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The binding of an insect-selective neurotoxin and saxitoxin to insect neuronal membranes

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(1) The radioiodinated insect-selective toxin derived from the venom of the scorpion *Androctonus australis* ^{125}I-labeled AaIT (^{125}I-AaIT) was used in a series of equilibrium-saturation binding assays to synaptosomal plasma membrane vesicles derived from locust brains, locust ventral nerve cords, cricket central nervous system, fly larvae central nervous system and fly heads. AaIT binds to single classes of non-interacting binding sites of high affinities (K_d = 1.2–3 nM) and low capacities (0.5–2.0 pmol/mg of membrane protein), thus indicating the existence of an evident homogeneity in the AaIT-binding constants among various insect neuronal membranes. (2) Assays on the binding of tritiated saxitoxin to the locust central nervous system synaptosomal membrane vesicles indicate that it (a) binds to a single class of non-interacting binding sites with K_d = 0.14 nm and a capacity of 1.4 pmol/mg of membrane protein closely resembling the capacity of AaIT (1.2–2 pmol/mg protein), and (b) is competitively displaced with a high affinity (K_d = 0.35 nM) by tetrodotoxin. (3) The estimated binding capacity of AaIT to a crude preparation of fly heads (0.12–0.14 pmol/mg protein) is in accordance with previous data [31,32] concerning the binding of saxitoxin. The close resemblance in the binding capacity of saxitoxin and insect toxin to insect neuronal membranes supports the suggestion, previously given by electrophysiological studies, that the insect toxin's binding sites are related to the insect neuronal sodium channels.

Introduction

The AaIT is an insect-selective neurotoxic polypeptide (8 kDa) [1,2] derived from the venom of the scorpion *Androctonus australis*. The AaIT's excitatory paralysis of insects is caused by the induction of repetitive firing of motor nerves [3] attributable to a modification of the insect axonal sodium conductance [4,5]. The selectivity of the neurotoxic action of AaIT and related toxins was strongly emphasized through binding assays indicating the existence of a specific binding exclusively to insect nervous tissues and not to insect non-innervated (such as fat tissue or malphighian tubules), or mammalian and crustacean nervous tissues [6–8]. The recent development of a pharmacologically functional insect neuronal preparation in the form of locust synaptosomal plasma membrane vesicles [9,10] enabled a detailed study

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Abbreviations: AaIT, an excitatory insect toxin from the venom of the scorpion *Androctonus australis*; AmIT, an excitatory insect toxin from the venom of the scorpion *Androctonus mauretanicus*; $BjIT_1$ and $BjIT_2$, the excitatory and depressory insect toxins 1 and 2, respectively, from the venom of the scorpion *Buthotus judaicus*; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $LqqIT_1$ and $LqqIT_2$, the excitatory and depressory insect toxins 1 and 2, respectively, from the venom of the scorpion *Leiurus quinquestriatus quinquestriatus*.

of the interaction of AaIT with insect neuronal membranes through a series of kinetic and equilibrium-binding assays [11]. It has been shown AaIT binds specifically to a single class of non-interacting binding sites of high affinity (K_d = 1.2–3 nM) and low capacity (1.2–2 pmol/mg protein) independently of the membrane potential [11].

The present work has a double purpose: (a) to examine whether the binding constants of AaIT in the locust neuronal membrane represent a general feature of insect neuronal tissues, and (b) to demonstrate the possible relation between the AaIT-binding sites and the insect neuronal sodium channels.

Materials and Methods

Materials. AaIT was purified according to a previously published method [1]. Veratridine and tetrodotoxin were purchased from Sigma (U.S.A.). Saxitoxin was obtained from the National Institute of Health. ^{125}I-labeled AaIT (^{125}I-AaIT) was prepared according to a previously described procedure [11] and was purified with the aid of a specific antiserum [12]. The preparation used possessed a specific radioactivity in the range of 320–400 Ci/mmol. [^{3}H]Saxitoxin was prepared by the specific exchange procedure [13] and purified and characterized according to a previously described method [14]. The preparation had a specific radioactivity of 12 Ci/mmol.

Collection of insect central nervous system. In this study, we have employed neuronal tissue derived from the central nervous system of the following laboratory-bred insects: (1) adult locusts (*Locusta migratoria migratorioides*, Acrididae, Orthoptera), (2) adult crickets (*Gryllus bimaculatus*, Gryllidae, Orthoptera), (3) fleshflies (*Sarcophaga falculata*, Calliphoridae, Diptera), (4) larvae 6–8 days old of the above *Sarcophaga* flies. The locust central nervous system was collected in two separate batches: (a) brains (including optic lobes) and the sub-esophageal ganglia, and (b) the ventral nerve cords including the thoracic and the abdominal ganglia. With the crickets, the whole central nervous system was included. In adult flies, the whole heads were employed. The central nervous system of the fly larva is condensed in a single mass located between the third and fourth segments of the body and it was collected by cutting the animal between segments 4 and 5.

Insect synaptosomal plasma membrane vesicles. Insect synaptosomes and their derived membrane vesicles (mvP_2L preparation) were prepared from homogenates of the different central nervous system according to previously described methods [9,11]. All manipulations were performed at 4°C in the mannitol buffer (0.25 M D-mannitol/1 mM EDTA (pH 7.3)). Fly heads, suspended in the mannitol buffer, were first treated by a blender in order to break the head capsules. The suspension of crushed heads was filtered through a dense nylon sieve in order to separate the cuticle fragments from the head tissues which were then homogenized.

Briefly, insect central nervous system homogenates were centrifuged for 10 min at $12000 \times g$ and the supernatants were collected and centrifuged for 20 min at $27000 \times g$. The pellets (P_2 preparation) were resuspended in the mannitol buffer and 20% Ficoll solution was added up to a concentration of 10% Ficoll for locust central nervous system and 12.5% for other preparations. The mixture was centrifuged for 75 min at $12100 \times g$ resulting in the floating pellicle (P_2L preparation), representing the enriched synaptosomal fraction and the pellet (P_2H). The synaptosomal plasma membrane vesicles (mvP_2L) were prepared by osmotic shock of the P_2L preparations by suspension in 5 mM Tris-HCl/1 mM EDTA (pH 7.4) medium. The membrane vesicles (mvP_2L) were collected by centrifugation for 20 min at $27000 \times g$ and loaded (5 min, 37°C) with the loading medium composed of 0.1 M potassium phoshate (pH 6.8)/1 mM $MgSO_4$ (the indicated molarity corresponds to the anion) in the binding assays with AaIT or 0.1 M choline chloride/20 mM Hepes-Tris (pH 7.4) in the binding assays with saxitoxin.

Binding assays. Binding experiments with the ^{125}I-AaIT were routinely performed as follows; 10 µl of the mvP_2L preparation (20–60 µg of membrane protein) were added to 190 µl of the standard binding medium (0.15 M choline chloride/1 mM $MgSO_4$/2 mM $CaCl_2$/0.1% bovine serum albumin), containing 1.0–1.5 nM of ^{125}I-AaIT. The buffer capacity of the external medium was provided by carryover of the loading buffer (final concentration of 5 mM) upon dilution of the mem-

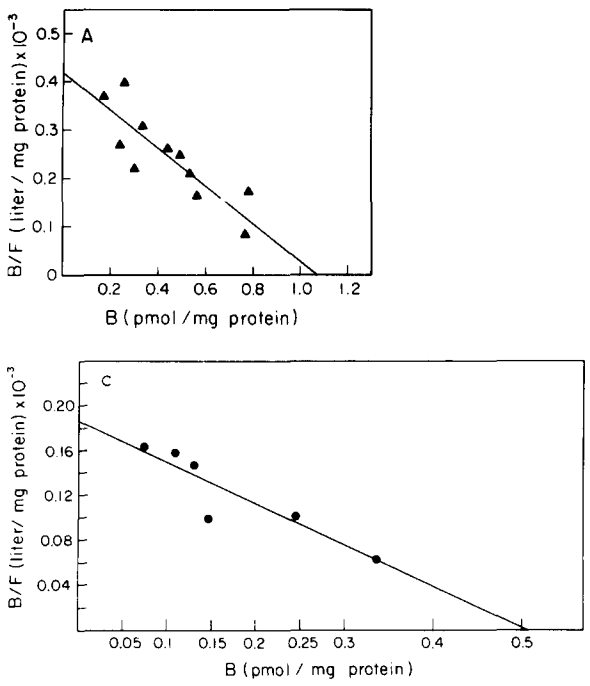

Fig. 1. Scatchard plots of specific binding data obtained by equilibrium saturation-binding assays of the ^{125}I-AaIT ([^{125}I]AaIT) to synaptosomal membrane vesicles (mvP_2L) derived from locust brains and sub-esophageal ganglia (A), locust ventral nerve cord (B) and fly larvae central nervous system (C). The binding was measured after 30 min of incubation in the standard binding medium at 20°C with increasing concentrations of labeled toxin in reaction mixtures containing 40, 36 and 10.8 μg of membrane protein in A, B, and C, respectively. The non-specific binding was determined at each concentration of the ^{125}I-AaIT in the presence of 1 μM of the unlabeled toxin.

brane vesicles in the binding medium. The membranes were incubated for 30 min at 20°C. To terminate the reaction and to separate the membrane bound from the free ^{125}I-AaIT, the reaction mixture was rapidly diluted with 2 ml of ice-cold wash medium (0.15 M choline chloride/0.5% bovine serum albumin), filtered through a Millipore EH (0.5 μm) filter, and washed twice with 2 ml of the same solution. Stopping the reaction, filtration and washing took 12–15 s. The binding of labeled toxin measured in the presence of a large excess of unlabeled toxin (1 μM) was defined as the non-specific binding.

The binding assays with [^{3}H]saxitoxin were essentially performed in the same manner with the following changes: (1) The loading medium (see

TABLE I

THE BINDING CONSTANTS OF ^{125}I-AaIT TO SYNAPTIC PLASMA MEMBRANE VESICLES DERIVED FROM DIFFERENT NEURONAL PREPARATIONS

[a] The protein yield of the mvP_2L derived from the brain (and esophageal ganglia) and ventral nerve cords corresponds to 50–54% and 46–50%, respectively, when compared to the mvP_2L preparation derived from the total central nervous system of the locust. CNS, central nervous system; VNC, ventral nerve cord.

Origin of neuronal preparation	K_d (nM)	Binding-site capacity (pmol/mg protein)	Reference
Locust CNS [a]	1.2–3	1.2–2.0	11
Locust brains and sub-esophageal ganglia [a]	2.6	1.1	Fig. 1A
Locust VNC [a]	2.7	1.2	Fig. 1B
Cricket CNS	2.9	1.8	Fig. 2A
Fly heads	2.2	0.7	Fig. 2B
Fly larvae CNS	2.7	0.5	Fig. 1C

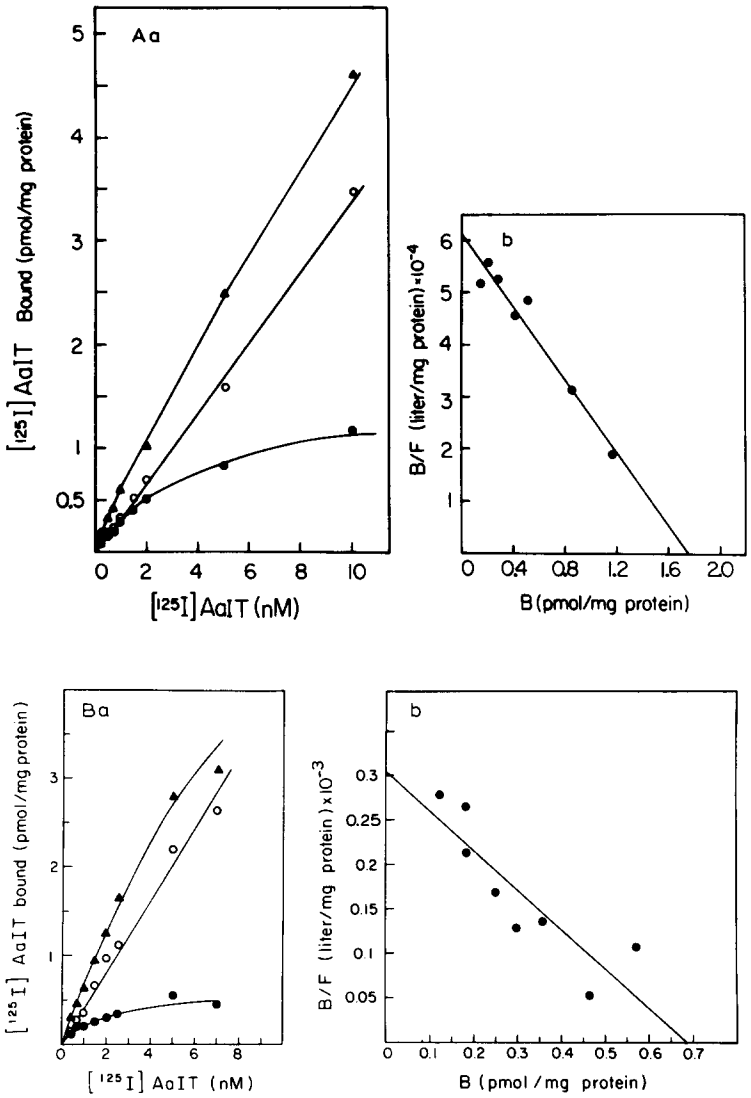

Fig. 2. Binding of ^{125}I-AaIT ([^{125}I]AaIT) to the cricket central nervous system (A) and fly head (B) synaptosomal membrane vesicles (mvP_2L). The binding was measured after 30 min of incubation in the standard binding medium at 20°C with increasing concentrations of labeled toxin in reaction mixtures containing 44 (A) and 31.4 (B) μg membrane protein, in the absence (▲) and presence (○) of 1 μM unlabeled AaIT. The specific binding (●) is the difference between the two curves. (a) Saturation binding assays; (b) Scatchard plots of specific binding data.

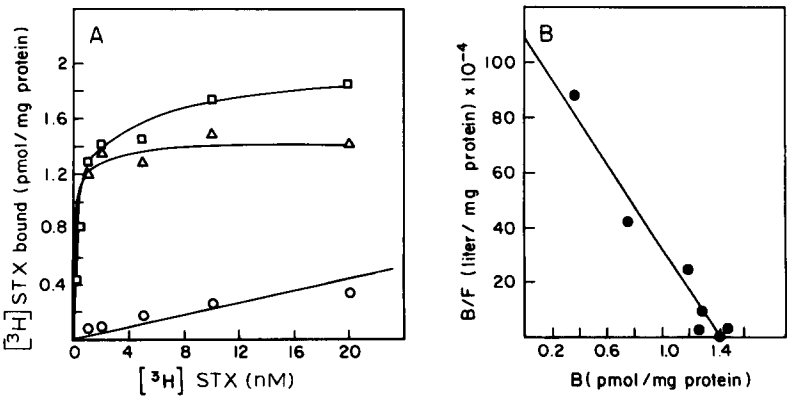

Fig. 3. Binding of [^{3}H[saxitoxin (STX) to the locust synaptosomal membrane vesicles. (A) The binding was measured after 15 min at 2°C in reaction mixtures (175 μl) composed of 100 mM choline chloride/20 mM Hepes-Tris (pH 7.4) in the presence of 75 μg of membrane protein and increasing concentrations of [^{3}H]saxitoxin in the absence (□, total binding) or the presence (○, non-specific binding) of 1 μM tetrodotoxin. The specific binding (△) is the difference between the two curves. (B) Scatchard plot of specific binding data as taken from A. The calculated K_d equals to 0.138 nM and the maximal capacity is equal to 1.43 pmol/mg of membrane protein.

above) was also employed as the binding medium. (2) Reaction was initiated by addition of 15 μl of the mvP_2L preparation (75 μg protein) to the binding medium containing the [^{3}H]saxitoxin (final volume 175 μl). (3) Incubation was performed during 15 min over ice (0–2°C). (4) The non-specific binding was determined in the presence of 1 μM tetrodotoxin. (5) For the termination of reaction and washing, 0.1 M choline chloride was employed. (6) Whatman GF/c filters were used for filtration.

Radioactivity was counted by a Packard Liquid Scintilation Spectrometer in the presence of Packard Insta-Gel II scintillation liquid.

Protein determination. Tissue protein was determined by the procedure of Lowry et al. [33] using bovine serum albumin for standards.

Results

Binding assays with ^{125}I-AaIT

In these assays, the binding properties of ^{125}I-AaIT to synaptosomal plasma membrane vesicles derived from locust brains, locust ventral nerve cord, central nervous system of crickets, fly heads and central nervous system of fly larvae were compared through a series of equilibrium saturation studies. The assays are presented in Figs. 1 and 2 and their resulting equilibrium dissociation constants (K_d) and maximal binding capacities are presented in Table I.

In the different dissected central nervous system tissues, the protein yields and binding capacities of the intermediate stages (P_2, P_2L, P_2H) of the preparation of the membrane vesicles (mvP_2L) resembled the previous data obtained with the locust central nervous system [11]. The fly heads are substantially occupied by non-neuronal tissues heavily contaminating the P_2 preparation. Batches of 500 *Sarcophaga* heads yield about 42–47 mg of protein at the stage of the P_2 preparation (see Materials and Methods) which is further subdivided to the P_2L and P_2H (Materials and Methods and Ref. 9) comprising 12–15 and 85–88% of its protein yield, respectively. The final mvP_2L preparation possesses the whole ^{125}I-AaIT binding activity of its preceding P_2L fraction and about 60% of its protein yield. The specific binding activity of the mvP_2L preparation was 5-times higher than that of mvP_2H.

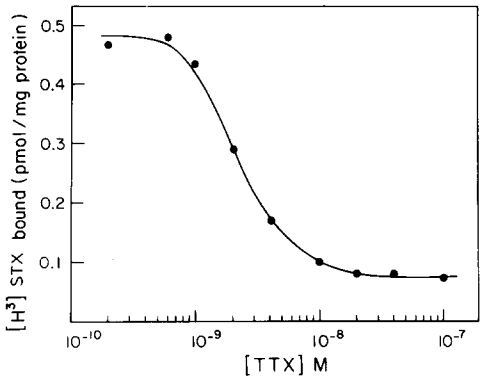

Fig. 4. Competitive displacement curve of [^{3}H]saxitoxin (STX) from the locust synaptosomal vesicles by tetrodotoxin. Reaction mixtures of the same composition as in Fig. 3 were incubated (15 min, 2°C) in the presence of 0.6 nM of [^{3}H]saxitoxin and increasing concentrations of tetrodotoxin. The apparent concentration of tetrodotoxin which displaces the specific binding by 50% ($K_{0.5}$) corresponds to 2 nM. The low specific radioactivity of [^{3}H]saxitoxin demanded the employment of relatively high concentrations of the labeled toxin and membrane protein. Thus, in order to calculate the true dissociation constant (K_d) for tetrodotoxin, the following equation [15] (which is a modification of that in Ref. 16) was employed:

$$K'_d = \frac{K^*_d \times (RL)}{(L_T)-(RL)} \times \left[K_{0.5} - (R_T) + (RL)\left(1 + \frac{K^*_d}{(L_T)-(RL)}\right)\right] \times \left[(R_T)-(RL)\left(1+\frac{K^*_d}{(L_T)-(RL)}\right)\right]^{-1}$$

$$= 0.35 \text{ nM}$$

K'_d, dissociation constant for tetrodotoxin-receptor; K^*_d, dissociation for [^{3}H]saxitoxin as determined by the above assay (Fig. 3, 0.14 nM); R_T, total concentration of receptor in reaction mixture (612.9 pM, estimated on the basis of the maximal specific binding capacity determined in the previous assay (Fig. 3); RL, concentration of the complex [^{3}H]saxitoxin-receptor (203.6 pM, estimated from the data of the maximal binding in the absence of tetrodotoxin); L_T, the concentration of [^{3}H]saxitoxin (0.6 nM); $K_{0.5}$ = the apparent concentration of tetrodotoxin which displaces [^{3}H]saxitoxin by 50% (2 nM).

Assays on [^{3}H]saxitoxin binding

Saturation and displacement binding assays with locust central nervous system synaptosomal membrane vesicles are presented in Figs. 3 and 4 and they indicate that saxitoxin binds to a single class of non-interacting binding sites of a high

affinity ($K_d = 0.14$ nM) and low capacity (1.43 pmol/mg protein) (Fig. 3). Tetrodotoxin demonstrated a high affinity of the same magnitude ($K_d = 0.35$ nM, Fig. 4).

Discussion

The data presented in Table I indicate that synaptosomal membrane vesicles derived either from different parts of the nervous system of a given insect (locust) or from nervous systems of diverse insects (locusts, crickets, flies and fly larvae) possess closely resembling binding properties of the insect-selective toxin AaIT. They all demonstrate single classes of non-interacting binding sites of high affinities (K_d values in the range of 1.2–3 nM) and low capacities (in the range of 0.5–2.0 pmol/mg of membrane protein).

The possession of an AaIT-binding site appears to be a unique property of the insect neuronal membrane (see Introduction). The present data demonstrating an obvious homogeneity in the AaIT receptors among the diverse insect neuronal preparations, emphasize the necessity to clarify the pharmacologic significance of these receptors. The scorpion venom toxins affecting vertebrates, which strongly resemble the insect toxins' in their binding constants, were shown to interact with the mammalian sodium channels and play an essential role in the pharmacological and chemical characterization of these channels [17–23,11]. So far, the only evidence indicating the relation between the insect toxins' binding site and the insect sodium channels, was supplied by electrophysiological data.

Buthid scorpion venoms possess two kinds of insect-selective neurotoxins, namely the excitatory (such as the AaIT [1], AmIT [7], $BjIT_1$ [24] and $LqqIT_1$ [25]) and depressory (such as the $BjIT_2$ [24] and $LqqIT_2$ [25]) neurotoxins. Both groups of toxins were shown to affect exclusively sodium conductance in insect axons (although in a different manner) [4,5,24,25] and competitively displace the ^{125}I-AaIT with a high affinity ($K_d = 1.0$–2.2 nM) [11,25,26]. This may indicate the existence of a common binding site to the above two groups of toxins possibly related to the insect neuronal sodium channel.

It was previously shown in mammalian neuronal systems [18] that both the water-soluble heterocyclic guanidines tetrodotoxin and saxitoxin and the scorpion venom toxins specific to vertebrates interact with the mammalian action potential sodium channels but, however, possess two distinct and separate binding sites [18]. Most recently, it was shown that tetrodotoxin (which is pharmacologically identical to saxitoxin) did not affect the binding of ^{125}I-AaIT to the locust synaptosomal membrane vesicles [11]. It may be thus concluded that, like in the mammalian, the insect neuronal system possesses tetrodotoxin-saxitoxin-binding sites which are distinct from those that bind the scorpion venom insect toxins both, presumably, are associated to the insect neuronal sodium channel

The present data concerning the binding of the [^{3}H]saxitoxin supply additional evidence supporting the possibility of a relation between the sodium channels and the insect toxins' binding site.

The existence of saxitoxin- and tetrodotoxin-binding sites, which represent the Na^+ channels in excitable tissues, is considered as the most characteristic property of neuronal tissue and serves as a quantitative indication of these channels [13,28,29]. The present binding constants of [^{3}H]saxitoxin to the insect synaptosomal vesicles ($K_d = 0.14$ nM, capacity 1.4 pmol/mg protein) resemble those previously obtained with rat brain synaptosomes ($K_d = 0.6$–2 nM, capacity 3–4 pmol/mg protein) [30,14]. In other words, it appears that the rat brain and locust central nervous system synaptosomal membranes possess very similar densities of sodium channels. The close resemblance between the above binding capacities and the AaIT-binding sites to the locust neuronal membrane may indicate their relation to the insect sodium channels. This aspect is further supported by some recent reports demonstrating that a crude preparation of *Drosophila* fly heads (which parallel the P_2 preparation of our procedure (see Materials and Methods)) possessed a binding capacity in the range of 0.1–0.13 pmol/mg protein for [^{3}H]saxitoxin [31,32]. On the basis of the above (see Results, and Fig. 2B) protein yields and recovery of binding activity of the ^{125}I-AaIT to the fly head preparation, it may be concluded that the maximal binding capacity of the present fly heads P_2 preparation to ^{125}I-AaIT corresponds to about

0.12–0.14 pmol/mg protein which closely resembles the above [31,32] saxitoxin binding data to *Drosophila* fly heads.

To summarize, the accordance between the binding capacities of AaIT and saxitoxin in two different neuronal preparations derived from diverse insects is not coincidental and it further suggests that the AaIT-binding sites are related to the insect sodium channels as previously indicated by current and voltage clamp studies in an isolated insect axon [4,5,24].

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