3}H]PbTx-3-receptor complex were followed by diluting the incubation medium 10-fold after 2 h of incubation at 4°C with 25 nM [^{3}H]PbTx-3. The dilution was monitored in the absence or presence of the same concentration of 0.2 ng/ml of CgTx.

3. RESULTS AND DISCUSSION

Both PbTxs [8] and CgTx [7] are effectors of the voltage-dependent Na^+ channel. Electrophysiological properties of their action seem to be very similar [7,10] and both toxins stimulate $^{22}Na^+$ influx through TTx-sensitive Na^+ channels in excitable cells in culture [7,8]. Moreover, both toxins have been claimed [7,8] to have receptor sites distinct from those previously identified for the large number of other toxins that are also known to be specific for the Na^+ channel (table 1). Therefore the question naturally arises to know whether the two toxins can be active on the same receptor site as previously observed for other toxin families including TTx and saxitoxin [16] (table 1) or veratridine, aconitine, grayanotoxins and batrachotoxin [17] (table 1).

The properties of the PbTx receptor have recently been identified using [^{3}H]PbTx-3 [14]. Moreover PbTx binding to its receptor is known to allosterically alter the binding of [^{3}H]BTX-B in the direction of an enhancement of the binding activity because of a PbTx-induced increase of the affinity of [^{3}H]BTX-B at its own binding site [8].

Results presented in fig.1 show that both CgTx and PbTx enhance [^{3}H]BTX-B binding to brain membranes (fig.1). In the absence of As_2, CgTx

Table 1

Neurotoxin receptor sites associated with the sodium channel

Receptor site	Ligand	Physiological effect
1	tetrodotoxin saxitoxin	block transport
2	batrachotoxin veratridine aconitine grayanotoxin	produce persistent activation
3	α-scorpion toxins sea anemone toxins	inhibit inactivation enhance persistent activation
4	β-scorpion toxins	repetitive firing shift activation
5	brevetoxins ciguatoxin	repetitive firing enhance persistent activation
6	pyrethroids DDT	repetitive firing enhance persistent activation

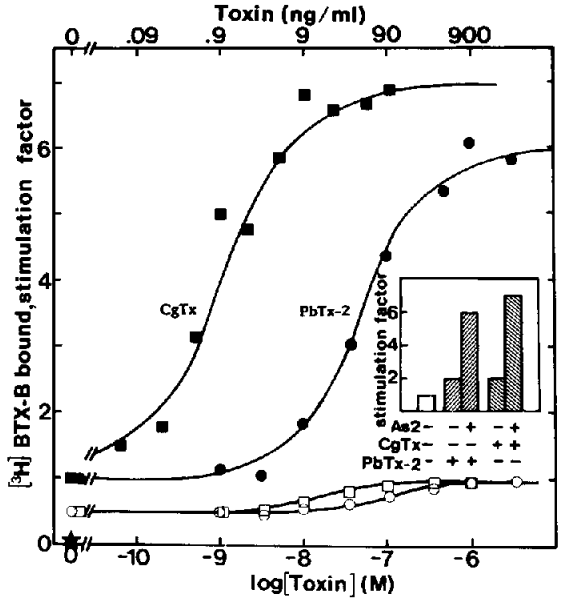

Fig.1. Enhancement of [^{3}H]BTX-B binding to rat brain membrane by CgTx and PbTx-2. (Inset) Comparison of the effect of As_2, CgTx and PbTx-2 on the binding of 2 nM of [^{3}H]BTX-B to rat brain membranes. (Main panel) [^{3}H]BTX-B (2 nM) was incubated with rat brain membranes (300 μg/ml) in the absence (□ ,○) or in the presence (■ ,●) of 40 μM As_2 with increasing concentration of CgTx (■ , □) or PbTx-2 (●,○). After 1 h of incubation at 22°C, two aliquots were filtered through GF/C filters and radioactivity measured. Non-specific binding was measured in the presence of 100 μM veratridine (*).

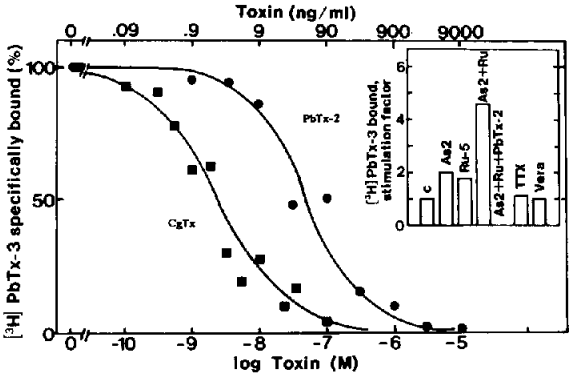

Fig.2. Effect of Na^+ channel neurotoxins on the binding of [^{3}H]PbTx-3 to rat brain membranes. (Inset) The binding of 25 nM [^{3}H]PbTx-3 in the presence of 40 μM As_2, 10 μM RU 39568, 40 μM As_2 + 10 μM RU 39568, 40 μM As_2 + 10 μM RU 39568 + 10 μM PbTx-2, 1 μM TTX or 100 μM veratridine were compared and the efficiency of these drugs to modify this binding was measured by radioactivity bound relative to control experiment. (Main panel) Inhibition of the binding of [^{3}H]PbTx-3 by CgTx and PbTx-2. In the conditions of maximal stimulation of the [^{3}H]PbTx-3 binding (40 μM As_2 + 10 μM RU 39568), CgTx (■) and PbTx-2 (●) inhibit specific binding of [^{3}H]PbTx-3 with increasing concentration of these toxins. After 2 h at 4°C of incubation, two aliquots were filtered through GF/C filters and radioactivity measured.

and PbTx-2 produced a 2-fold enhancement of the binding of [^{3}H]BTX-B as also observed for As_2 alone (control point). In the presence of 40 μM As_2 the enhancement was higher and reached values of 6- and 7-fold for PbTx-2 and CgTx, respectively (inset fig.1). Dose-response curves for the enhancing effect of PbTx-2 and CgTx on [^{3}H]BTX-B binding gave $K_{0.5}$ values of 50 nM (45 ng/ml) for PbTx-2 and 0.9 ng/ml (0.8 nM) for CgTx (fig.1, main panel). Synergies of the two toxins with As_2 and pyrethroids were also very similar.

CgTx, like PbTx-2, inhibited [^{3}H]PbTx binding to the Na^+ channel (fig.2). The inset of fig.2 shows that the binding of [^{3}H]PbTx-3 was increased in the presence of 40 μM As_2 (2-fold), but also in the presence of 10 μM of the pyrethroid RU 39568 (1.8-fold) and in the presence of a mixture of these two Na^+ channel effectors (4.6-fold). This specific binding of [^{3}H]PbTx-3 was totally inhibited by 10 μM PbTx-2. TTx and veratridine that are known to bind to different receptor sites did not significantly modify the specific binding of [^{3}H]PbTx-3. The main panel of fig.2 shows that the specific binding of [^{3}H]PbTx-3 measured in the presence of both As_2 and RU 39568 is inhibited by increasing concentrations of CgTx or PbTx-2 with $K_{0.5}$ values of 1.4 ng/ml (1.2 nM) and 45 ng/ml (50 nM), respectively. The corresponding calculated K_i values were 0.16 ng/ml (0.14 nM) for CgTx and 5 ng/ml (5.6 nM) for PbTx-2. The only difference between the two toxins is potency. CgTx appeared to be much more potent than PbTx. The same difference in potency was also seen by comparing the toxicities of these two compounds using the same type of intraperitoneal injection in mice. The LD_{50} for CgTx was 0.45 μg/kg (4×10^{-10} mol/kg) [3] and the LD_{50} for PbTx-2 was 180 μg/kg (2×10^{-7} mol/kg) [18]. In such assays CgTx was 400–500-fold more active than PbTx-2.

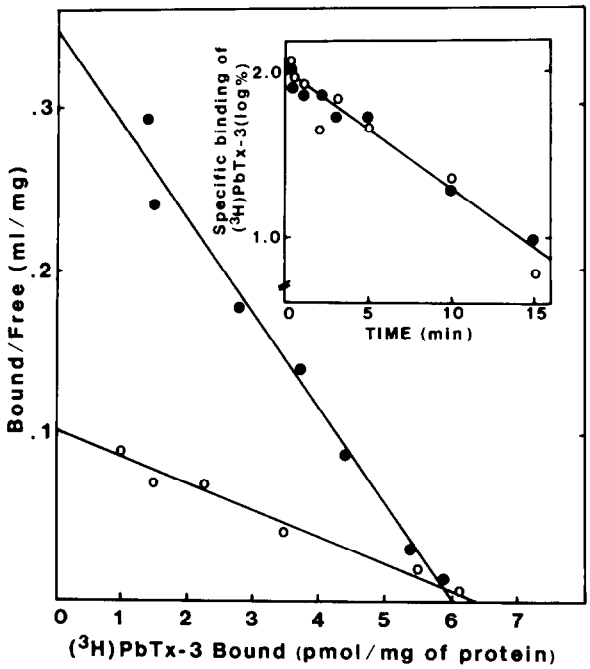

Fig.3. Competitive inhibition of [^{3}H]PbTx-3 binding by CgTx. The binding of [^{3}H]PbTx-3 was carried out in the presence of 40 μM As_2 and 10 μM RU 39568 (●). The effect of 0.2 ng/ml of CgTx was measured under the same conditions (○). Incubation conditions are those described for fig.2. (Inset) The remaining binding of [^{3}H]PbTx to its sites as a function of time after a 10-fold dilution in the absence (●) or presence (○) or 0.2 ng/ml of CgTx. The dilution was carried out after 2 h incubation of 25 nM [^{3}H]PbTx-3 at 4°C on rat brain membranes. In the dilution medium, concentrations of As_2 and RU 39568 were maintained to eliminate any possible modification of the conformation of their receptors that could modify the interaction of PbTx to its own binding sites.

In the experiments reported here CgTx had a 30–50-fold higher affinity for the Na^+ channel than PbTx-2. The high affinity of CgTx for the Na^+ channel is consistent with the observation that the toxin is active on isolated guinea pig ileum at concentrations as low as a few picomoles [19]. Plots presented in fig.3 show [^{3}H]PbTx-3 binding to a single family of binding sites with a K_d of 17 nM that is slightly higher than previously reported [14], and a B_{max} value of 6.0 pmol/mg of protein. In the presence of 0.2 ng/ml of CgTx the B_{max} value was almost identical 6.4 pmol/mg of protein whereas the K_d value was significantly decreased to 63 nM (a 3.7-fold lowering). Therefore CgTx seems to act as a competitive inhibitor at the PbTx binding site. Analysis of dissociation kinetics of the [^{3}H]PbTx-3-receptor complex confirmed this view (inset of fig.3). The half-life of dissociation of the PbTx-receptor complex ($t_{1/2}$ ~ 4.0 min) was almost the same in the presence and absence of CgTx.

Results presented in this paper not only show that PbTxs and CgTx are to be put in the same class of compounds acting on the Na^+ channel, they also confirm that this class of toxins binds to a specific receptor distinct from all other toxin receptors. Stimulating effects on [^{3}H]BTx binding show that the CgTx/PbTx receptor is distinct from the receptor of toxins of class 2 (table 1), synergism with sea anemone toxins and pyrethroids show that the CgTx/PbTx receptor is distinct from receptors for toxins of class 3 and class 6 (table 1). It has been shown [7] that CgTx had no interaction at sites 1 and 4 that are those recognized by TTx and β-scorpion toxins, respectively.

All these results taken together indicate that CgTx and PbTx share a common and unique receptor site on the voltage-dependent Na^+ channel (table 1). CgTx because of its very high affinity could become a new useful pharmacological tool as are TTx, saxitoxin, BTx and *Tityus serrulatus* toxin γ [20] to study the Na^+ channel. Moreover assays which have been described in this paper could be used analytically for the detection of CgTx in fishes associated with coral reefs.

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