Biochimica et Biophysica Acta, 778 (1984) 349–358
Elsevier

BBA 72310

THE BINDING OF THE INSECT SELECTIVE NEUROTOXIN (AaIT) FROM SCORPION VENOM TO LOCUST SYNAPTOSOMAL MEMBRANES

DALIA GORDON *, EMMANUEL JOVER, FRANCOIS COURAUD and ELIAHU ZLOTKIN *,**

Laboratoire de Biochimie, INSERM U 172, Faculté de Médecine, Nord-Marseille (France)

(Received June 20th, 1984)

Key words: Membrane-toxin interaction; Neurotoxin; Insect selective toxin; Membrane potential; Synaptosome; (Scorpion, Locust)

The binding of the radioiodinated insect selective neurotoxin from the venom of the scorpion *Androctonus australis* (AaIT), to synaptic plasma membrane vesicles derived from osmotically shocked insect synaptosomes was studied under kinetic and equilibrium conditions. The integrity of these vesicles and the existence of membrane potential and its modifiability were demonstrated by assays of the uptake of the lipophilic cation tetraphenylphosphonium. It has been shown that ^{125}I-labeled AaIT binds specifically and reversibly to a single class of noninteracting binding sites of high affinity (K_d* = 1.2–3 nM) and low capacity (1.2–2.0 pmol/mg protein). The values of the rate association and dissociation constants k_1 and k_{-1} are, respectively, $1.36 \cdot 10^6$ M$^{-1} \cdot$ s^{-1} and $1.9 \cdot 10^{-3}$ s^{-1}, and are in a good accordance with the equilibrium constant. The use of various ionophores and changes in external potassium concentration shown to modify the membrane potential of the present neuronal preparation, did not affect the binding of ^{125}I-AaIT, thus indicating its voltage-independence. Veratridine, tetrodotoxin, sea anemone toxin and the α and β scorpion toxins specific for vertebrates did not affect the binding of ^{125}I-AaIT. Furthermore, the above scorpion toxins were devoid of specific binding to the present insect neuronal preparation. Two additional insect toxins derived from the venom of the scorpion *Buthotus judaicus*, BjIT$_1$ (spastic-excitatory toxin, homologus to the AaIT) and BjIT$_2$ (flaccidity inducing-depressory toxin), were both shown to displace the ^{125}I-AaIT with a high affinity (K_d = 2.2 and 1.3 nM, respectively). These data are compared and discussed in light of the information concerning the interaction of scorpion venom toxins affecting vertebrates with mammalian neuronal tissues.

Introduction

The insect toxin derived from the venom of the North African scorpion *Androctonus australis* Hector (AaIT) is a single-chained polypeptide composed of 70 amino acids crosslinked by four disulfide bridges [1,2] with a unique position when compared with the scorpion toxins active on vertebrates [1,2]. Its most remarkable properties are its potent neurotoxicity and selectivity to insects. The pharmacology of this toxin has been established through series of studies indicating that

* Present address: Institute of Life Sciences, Department of Zoology, Hebrew University of Jerusalem, Jerusalem 91904, Israel.

** To whom correspondence should be addressed.

Abbreviations: AaIT, insect toxin from the venom of *Androctonus australis*; AmIT, insect toxin from the venom of *A. mauretanicus*; BjIT$_1$ and BjIT$_2$, insect toxins 1 and 2 from the venom of *Buthotus judaicus*; LqIT$_1$ and LqIT$_2$, insect toxins 1 and 2 from the venom of *Leiurus quinquestriatus*; AaH II, α-toxin II from the venom of *A. australis*; Css II, β-toxin II from the venom of *Centruroides suffusus suffusus*; Lqq V, α-toxin V from the venom of *Leiurus quinquestriatus*; TPP$^+$, tetraphenyl phosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

the AaIT: (a) induces a fast spastic paralysis of insects and does not affect mammals or closely related groups of arthropods [3]; (b) activates the insects' skeletal musculature through a presynaptic excitatory effect on motor nerves [4], and does not affect neuromuscular preparations of other arthropods or mammals [5]; (c) binds exclusively to insect nervous tissue and not to insect noninnervated tissues or crustacean nervous tissues [6]; (d) causes a specific modification in the sodium conductance in an insect axonal preparation [7,8].

It was recently shown that buthid scorpion venoms possess two kinds of insect selective neurotoxins, namely the excitatory neurotoxins (such as the AaIT [8] AmIT [9] $BjIT_1$ [10] and $LqIT_1$ (unpublished data) and depressory neurotoxins (such as the $BjIT_2$ [10] and LqIT (unpublished data). Both groups of toxins were shown to affect exclusively sodium conductance in insect axons, although in a different manner [8,10].

In the present study, the use of pharmacologically functional insect neuronal preparation, in the form of the locust synaptosomal plasma membrane vesicles [11], leads to a further clarification of the neuropharmacological significance of the scorpion venom insect toxins.

Materials and Methods

Materials. Carrier-free $Na^{125}I$ was obtained from Amersham International, Amersham, U.K. Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), veratridine, *Anemonia sulcata* toxin II, tetrodotoxin, D-mannitol, Ficoll (type 400), bovine serum albumin fraction V and choline chloride were purchased from Sigma (U.S.A.) and tritiated tetraphenylphosphonium bromide ($[^3H]TPP^+$) from the Nuclear Research Centre, Negev, Israel. The scorpion venom toxins AaIT, $BjIT_1$, $BjIT_2$ AaH II and Css II were purified according to Refs. 2, 10, 10, 12 and 13, respectively.

Radioiodination. The toxins AaIT, AaH II and Css II were iodinated using the lactoperoxidase method for $^{125}I^-$ oxidation and purified either by immunoprecipitation with a monospecific antiserum prepared against the native toxin [14] or by a batchwise resuspension with Dowex 1-X8 (Merck, F.R.G.) anion exchanger [6]. The presence of free iodine (3–5%) was examined by ascending paper chromatography on Whatman No. 1 paper using methanol as solvent. Specific radioactivities of about 50–70 $\mu Ci/\mu g$ (375-530 Ci/mmol) were routinely obtained from a reaction of 5 μg of scorpion toxin in the presence of 0.5 mCi of carrier-free $Na^{125}I$.

Insect and rat neuronal preparations. Insect synaptosomes and their derived membrane vesicles were prepared from homogenates of dissected ventral nerve cords, brains and subesophageal ganglia from the locust *Locusta migratoria* (Insektarium Dr. Frieshammer, Jaderberg, F.R.G. and 'Appats Express', Chatonnay, France), according to methods previously described [11].

All manipulations were performed at 4°C in 0.25 M mannitol, 10 mM Tris-HCl (pH 7.4). Briefly, the homogenates were centrifuged for 10 min at $1200 \times g$, and the supernatant was collected and centrifuged for 20 min at $27000 \times g$. The pellet (P_2) was resuspended and 20% Ficoll solution was added up to a concentration of 10% Ficoll. The mixture was centrifuged for 75 min at $12100 \times g$ resulting in the floating pellicle (P_2L), representing the enriched synaptosomal fraction and the pellet (P_2H). The preparations P_2, P_2L and P_2H were occasionally used (Table I). The membrane vesicular preparation (mvP_2L), which was routinely used, was prepared by osmotic shock of the P_2L fraction by suspension in 5 mM Tris-HCl (pH 7.4) medium. The membrane vesicles (mvP_2L) were collected by centrifugation for 20 min at $27000 \times g$. The membrane vesicles were routinely loaded (5 min, 37°C) with standard loading medium (0.1 M potassium phosphate (pH 6.8)/1 mM $MgSO_4$, the indicated molarity corresponds to the anion). Thus, under the present pH conditions, the actual molarity of potassium ions is 150 mM).

It was noted that the membrane vesicles suspended in the loading solution (prior to the loading step) could be stored in liquid nitrogen without loss of activity for at least 4 months. Rat brain synaptosomes were prepared according to the procedure given by Kanner [15].

Binding assays. Binding experiments were routinely performed as follows. 10 μl of the mvP_2L preparation (20–60 μg of membrane protein) were added to 190 μl (unless stated otherwise) of the standard binding medium (0.15 M choline chloride, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1% bovine

serum albumin and 1.0–1.5 nM ^{125}I-AaIT. The buffer capacity of the external medium was provided by carry-over of the loading buffer (final concentration of 5 mM) upon dilution of the membrane vesicles in the binding medium. The membranes were incubated for 30 min at 20–22°C. To terminate the reaction and to separate the bound membrane 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. To determine the association kinetics, the binding medium containing ^{125}I-AaIT with or without the unlabeled toxin was used. The reaction was initiated by the addition of the mvP$_2$L suspension followed by sampling of 250-μl portions at different time intervals. Each sample was rapidly diluted (2.5 ml of the above ice-cold wash medium) filtered and washed according to the above procedure (Fig. 1). the same incubation mixtures were used in the experiments on dissociation kinetics. In these experiments, membranes were first incubated for 30 min at 20°C, and then dissociation was initiated by the addition of the unlabeled toxin (1 μM) followed by sampling (250 μl) and rapid filtration at different time intervals (Fig. 2).

In all cases, the binding of labeled toxin measured in the presence of a large excess of unlabeled toxin (1 μM) was defined as the nonspecific binding. Radioactivity was counted in an Auto Gamma Spectrometer (Packard, model A 500 C) with a counting efficiency for ^{125}I of 60%.

Uptake of tetraphenylphosphonium (TPP$^+$). 5 μl of mvP$_2$L suspension (50–100 μg protein) were added to 195 μl of a medium composed of 0.15 M choline chloride, 1 mM $MgSO_4$ and 4.4 or 6.28 μM TPP$^+$, which included 1 or 5 μCi of [^{3}H]TPP$^+$ (5.7 Ci/mmol). Other additions were not greater than 1% of the total assay volume. Reactions were initiated by adding the membrane suspension and terminated by the addition of 2 ml of ice-cold 0.15 M choline chloride followed by rapid filtration and washing twice with 2 ml of the same solution. Corrections for the nonspecific adsorption to filters were made by diluting the reaction mixture with 0.15 M choline chloride medium prior to addition of the membranes, followed by addition of the membranes, filtration and washing. Values obtained in this manner were subtracted from the data presented. Radioactivity was measured by liquid scintillation spectrometry after addition of Beckman Ready Solv scintillation fluid in a Packard TRI-Carb 460 C counter.

The internal concentration of TPP$^+$ ([TPP$^+$]$_{in}$) was calculated by using an intravesicular volume of 5.6 ± 0.2 μl/mg protein [11]. External TPP$^+$ concentration ([TPP$^+$]$_{out}$) was calculated by subtracting the amount taken up from the concentration of TPP$^+$ originally present in the reaction mixture. Concentration gradients of TPP$^+$ were calculated as [TPP$^+$]$_{in}$/[TPP$^+$]$_{out}$ [16].

Protein determination. Tissue protein was determined by the Lowry procedure [17], using bovine serum albumin for standards.

Results

Binding capacity of different neuronal preparations

Table I presents the binding of ^{125}I-AaIT to various insect synaptosomal fractions compared to their capacity to actively accumulate γ-[^{3}H]aminobutyric acid. It is evident that the membrane vesicles (mvP$_2$L), which comprise only about 22% of the protein content of the crude synapto-

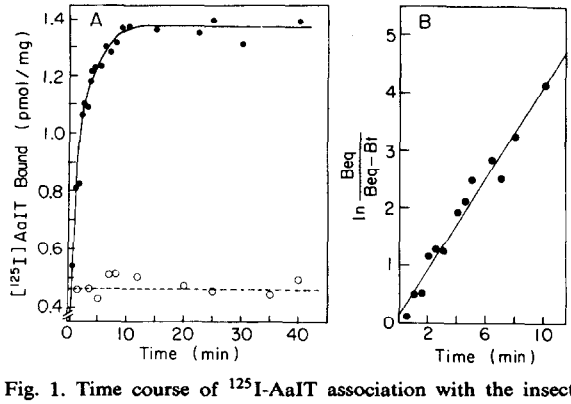

Fig. 1. Time course of ^{125}I-AaIT association with the insect synaptosomal membrane vesicles (mvP$_2$L). (A) mvP$_2$L membranes (32 μg of protein per sample of 250 μl) were incubated for the indicated times, at 20°C in the standard binding medium, containing 1.5 nM ^{125}I-AaIT in the absence (●) or presence (○) of 1 μM unlabeled AaIT. (B). The kinetic of the ^{125}I-AaIT binding was linearized according to the equation of a pseudo-first-order reaction, as explained in the text.

TABLE I

BINDING OF ^{125}I-AaIT TO DIFFERENT SYNAPTOSOMAL FRACTIONS [11] DERIVED FROM THE CENTRAL NERVOUS SYSTEM OF *LOCUSTA MIGRATORIA* COMPARED TO THEIR γ-[^{3}H]AMINOBUTYRIC ACID (GABA) UPTAKE

The binding assays were performed in the presence of 1.27 nM ^{125}I-AaIT at 20°C for 30 min. Nonspecific binding was determined in the presence of 1 μM of unlabeled AaIT. P_2, crude synaptosomal fraction; P_2L, enriched synaptosomal fraction, which is the floating pellicle obtained from P_2; P_2H, the pellet obtained from P_2; mvP_2L, membrane vesicles derived from the P_2L. The binding medium for P_2, P_2L and P_2H was composed of 125 mM choline chloride, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) adjusted to pH 7.3 with Tris base, 2 mM $CaCl_2$, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl and 0.1% bovine serum albumin. With the mvP_2L the standard binding medium was used. In the binding experiments the amount of membrane protein employed per assay for each neuronal fraction was 210, 73, 73 and 53 μg of membrane protein for P_2, P_2L, P_2H and mvP_2L, respectively. These amounts correspond to the various fractions obtained from the neuronal tissue of about two locusts. The data on γ-[^{3}H]aminobutyric acid uptake were taken from Gordon et al. [11] and they represent the steady-state conditions following 5 min of incubation.

Neuronal preparation	Protein yield (%)	Specific binding (pmol/mg prot.)	Recovery of specific binding (%)	Relative binding capacity	[^{3}H]GABA uptake (pmol/mg prot.)	Relative GABA uptake
P_2	100	220	100	1.0	19.2	1.0
P_2L	39	400	71	1.82	49.7	2.6
P_2H	33.1	75	11.3	0.34	1.0	0.05
mvP_2L	21.5	720	70	3.3	106.3	5.5

somal fraction (P_2), account for 70% of its binding capacity with an increase of 3.3-fold in its specific binding capacity. All four preparations have revealed closely similar affinities (data not shown). Similarly, the mvP_2L fraction was shown (Table I) to possess the highest ability to actively accumulate γ-[^{3}H]aminobutyric acid. On the basis of these data, the mvP_2L preparations was chosen for further studies.

Kinetics of association

Fig. 1 presents the associations kinetics of ^{125}I-AaIT to the mvP_2L preparation. The specific binding increased with time and reached a plateau at about 9 min (Fig. 1A), the nonspecific binding remaining constant throughout. The interaction of ^{125}I-AaIT with mvP_2L membranes can be analyzed [18] as a pseudo-first-order reaction by the equation:

$$\ln \frac{[B_{eq}]}{[B_{eq}]-[B_t]} = k_1 t \frac{[L]_T[R]_T}{[B_{eq}]}$$

in which $[B_{eq}]$ is the concentration of bound ligand at equilibrium (53.67 pM); $[B_t]$ is the concentration of bound ligand at a given time t; $[L]_T$ is the total concentration of the ligand (1500 pM); $[R]_T$ is the total concentration of the binding sites (174 pM, based on data presented in Fig. 3). The slope of the pseudo-first-order plot (Fig. 1B) is equal to $k_1[L]_T[R]_T/[B_{eq}]$. Thus, under the present conditions, k_1 equals $1.36 \cdot 10^6\ M^{-1} \cdot s^{-1}$. The pseudo-first-order conditions were satisfied, since the value

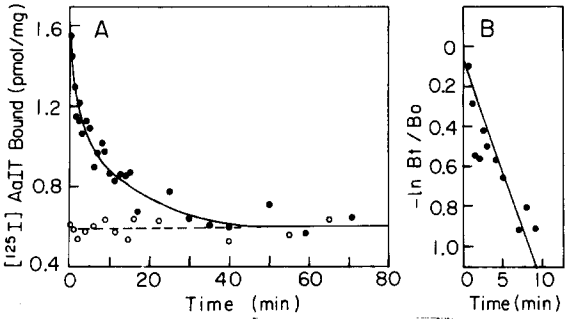

Fig. 2. Time course of dissociation of ^{125}I-AaIT from the insect synaptosomal membrane vesicles (mvP_2L). (A) mvP_2L membraners were first incubated with 1.2 nM of ^{125}I-AaIT in the standard binding medium. After 30 min, 1 μM of unlabeled AaIT was added and samples of 250 μl (39 μg of protein) were removed for the determination of binding at the indicated time intervals (●). The nonspecific binding (○) was determined by the same procedure in a parallel experiment, except that 1 μM of unlabeled AaIT was present in the incubation medium. After 30 min, the concentration of the unlabeled toxin was increased to 2 μM followed by the above samplings at the indicated time intervals. (B) Dissociation was linearized according to the equation of a first-order-kinetic reaction as explained in the text.

of $[B_{eq}]$ corresponded to only 3.6% of the $[L]_T$ value [18]. When employing the integrated second-order-rate equation (Eqn. 4 in Ref. 18) the value of k_1 was calculated as $7.13 \cdot 10^5\ M^{-1} \cdot s^{-1}$.

Kinetics of dissociation

The dissociation of the ^{125}I-AaIT from the membrane vesicles was initiated by addition of a large excess of unlabeled toxin to a finally equilibrated reaction mixture at 20°C. The value of k_{-1} was determined by measuring the dissociation of ^{125}I-AaIT (Fig. 2A). Dissociation was linear when plotted as a first-order-reaction according to the following equation [18]:

$$\ln\frac{[B_t]}{[B_o]} = k_{-1}t$$

where $[B_o]$ is the concentration of bound toxin at time zero, just prior to addition of the competing unlabeled toxin, $[B_t]$ the concentration of the bound toxin at time t after initiation of the dissociation. The slope calculated from the dissociation curve gave $k_{-1} = 1.89 \cdot 10^{-3}\ s^{-1}$. The half-time ($t_{1/2}$) for dissociation of the specifically bound radioligand was 6 min. From the values of the rate constants, the equilibrium dissociation constant was calculated $K_d^* = k_{-1}/k_1 = 1.39$ nM. This value is in a good agreement with the dissociation constant obtained by the equilibrium experiments (Fig. 3).

Binding at equilibrium conditions

The binding of ^{125}I-AaIT to the mvP_2L preparation is a saturable phenomenon (Fig. 3A). Scatchard plot (Fig. 3B) revealed a single class of noninteracting binding sites, the dissociation constant (K_d^*) being 1.2 nM. The dissociation constant (K_d) for unlabeled AaIT obtained by displacement of the ^{125}I-AaIT by increasing concentrations of unlabeled AaIT was very similar (Fig. 6, Table V) resulting in a $K_d = 1.15 \pm 0.15$ nM ($n = 6$). The calculated binding site capacity (Fig. 3B) was 1.37 pmol/mg protein which varied in the range of 1.2–2.0 pmol/mg protein.

Assays on the uptake of tetraphenylphosphonium (TPP^+)

Fig. 4 demonstrates the dependence of the uptake of TPP^+ on external potassium concentration in mvP_2L loaded with 0.1 M potassium phosphate buffer, in the presence of the potassium ionophore valinomycin. The uptake was relatively rapid and the steady-state level of accumulation was achieved

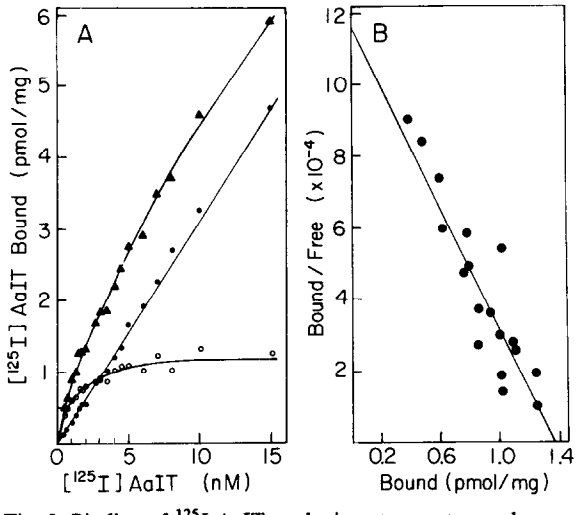

Fig. 3. Binding of ^{125}I-AaIT to the insect synaptosomal membrane vesicles (mvP_2L). (A) The binding was measured after 30 min of incubation in the standard binding medium at 20°C with increasing concentrations of labeled toxin in reaction mixtures containing 40 μg of membrane protein, in the absence (▲) and presence (●) of 1 μM unlabeled AaIT. The specific binding (○) is the difference between the two curves. (B) Scatchard plot of specific binding data as taken from (A).

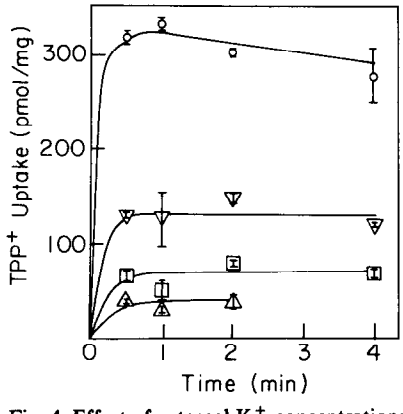

Fig. 4. Effect of external K^+ concentrations on TPP^+ accumulation in the insect synaptosomal membrane vesicles (mvP_2L). Membrane vesicles (96 μg protein per assay) were incubated in 150 mM choline chloride, 1 mM $MgSO_4$ in the presence of 6.28 μM TPP^+, 1 μCi of [^{3}H]TPP^+ and 5 μM valinomycin. The external K^+ concentration of the medium was 3.8 mM (○), 18.8 mM (▽), 63.8 mM (□) and 150 mM (△), and the choline concentration was varied reciprocally.

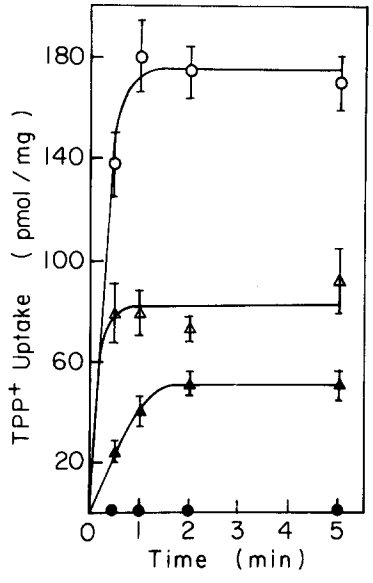

Fig. 5. Effect of ionophores on TPP^+ accumulation in the insect synaptosomal membrane vesicles. Membrane vesicles (50 μg protein per assay) were incubated in the presence of 4.4 μM of [^{3}H]TPP^+ in a medium either composed of 0.15 M choline chloride, 1 mM $MgSO_4$ in the absence of ionophores (△), in the presence of 5 μM valinomycin (○) or 5 μM CCCP (▲), or in medium composed of 0.1 M potassium phosphate (pH 6.8), 1 mM $MgSO_4$ in the presence of 5 μM valinomycin (●).

in less than 1 min. With increasing external K^+ concentrations the concentration gradient of TPP^+ established at the steady state decreased from about 8 at $[K^+]_{out}$ of 3.8 mM to about 1 at $[K^+]_{out}$ of 150 mM (Fig. 4). Fig. 5 demonstrates the effect of ionophores on the TPP^+ uptake in the mvP_2L preparation. The ionophore valinomycin selectively enhances the membrane permeability to K^+. Under the conditions of the experiments with $[K^+]_{in} > [K^+]_{out}$, valinomycin is expected to enhance the magnitude of the membrane potential (interior negative) and this increases the TPP^+ concentration gradient ($[TPP^+]_{in}/[TPP^+]_{out}$) from 3.2 to 7.1 in the presence of valinomycin (Fig. 5). When the protonophore CCCP was added, the TPP^+ accumulation decreased and its concentration gradient corresponded to about 2. Although the potential difference across the membrane is due primarily to K^+ diffusion potential, enhanced membrane permeability to cations other than K^+ (in this case H^+) should cause, to a certain extent, charge neutralization across the membrane [19],

TABLE II

VALUES OF MEMBRANE POTENTIAL IN THE INSECT SYNAPTOSOMAL MEMBRANE VESICLES CALCULATED ACCORDING TO THE CONCENTRATION GRADIENT OF TPP^+ COMPARED TO THE THEORETICAL VALUES AS BASED ON THE INITIAL K^+ GRADIENTS

Based on the data presented in Fig. 4, in the presence of 5 μM valinomycin. The estimation of $[TPP^+]_{in}$ and $[TPP^+]_{out}$ was performed as described in Materials and Methods. $\Delta\psi$ was calculated according to the Nernst equation: $\Delta\psi = -58 \log[ion^+]_{in}/[ion^+]_{out}$.

$[K^+]_{out}$ (mM)	$\frac{[TPP^+]_{in}}{[TPP^+]_{out}}$	$\Delta\psi$ (mV)	$\frac{[K^+]_{in}}{[K^+]_{out}}$	$\Delta\psi$ (mV)
3.8	8.01	−52.4	39.47	−92.6
18.3	2.87	−26.6	8.20	−53.0
63.8	1.03	−0.72	2.36	−21.5
150	0.96	+1.03	1.00	0

which is expressed in the decrease in TPP^+ accumulation (Fig. 5). The equalization of the internal and external medium (0.1 M potassium phosphate buffer inside and outside the vesicles) is expected to abolish the potential difference across the membrane as reflected in the complete prevention of the TPP^+ accumulation (Fig. 5). However, there exists a difficulty in the determination of the absolute values of membrane potential, due to the disagreement between the values of $\Delta\psi$ obtained through the expected initial K^+ concentrations and those which follow from the TPP^+ concentration gradients (Table II). This disaccordance may result from difficulties such as that concerning the actual value of $[K^+]_{in}$ (less than the expected 150 mM) and a relatively limited permeability of the membrane to K^+. There is no doubt, however, that the different degrees of TPP^+ accumulation monitor different levels of membrane potential.

Binding of ^{125}I-AaIT to the insect synaptosomal membrane vesicles at different membrane potential conditions

Table III presents the binding of ^{125}I-AaIT to mvP_2L incubated in different external media, shown to modify the TPP^+ uptake (Fig. 5 and Table III), thus indicating differences in their transmembrane potential. As shown (Table III), this did not modify the binding capacity of the synaptosomal vesicles to ^{125}I-AaIT, indicating its voltage-independence.

TABLE III

TPP$^+$ UPTAKE AND THE BINDING OF ^{125}I-AaIT TO THE INSECT SYNAPTOSOMAL MEMBRANE VESICLES IN DIFFERENT MEDIA SHOWN (Fig. 5) TO MODIFY THE MEMBRANE POTENTIAL

The mvP$_2$L (20 μg protein per assay) loaded with the standard loading medium (0.1 M potassium phosphate, 1 mM $MgSO_4$ (pH 6.8)) were incubated with ^{125}I-AaIT (1 nM) in the different external media. The data on the TPP$^+$ are taken from the experiment presented in Fig. 5 and they represent the level of uptake at steady state after 2 min of incubation. The data correspond to mean ± S.D. (n = number of assays). BSA, bovine serum albumin.

Composition of external medium	Specific binding of ^{125}I-AaIT (fmol/mg protein)	Uptake of [^{3}H]TPP$^+$ (pmol/mg protein)
Standard loading medium + 0.1% BSA + valinomycin (5 μM)	563 ± 107 (3)	0 ± 0.07 (9)
Standard binding medium	562 ± 61 (3)	80.6 ± 17.8 (3)
Standard binding medium + valinomycin (5 μM)	569 ± 162 (3)	174.4 ± 26.6 (3)

TABLE IV

THE BINDING OF THE AaIT AND THE α (AaH II) AND β (Css II) SCORPION TOXINS TO INSECT AND MAMMALIAN SYNAPTOSOMAL PREPARATIONS

The ^{125}I-AaIT and ^{125}I-AaH II were employed in a concentration of 1.5 nM in the standard binding medium at 20°C, the ^{125}I-Css II was used in 6 nM in a medium composed of 0.15 M choline chloride, 0.1% bovine serum albumin at 10 and 20°C. For the determination of the nonspecific binding, the respective unlabeled toxins were applied in a concentration of 2 μM. –, none.

Preparation	Toxin	Binding site capacity (fmol/mg protein)	Ref.
Rat brain synaptosomes	AaH II	360	27
	Css II	1200	27
	AaIT	–	
Insect mvP$_2$L	AaH II	–	
	Css II	–	
	AaIT	1370	

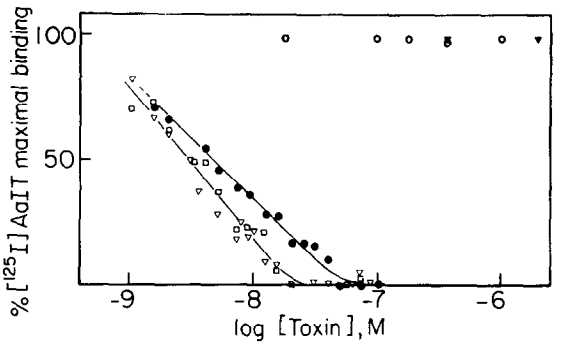

Fig. 6. Displaceability of the ^{125}I-AaIT by several insect and vertebrate toxins. The mvP$_2$L vesicles were incubated under the standard conditions (see also legend to Fig. 3) with ^{125}I-AaIT (1.5 nM) and increasing concentrations of each of the following unlabeled toxins: AaIT (□), BjIT$_1$ (●), BjIT$_2$ (▽), AaH II (▼), and Css II (○).

Effects of different neurotoxins on the binding of ^{125}I-AaIT to the insect synaptosomal membrane vesicles

As shown in Table IV, the AaH II and the Css II did not reveal any detectable specific binding to the mvP$_2$L preparation. In a reciprocal experiment the ^{125}I-AaIT was devoid of any detectable binding to membrane vesicles prepared from rat brain synaptosomes (Table IV).

In assays on competitive displacement presented in Fig. 6 the AaH II and CssII toxins, applied in high concentrations, up to 2.6 μM, did not affect the binding of the ^{125}I-AaIT to the

TABLE V

THE BINDING AFFINITY OF THE UNLABELED AaIT, BjIT$_1$ AND BjIT$_2$ TO THE INSECT SYNAPTOSOMAL MEMBRANE VESICLES

These data were obtained from the experiment presented in Fig. 6. The K_d values were calculated according to the following equation [18]: $K_d = K_{0.5}/(1 + ([L^*]/K_d^*))$ where the $K_{0.5}$ is the concentration of the competing unlabeled compound which inhibits specific binding by 50% at equilibrium, [L*] is the concentration of free labeled ligand and K^* is the equilibrium dissociation constant of the labeled ligand which is 1.2 nM for the ^{125}I-AaIT (Fig. 3).

	$K_{0.5}$ (nM)	K_d (nM)
AaIT	2.75	1.30
BjIT$_1$	4.6	2.2
BjIT$_2$	2.75	1.30

mvP_2L preparation (Fig. 6). Similarly, blockers and modifiers of sodium channels, such as tetrodotoxin (2 μM), veratridine (50–500 μM) and *Anemonia sulcata* toxin II (5 μM), did not modify the binding of the ^{125}I-AaIT. The binding, however, was readily displaced by two other insect toxins, $BjIT_1$ and $BjIT_2$ (Fig. 6). Table V presents the values of $K_{0.5}$ and K_d of the *B. judaicus* toxins compared to those of the unlabeled AaIT. As shown, the three insect toxins possess very similar affinities to the insect neuronal preparation.

Discussion

The selective neurotoxicity of AaIT (see Introduction) prompted us to adopt a chemical-neuropharmacological approach to the study of its interaction with receptor sites on the insect neuronal membrane. Such an approach required the development of an appropriate insect neuronal preparation. The suitability of the present insect synaptosomal membrane vesicles (mvP_2L) to fulfill this role was assessed by morphological [11,22], enzymatic [22] and neurotransmitter active transport [11,23] criteria. Concerning the latter, it was shown that the transport of γ-aminobutyric acid [11] and that of choline [23] by the mvP_2L preparation are high-affinity electrogenic processes, both driven by Na^+ and Cl^- gradients. The data presented in Table I indicate a close correlation between the abilities of the different insect synaptosomal fractions to actively transport γ-aminobutyric acid and to bind the AaIT. The mvP_2L preparation has demonstrated the highest specific activity in both respects.

In the present work, the pharmacological functionality of this preparation was further assessed by its ability to maintain a membrane potential in a modifiable manner. It has been shown that the membrane potential, as monitored by TPP^+ accumulation, is modified by various external concentrations of K^+ (Fig. 4) and by the ionophores valinomycin and CCCP(Fig. 5, Table III). Notwithstanding the difficulty in the $\Delta\psi$ determination (see Results), the variations in TPP^+ accumulation reflect different levels of membrane potential, and thus its effect on the binding of AaIT can be clarified.

The scorpion venom toxins affecting vertebrates, which play an essential role in the pharmacological and chemical characterization of Na^+ channels in mammalian excitable tissues [20,21,24–28], are presently subdivided into two categories [20,26,27]. The α toxins, such as the AaH II or Lqq V affect sodium inactivation, possess a voltage-dependent binding ability and positive cooperativity with the lipid-soluble alkaloids, such as veratridine [24–30]. The β toxins (derived from the venoms of *Centruroides* and *Tityus* scorpions) such as the Css II affect sodium activation, possess potential independent binding sites distinct from those of the α toxins, and do not interact synergically with veratridine [20,27]. The binding sites of both groups of toxins were shown to be distinct from those of tetrodotoxin, saxitoxin and alkaloids, such as veratridine and batrachotoxin [20,24–27].

The binding of the AaIT to the insect synaptosomal membrane vesicles reveals several similarities to that of the scorpion toxins affecting vertebrates.

(1) Like the vertebrate toxins (Refs. 21, 24, 27–30 and Table IV) the ^{125}I-AaIT binds specifically to a single class of noninteracting binding sites of a high affinity ($K_d^* = 1.2$ nM) and low capacity (1.37 pmol/mg protein).

(2) The binding of AaIT was unaffected in the presence of high concentrations of tetrodotoxin and veratridine. This indicates that, like with the vertebrate toxins, its binding site is distinct from those of the above Na^+-channel effectors.

(3) As shown (Fig. 5, Table III), the employment of insect synaptosomal membrane vesicles possessing three different levels of experimentally induced membrane potentials (as monitored by TPP^+ uptake) did not modify the binding of the ^{125}I-AaIT, indicating its potential independence. In this respect, the AaIT differs from the α-toxins, but strongly resembles the β-toxins, as exemplified by the CssII [20,21,27].

The AaIT and Css II share additional common features, namely (a) the induction of repetitive firing [7,27] and (b) the absence of positive cooperativity with veratridine [27], in the respective neuronal preparations.

In spite of the basic similarities of the binding properties of AaIT to those of the scorpion vertebrate toxins, and mainly the β toxins, the AaIT is unique in its selective neurotoxicity to

insects (see Introduction). This aspect of selectivity was further emphasized in the present study. As shown (Table IV), the ^{125}I-AaIT did not reveal any specific binding to the respective mammalian neuronal preparation, and the radioiodinated AaH II and Css II did not bind specifically to the insect mvP_2L preparation. Furthermore the above toxins when applied in high concentrations did not affect the binding of the ^{125}I-AaIT to the insect synaptosomal membrane vesicles (Fig. 6).

The possession of an AaIT binding site appears to be a unique property of the insect neuronal membrane. Presently, there exist three pieces of evidence indicating a probable relation between the AaIT binding site and sodium channels: (a) the very resemblance (see above) in the binding properties of the AaIT to those of the scorpion venom vertebrate toxins, which are well known markers of sodium channels [24–27]; (b) the accordance between the binding capacity of the ^{125}I-AaIT (Fig. 3) and that of [^{3}H]saxitoxin (1.43 pmol/mg protein) to the same mvP_2L preparation (unpublished data), and (c) neurophysiological data indicating a specific effect of the AaIT on sodium conductance in an isolated insect axonal preparation [7,8].

These considerations direct attention to the two insect toxins derived from the venom of the scorpion *B. judaicus*. $BjIT_1$ mimics the AaIT by the induction of a fast spastic excitatory paralysis of the intact insect and repetitive firing and a slight increase of the sodium peak current in the isolated insect axon [7,10]. $BjIT_2$ induces a slow flaccid-paralysis of the insect and a depolarization (reversed by saxitoxin), action potential blockage and partial suppression of the Na^+ current in the isolated insect axon [10]. Both toxins do not affect potassium current and their effect is restricted to sodium conductance. The present data (Fig. 6, Table IV) indicate that these two toxins share an additional common property: they are able to competitively displace the ^{125}I-AaIT with K_d values closely resembling those of the labeled and unlabeled AaIT (Table V). This suggests that the two different kinds of insect toxins ('spastic' and 'flaccid') either share a common binding site or possess separate, but mutually related, interacting binding sites, probably associated to the insect neuronal sodium channel. Further experimental clarification is required to decide between these possibilities.

Acknowledgements

The authors are grateful to Prof. H. Rochat (Biochemistry, Medical School, Marseille, France) for his help, hospitality and encouragement and to Dr. B. Kanner (Biochemistry, Medical School, Hebrew University, Jerusalem) and Dr. Z. Teitelbaum (Radiochemistry, Nuclear Research Centre, Negev, Israel) for their fruitful consultations. This study was supported by fellowships from INSERM, France, granted to E.Z. and D.G., and by grant No. 2461/81 from the U.S.-Israel Binational Science Foundation (BSF) Jerusalem.

References

1 Zlotkin, E., Rochat, H., Kopeyan, C., Miranda, F. and Lissitzky, S. (1971) Biochimie (Paris) 53, 1073–1078
2 Darbon, H., Zlotkin, E., Kopeyan, C., Van Rietschoten, J. and Rochat, H. (1982) Int. J. Peptide Protein Res. 20, 320–330
3 Zlotkin, E., Miranda, F and Lissitzky, S. (1972) Toxicon 10, 207–210
4 Walther, C., Zlotkin, E. and Rathmayer, W. (1976) J. Insect. Physiol. 22, 1187–1194
5 Rathmayer, W., Ruhland, M., Tintpulver, M., Walther, C. and Zlotkin, E. (1978) in Toxins: Animal, Plant and Microbial (Rosenberg, P., ed.), pp. 629–637, Pergamon, Oxford
6 Teitelbaum, Z., Lazarovici, P. and Zlotkin, E. (1979) Insect Biochem. 9, 343–346
7 Pelhate, M. and Zlotkin, E. (1981) J. Physiol. Lond. 319, 30–31
8 Pelhate, M. and Zlotkin, E. (1982) J. Exp. Biol. 97, 67–77
9 Zlotkin, E., Teitelbaum, Z., Rochat, H. and Miranda, F. (1979) Insect Biochem. 9, 347–354
10 Lester, D., Lazarovici, P., Pelhate, M. and Zlotkin, E. (1982) Biochim. Biophys. Acta 701, 370–381
11 Gordon, D., Zlotkin, E. and Kanner, B. (1982) Biochim. Biophys. Acta 688, 229–236
12 Miranda, F., Kopeyan, C., Rochat, C., Rochat, H. and Lissitzky, S. (1970) Eur. J. Biochem. 16, 514–523
13 Garcia, L.G. (1976) Etude des Neurotoxines du Venin du Scorpion Mexicain *Centruroides suffusus suffusus*, Thèse d'Université, Faculté des Sciences, Nice, France
14 Rochat, H., Tessier, M., Miranda, F. and Lissitzky, S. (1977) Anal. Biochem. 82, 532–548
15 Kanner, B.I. (1978) Biochemistry 17, 1207–1211
16 Rames, S., Grollman, E.F., Lazo, P.S., Dyer, S.A., Habig, W.H., Hardegree, M.C., Kaback, H.R. and Kohn, L.D. (1979) Proc. Natl. Acad. Sci. USA 76, 4783–4787
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

18 Weiland, G.A. and Molinoff, P.B. (1981) Life Sci. 29, 313–330
19 Lichtstein, D., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 650–654
20 Jover, E., Couraud, F. and Rochat, H. (1980) Biochem. Biophys. Res. Commun. 95, 1607–1614
21 Jover, E., Bablito, J. and Couraud, F. (1984) Biochemistry, in the press
22 Breer, H. and Jeserich, G. (1980) Insect Biochem. 10, 457–463
23 Breer, H. (1983) FEBS Lett. 153, 345–348
24 Catterall, W.A. (1980) Annu. Rev. Pharmacol. Toxicol. 20, 15–43
25 Catterall, W.A. (1982) Trends Neurosci. 5, 303–306
26 Couraud, F. and Jover, E. (1984) in Handbook of Natural Toxins (Tu, A.T., ed.), Marcel Dekker, New York, in the press
27 Couraud, F., Jover, E., Dubois, J.M. and Rochat, H. (1982) Toxicon 20, 9–16
28 Jover, E., Martin-Moutot, N., Couraud, F. and Rochat, H. (1980) Biochemistry 19, 463–467
29 Couraud, F., Rochat, H. and Lissitzky, S. (1978) Biochem. Biophys. Res. Commun. 83, 1525–1530
30 Catterall, W.A., Morrow, C.S. and Hartshorne, R. (1979) J. Biol. Chem. 254, 11379–11387