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PHOTOAFFINITY LABELING OF SCORPION TOXIN RECEPTORS ASSOCIATED WITH INSECT SYNAPTOSOMAL NA+ CHANNELS

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SUMMARY : Photoreactive and radioiodinated derivatives of several scorpion toxins acting on insect Na+ channels were prepared without loss of their pharmacological activities. Photoaffinity experiments were carried out on a synaptosomal fraction from the nerve cord of the cockroach Periplaneta americana: with all toxin derivatives, a single specifically labeled band was obtained with a molecular weight of 188,000 ± 12,000 (n = 17). These results indicate for the first time the molecular weight of the scorpion toxin receptor from the insect nervous system which is probably associated with voltage sensitive Na+ channels. One of these toxins, toxin VII from Tityus serrulatus venom, has been previously shown to be active both in mammals and in insects, in rat brain synaptosomes this toxin labeled a Mr = 31,000 ± 4,000 band in contrast, to observations in the insect preparation.

The structure of vertebrate voltage sensitive Na+ channels has been extensively studied during the last few years (for a review see 1). They appear to be formed by a large glycoprotein of M_r = 260,000 - 280,000, called the α-subunit, found in eel electroplax (2), in avian heart (3), in rat skeletal muscle (4) and brain (5). In the last two cases the α-subunit is associated with one or two smaller glycopeptides of M_r = 34,000 - 38,000, called β-subunits. The amino acid sequences of the α-subunits from eel electroplax (6) and rat brain (7) have been deduced from the sequence of the cloned cDNAs. In contrast, Na+ channels from invertebrates have not yet been purified and the only available information comes from the deduced partial amino acid sequence of a Drosophila gene isolated with a vertebrate Na+ channel cDNA (8).

In order to establish the molecular weight(s) of insect Na+ channel component(s), we have carried out photoaffinity labeling experiments using scorpion toxins as probes. Some of these toxins have been shown to specifically interact with mammalian Na+ channels : α-scorpion toxins bind to site 3 and β-scorpion toxins to

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site 4 (1, 9) and photoreactive derivatives have been used to covalently label channel components (10-12). These toxins ("mammal toxins") are inactive on insects (13). Proteins homologous to the "mammal toxins" have been purified, from scorpion venoms, that are inactive on mammals but active on insect Na^+ channels (14-17), and have been called "insect toxins" (IT). In the present report we describe the preparation of photoreactive and radioiodinated derivatives of several "IT", and we show the results of photoaffinity labeling experiments in synaptosomal preparations from the cockroach Periplaneta americana. In addition, in order to compare photoaffinity labeling in cockroach nerve cord and rat brain synaptosomes, we have used a toxin from the scorpion Tityus serrulatus (Ts VII) that has been shown to be active both in mammals and in insects (17-19).

MATERIALS AND METHODS

Materials. Molecular weight standards were from Bio-Rad. Succinimidyl-4-azido-2 nitrophenylaminoacetate (su-ANPAA) was a generous gift of Dr. Angelides (Gainesville-Florida, USA), N-succinimidyl-6 (4-azido-2' nitrophenylamino) hexanoate (su-ANPAH) and N-5-Azido 2 nitrobenzoyloxysuccinimide (ANB-NOS) were purchased from Pierce Chemical Co.(Rockford IL, USA), and carrier free $Na[^{125}I]$ from Amersham Co. All the other chemicals used were of analytical grade.

Iodination of toxins. The AaH IT from Androctonus australis Hector, Lqq IT_1 and IT_2 from Leiurus quinquestriatus quinquestriatus and Ts VII from Tityus serrulatus, were purified in the laboratory (13, 14, 20) and radioiodinated using either iodogen for Ts VII (17) or lactoperoxidase for AaH IT, Lqq IT_1 and Lqq IT_2 (21). Specific radioactivities of 500 to 1,000 Ci/mmol were obtained.

Preparation of the synaptosomal fractions. The nerve cord synaptosomal fraction from the cockroach was prepared according to Lummis & Sattelle (22) with some minor modifications. Abdominal and thoracic nerve cords were homogenized with a Potter-Elvejhem apparatus (800 rpm, 15 strokes with 1 min interval every 5 strokes in order to avoid heating) at 10 % (w/v) in an ice cold buffer (concentration in mM : 10 glucose, 140 choline chloride, 5.4 KCl, 0.8 MgSO4, 25 HEPES adjusted to pH 7.4 with Tris) and a mixture of protease inhibitors (final concentration in mM : 0.1 phenylmethylsulfonyl fluoride, 1.0 iodoacetamide, 1.0 (1,10)-phenantroline, 0.001 pepstatin A, 1.0 EDTA). The homogenate was centrifuged at 1,000 g for 10 min at 4°C (twice) and the pellet discarded. The supernatant was centrifuged at 30,000 g for 30 min and the pellet resuspended in the same buffer (1 ml/g nerve cords). The protein content was determined according to Peterson (23) with bovine serum albumin (BSA) as a standard. A synaptosomal fraction (P2) from rat brain was prepared as described (24).

Binding and photoaffinity labeling experiments The incubation of labeled toxins and synaptosomal membranes was carried out as previously described (17). The photoreactive derivatives of the radioiodinated toxins were synthetized as previously described (11) except that three different reagents, ANB-NOS, su-ANPAA and su-ANPAH were used, with a molar ratio of 1 molecule of reagent per amino group of toxin and BSA. These photoreactive and iodinated toxins were then incubated in the dark with cockroach or rat synaptosomal membranes in the homogenization buffer supplemented with 1.8 mM $CaCl_2$ and 0.2% BSA (without EDTA) for 90 min at 25°C. At the end of the incubation period synaptosomes were irradiated for 5 min with a Phillips UV lamp (λ max = 356 nm) placed 5 cm above the samples, then centrifuged for 15 min at 100,000 g. The pellet was washed with the ice-cold incubation buffer, then with the BSA-free ice cold incubation buffer. Membranes were resuspended in 30 μl of distilled and ice cold water and then denatured by addition of 45 μl of a 3% SDS, 70 mM Tris, 10 mM EDTA, 10% glycerol buffer at pH 9. In

some experiments, reduction of disulfide bridges was obtained by addition of β-mercaptoethanol at a final concentration of 10-50 mM. The solution was incubated at 100°C firstly 5 min, and then 2 min, after addition of 40-200 mM iodoacetamide. SDS polyacrylamide gel gradient (5-15%) electrophoresis was performed essentially as previously described (11), using the discontinuous gel system of Maizel (25). Autoradiography was carried out using a Kodak X-Omat S film with an intensifying screen.

RESULTS AND DISCUSSION

We have previously described the presence of specific binding sites for Ts VII and AaH IT in a house fly head synaptosomal preparation (17). As preliminary experiments showed that the binding capacities were too low to allow successful photoaffinity labeling, we have prepared a nerve cord synaptosomal fraction from the cockroach P. americana which has about seventy times more sites for AaH IT than the house fly head preparation (5,000 fmol instead of 70 fmol per mg of protein). Furthermore, the affinities were very similar to those measured in the fly synaptosomes, with dissociation constants of 0.04, 0.25, 0.50 and 0.55 nM for Ts VII, Lqq IT_1, Lqq IT_2 and AaH IT, respectively. In addition, all these toxins compete for the same binding site as previously shown in other insect synaptosomal preparations (14, 17). Figure 1 shows representative results obtained in photoaffinity experiments carried out with these four toxins : a single band of about M_r = 195,000 was specifically labeled with all the toxin derivatives using either the su-ANPAA or su-

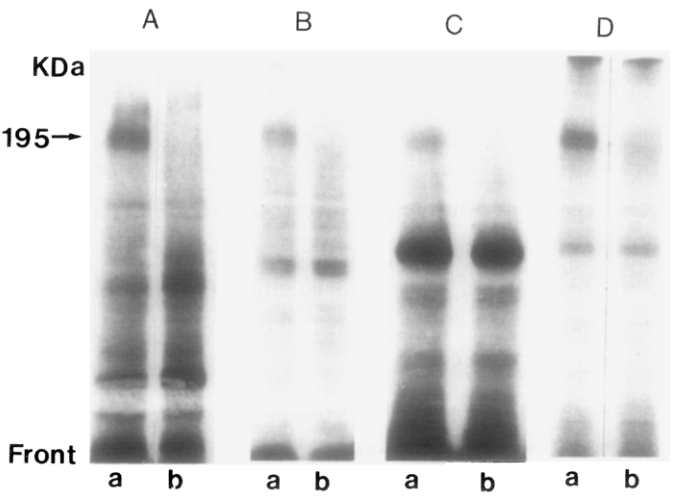


Figure 1. COMPARISON OF COVALENTLY LABELED POLYPEPTIDES OF COCKROACH (P. AMERICANA) NERVE CORD SYNAPTOSOMES USING PHOTOREACTIVE IODODERIVATIVES OF SCORPION TOXINS ACTIVE IN INSECTS.
Synaptosomes (250 to 500 μg protein per assay) were covalently labeled using the following derivatives : (A) 0.1 nM ANPAA[^{125}I]AaH IT ; (B) 0.5 nM ANPAH[^{125}I]-Lqq IT_1 ; (C) 1.0 nM ANPAH[^{125}I]Lqq IT_2 ; (D) 0.5 nM ANPAA[^{125}I]Ts VII, in the absence (a) or the presence (b) of an excess (1 μM) of the corresponding native toxins. Membrane proteins were denatured, reduced and analyzed on polyacrylamide gels as described in "Materials and Methods". The M_r indicated in the figure corresponds that of the receptor/ligand complex before subtraction of 7000, the contribution of the scorpion toxin.

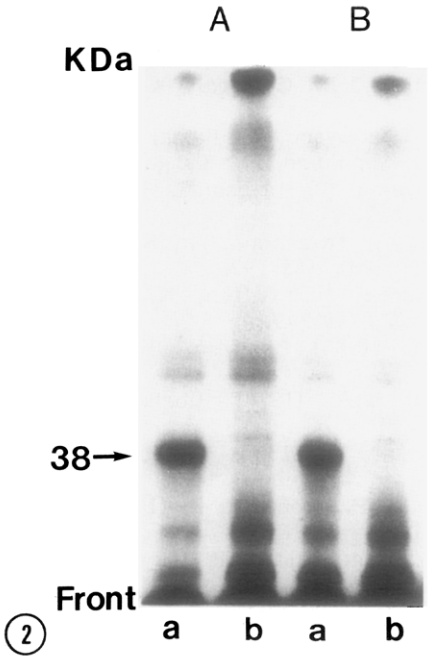
ANPAH reagent. After subtracting the toxin molecular weight the average molecular weight of the labeled component was 188,000 ± 12,000 (the mean values were 192,000 ± 15,000 with Ts VII, n = 7, 173,000 ± 2,000 with Lqq IT_1, n = 2, 177,000 ± 9,000 with Lqq IT_2, n = 2 and 188,000 ± 12,000 with AaH IT, n = 6).

Since the only high molecular weight component in the vertebrate Na^+ channel is the M_r = 260,000 α-subunit (1) and since an homologous protein has been shown to be present in Drosophila by cDNA techniques (8), we may assume that the component labeled in our experiments is homologous to the α-subunit described in vertebrates tissues. The difference in molecular weight between insects and vertebrate (M_r = 188,000 versus 260,000) may be due to : (i) proteolysis that occurs in insect tissue despite the presence of protease inhibitors; (ii) a much lower level of glycosylation of the α-subunit in insects; (iii) a difference in the size of the polypeptide chain.

Since Ts VII has been shown to be active both in mammals and in insects (17), we used the Ts VII photoreactive derivatives in photoaffinity labeling experiments on rat brain synaptosomes. In this preparation, a low molecular weight component of 31,000 ± 4,000 (after subtraction of the molecular weight of the toxin) was specifically labeled (Fig. 2). This confirms results previously obtained with other β-scorpion toxins on the same preparation (12). The non labeling of a similar subunit in the insect preparation may be interpreted as either the absence of this low molecular weight component in insect Na^+ channels as is the case in eel electroplax (2) or the non accessibility of this component to the toxin photoreactive group. In order to increase the probability of cross-linking we have used three photoreactive reagents that differ in the length of their carbon chain (1, 2 and 6 carbons for ANB-NOS, su-ANPAA and su-ANPAH, respectively). We consistently obtained the same results : only one band was specifically labeled with Mr = 195,000 ± 12,000. However, this result cannot exclude the presence of a low molecular weight component as, in other oligomeric receptors, it has been demonstrated that two different derivatives of a ligand can label different subunits (10, 26).

Figure 3 shows that the reduction of disulfide bridges by β-mercaptoethanol did not change the migration of the labeled component, as was observed in eel electroplax (2) or rat skeletal muscle (4) indicating that the β_2-subunit is not present in these latter tissues. In contrast, in rat brain synaptosomes, the $\alpha\beta_2$ complex was dissociated by reduction of disulfide bridges (5) resulting in an apparent change in the migration of the α-subunit. Our data seem to show that this β_2 subunit is also absent in the cockroach neuronal Na^+ channel.

Finally, photoaffinity labeling with arylazide derivatives of radioiodinated scorpion toxins gives a first indication of the molecular weight of the insect Na^+ channel present in the nerve cord of P. americana. This protein appears to contain a high molecular weight polypeptide that is significantly smaller than the subunit of the vertebrate Na^+ channel. Further investigations will be necessary to determine whether this results from pre or post translational differences.



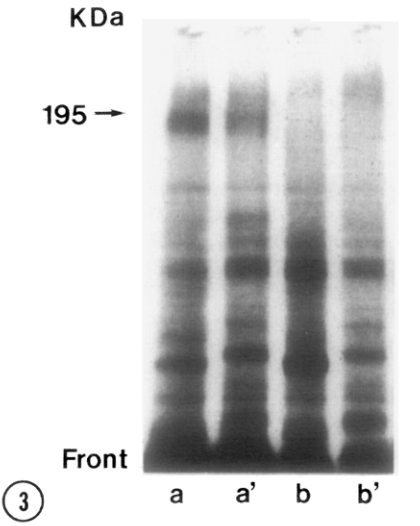


Figure 2. COMPARISON OF COVALENTLY LABELED POLYPEPTIDES OF RAT BRAIN SYNAPTOSOMES USING TWO PHOTOREACTIVE IODODERIVATIVES OF Ts VII.

Rat brain synaptosomes (500 μg protein per assay) were covalently labeled using the following derivatives : (A) 0.5 nM ANPAA[^{125}I]Ts VII and (B) 0.5 nM ANB[^{125}I]Ts VII, in the absence (a) or the presence (b) of 0.5 μM native toxin. Membrane proteins were denatured, reduced and analyzed on polyacrylamide gels as described in "Materials and Methods".The M_r indicated in the figure corresponds that of the receptor/ligand complex before subtraction of 7000, the contribution of the scorpion toxin.

Figure 3. ABSENCE OF EFFECT OF DISULFIDE BRIDGE REDUCTION ON THE MIGRATION OF THE 188 kDa COMPONENT SPECIFICALLY LABELED BY SCORPION TOXIN.

Synaptosomes from cockroach nerve cord (320 μg protein per assay) were covalently labeled using 1 nM ANPAA[^{125}I]AaH IT, in the absence (a and a') or the presence (b and b') of an excess (1 μM) of native toxin. Membrane proteins were denatured and disulfide bridges were reduced (a', b') by 50 mM β-mercaptoethanol. The labeling was then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The M_r indicated in the figure corresponds that of the receptor/ligand complex before subtraction of 7000, the contribution of the scorpion toxin.

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