Vol. 115, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 15, 1983 Pages 415-422

PHOTOAFFINITY LABELING OF α- AND β- SCORPION TOXIN RECEPTORS ASSOCIATED WITH RAT BRAIN SODIUM CHANNEL

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Received June 14, 1983

SUMMARY : Azido nitrophenylaminoacetyl [^{125}I]iodo derivative of toxin II from Centruroides suffusus suffusus, a β-toxin, and azido nitrophenylaminoacetyl [^{125}I]iodo derivative of toxin V from Leiurus quinquestriatus quinquestriatus, an α-toxin, have been covalently linked after binding to their receptor sites that are related to the voltage sensitive sodium channel present in rat brain synaptosomes. Both derivatives labeled two polypeptides of 253000 ± 20000 and 35000 ± 2000 mol. wt. Labeling was blocked for each derivative by a large excess of the corresponding native toxin but no cross inhibition was obtained. These results suggest that both α - and β - scorpion toxin receptors are located on or near the same two membrane polypeptides which may be part of the voltage dependent sodium channel.

Voltage sensitive sodium channels that are responsible for the rapid depolarization phase of the action potential in excitable cells possess different receptor sites for neurotoxins (1) : site 1 binds the heterocyclic guanidines, i.e tetrodotoxin and saxitoxin ; site 2 binds the lipid-soluble toxins, i.e. batrachotoxin, veratridine, aconitine and grayanotoxin ; and site 3 binds α-scorpion and sea anemone toxins (2,3). These binding sites were first described in neuroblastoma cells (1) and then in rat brain synaptosomes (4, 5). In rat brain synaptosomes the existence of a fourth binding site has been shown (2) which recognizes β-scorpion toxins. In contrast to the binding of β-scorpion toxins to site 4, binding of α-scorpion toxins (2-4) and sea anemone toxin (1-16) to site 3 was dependent on membrane potential.

The rat brain saxitoxin receptor has been purified and its molecular weight and subunit structure determined (6, 7). As far as the α -scorpion toxin receptor is concerned, only the

0006-291X/83 $1.50

molecular weight of its polypeptide components has yet been determined using photoaffinity methods (8).

In this paper, we describe the estimation of the molecular weight of the β-scorpion neurotoxin receptor by use of a photoactivable derivative of iodinated β-scorpion neurotoxin, i.e. toxin II from Centruroides suffusus suffusus and we compare it to that obtained by photoaffinity labeling of an α-scorpion neurotoxin, i.e. toxin V from Leiurus quinquestriatus quinquestriatus.

MATERIALS AND METHODS

Toxin II from Centruroides suffusus suffusus (Css II) was purified according to Garcia (9), and toxin V from Leiurus quinquestriatus quinquestriatus (Lqq V), according to Miranda et al. (10). γ toxin from Tityus serrulatus was purified according to Martin (to be published). Tris, bovine serum albumin (fraction V) and Hepes were from Sigma, lactoperoxidase from Worthington, carrier free $Na^{125}I$ from Amersham. Molecular weight standards were from Bio Rad. Succinimidyl-4-azido-2-nitrophenylaminoacetate (su-ANPAA) was a generous gift of Dr. Angelides, Gainesville, Florida.

The neurotoxins, either Lqq V or Css II were first iodinated using the lactoperoxidase immunoprecipitation method (11), then modified by the photoactivable reagent in a one-to-one molar ratio per total reactive amino group (scorpion toxin 2.10^{-7}M plus bovine serum albumin 7.6 10^{-6}M). The modification was carried out in the dark at 20°C for 3 hours in 1 ml 0.2 M Tris buffer, pH 8.8. The ANPAA[^{125}I] scorpion toxin was used immediatly for incubation with synaptosomes.

ANPAA[^{125}I] Css II (2 nM) was incubated with synaptosomes (500 µg) for 20 min at 10°C in 1 ml of medium containing 25 mM Hepes, 10 mM glucose, 140 mM choline chloride, 5.4 mM KCl, 0.25 % BSA at pH 6. Binding of ANPAA[^{125}I] Lqq V was done in the same way but incubation was for 30 min at 37°C in the above buffer containing 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, at pH 7.2.

At the end of incubation synaptosomes were irradiated for 5 min with a Phillips UV Lamp (λmax 356 nm) placed 10 cm above the sample, then centrifuged at 11000 g for 2 min. The pellet was washed twice with the ice-cold incubation buffer, then twice with a BSA-free ice-cold incubation buffer.

The membrane pellet was denatured by resuspending it in 30 µl of distilled water, then 45 µl of a 3 % SDS, 70 mM Tris, 10 mM EDTA, 10 % glycerol buffer pH 9 were added. Reduction was carried out in the presence of 5 µl of 150 mM β-mercaptoethanol. This solution was incubated 5 min at 100°C under nitrogen. Finally 8 µl of 400 mM iodoacetamide were added and the solution kept for 30 min under nitrogen at room temperature.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed essentially as previously described (12) and according to the figure legends.

Autoradiography was carried out using a Kodak X-Omat S film with an intensifying screen, and scanning was done at 550 nm on a Cello System apparatus.

RESULTS AND DISCUSSION

In order to test the reversibility of the specific component of ANPAA [^{125}I] Css II binding before and after irradiation, dissociation rates for the toxin receptor complex were measured at 10°C (Fig. 1). ANPAA [^{125}I] Css II (900 Ci/mmol) was able to bind specifically to its receptor site as association was blocked by an excess of unlabeled toxin. The half-time of release was found to be 40 sec and the dissociation rate constant 0.017 sec^{-1}. As for the [^{125}I] unmodified toxin, these data were found to be respectively 216 sec and 0.0032 sec^{-1} (13). After irradiation, the release was 55 % of that obtained without irradiation which means that 45 % of the total binding sites were covalently occupied. The same study, performed with ANPAA [^{125}I] Lqq V (Fig. 2), showed that 38 % of the binding sites were covalently occupied, the release data being 180

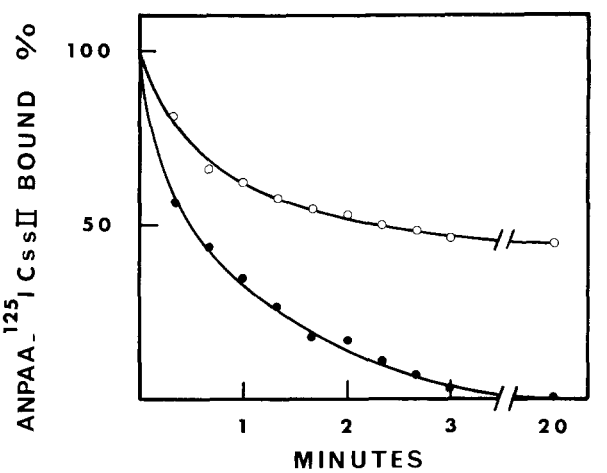

Fig. 1 : Dissociation of covalent and non covalent ANPAA [^{125}I] Css II receptor complexes. Synaptosomes were incubated with 2 nM ANPAA [^{125}I] Css II for 20 min at 10°C, then either irradiated (open symbols) or not (closed symbols). Finally, native Css II was added to a final concentration of 3.5 10^{-7}M. The synaptosomes (25 µg prot/assay) were incubated at 10°C for the periods of time indicated on the abscissa and filtered on GF/C Whatman filters. The filters were washed three times with the incubation buffer and the bound toxin was measured by counting the remaining radioactivity on the filter. In the illustrated experiment, total binding of ANPAA [^{125}I] Css II was 1864 fmol/mg prot. and non specific binding was 998 fmol/mg prot (maximum site capacity for [^{125}I] Css II was 2400 fmol/mg prot.).

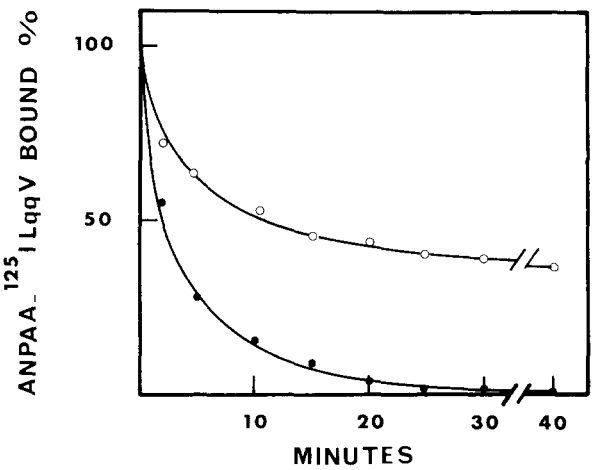

Fig. 2 : Dissociation of covalent and non covalent ANPAA[^{125}I] Lqq V receptor complexes. Experimental conditions were the same as in Fig. 1. except that ANPAA [^{125}I] Lqq V was incubated for 30 min at 37°C. Total binding of ANPAA [^{125}I] Lqq V was 570 fmol/mg prot. and non specific binding was 159 fmol/mg prot (maximum site capacity for [^{125}I]Lqq V was 430 fmol/mg prot.).

sec for the half-time of dissociation, and 0.0042 sec^{-1} for the dissociation rate constant, compared to 420 sec and 0.0017 sec^{-1} for [^{125}I]unmodified toxin.

In order to test a possible loss of specific binding upon UV treatment, an irradiation of synaptosomes was done under the same conditions but prior to addition of [^{125}I]Css II and it was shown that less than 6 % of the total binding sites were lost (results not shown).

Repeated analysis (6 independent experiments) by autoradiography of the membrane proteins after polyacrylamide gel electrophoresis revealed two major protein components of 260 ± 20 KD and 42 ± 2 KD that were labeled by ANPAA[^{125}I] Css II (Fig. 3A). Assuming that only one molecule of toxin was bound to each subunit, their real molecular weights were 253 ± 20 KD and 35 ± 2 KD. The scanning of these autoradiographies (Fig. 3B) showed that the labeling of the low molecular weigh band was 5 times more intense than that of the high one.

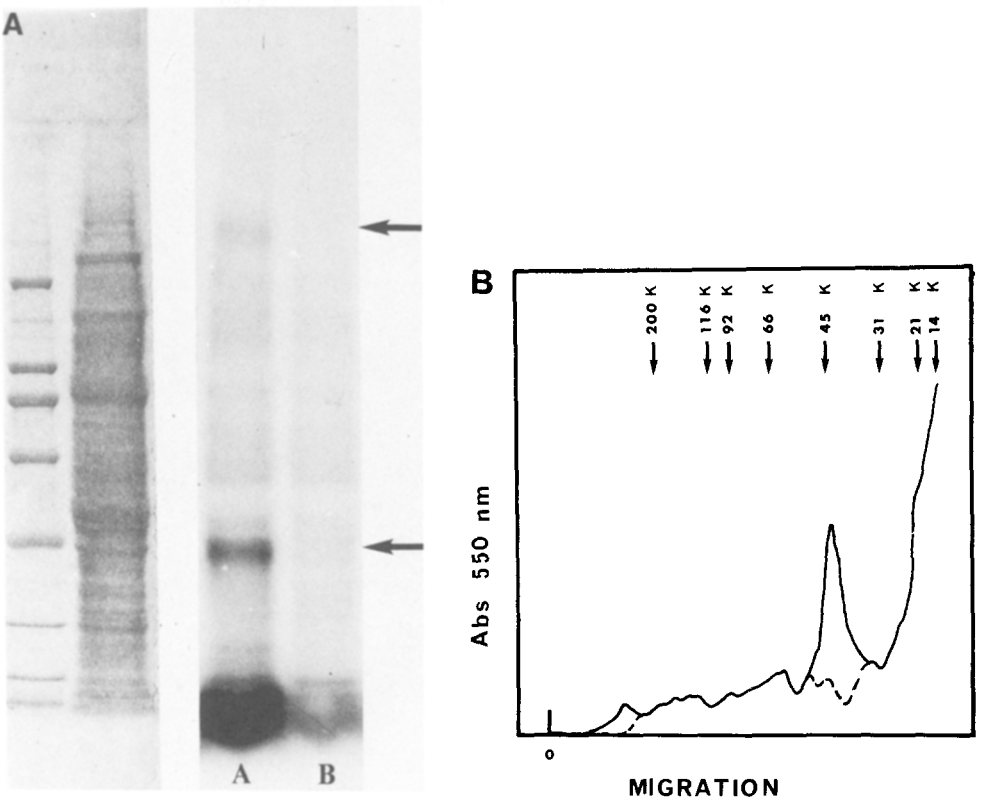

Fig. 3 : Analysis of covalently labeled polypeptides of synaptosomes by SDS gel electrophoresis using a linear gradient of acrylamide from 5% to 15%. Synaptosomes were covalently labeled by using 2 nM ANPAA [^{125}I]Css II (lane a) or 2 nM ANPAA [^{125}I]Css II plus 200 nM Css II (lane b). 500 µg of synaptosomal protein was applied to each gel track, after denaturation. An example of coomassie blue stained gel track is presented. The scanning of lanes a and b at 550 nm is shown (3B). The migration of standard molecular weight proteins is indicated on the scanning.

The same two bands were also obtained with ANPAA[^{125}I]Lqq V, but it appeared that unlabeled Lqq V was unable to decrease the intensity of the Css II derivative labeled band and reverse (Fig. 4). Another β - scorpion toxin, toxin γ from Tityus serrulatus was able to decrease the intensity of the two bands labeled by Css II derivative only.

Hartshorne et al. (6) have found that the saxitoxin receptor is composed of at least three subunits named α (Mr ∿ 270 KD), β_1 (Mr ∿ 39 KD) and β_2 (Mr ∿ 37 KD). They have demonstrated that

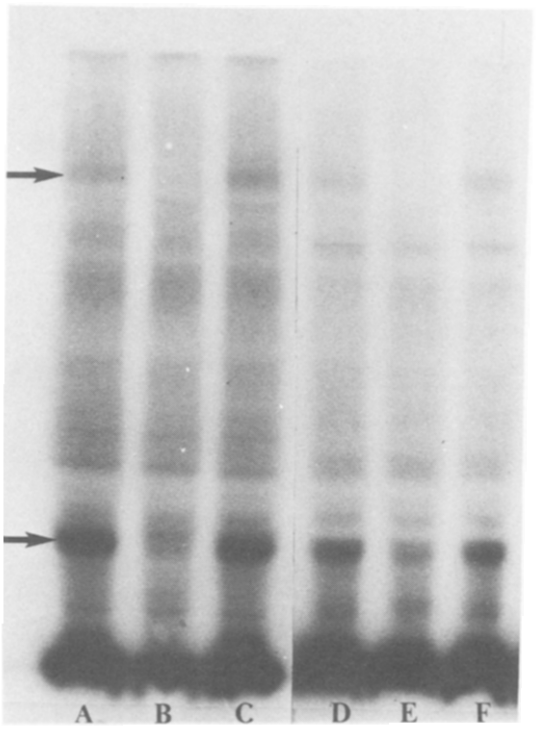

Fig. 4 : Comparison of covalently labeled polypeptides of synaptosomes by SDS gel electrophoresis using a linear gradient of acrylamide from 5% to 12%. Synaptosomes were covalently labeled using 2 nM ANPAA[^{125}I]Css II (lane a), 2 nM ANPAA[^{125}I]Css II plus 200 nM Css II (lane b), 2 nM ANPAA[^{125}I]Css II plus 200 nM Lqq V (lane c), 2 nM ANPAA[^{125}I]Lqq V (lane d), 2 nM ANPAA[^{125}I]Lqq V plus 200 nM Lqq V (lane e), 2 nM ANPAA[^{125}I]Lqq V plus 200 nM Css II (lane f). Other conditions were the same as in Fig. 3.

α- and β_2- are covalently attached by disulfide bridges, and that only α - and β_1 - subunits were labeled by a photoactivable derivative of iodinated Lqq V, i.e. azido nitro benzoyl[^{125}I]Lqq V. Our results obtained by electrophoresis of the membrane polypeptides labeled by both ANPAA[^{125}]Lqq V and ANPAA[^{125}I]Css II done without reduction by mercaptoethanol revealed a labeling pattern identical to that obtained with reduction. This result lead us to conclude that ANPAA[^{125}I]Css II labeled both α- and β_1-subunits as did ANPAA[^{125}]Lqq V. Meanwhile the low molecular weight determined was somewhat different to that of β_1- subunit, but this difference may be due to the difficulty in determining the exact molecular weight by SDS electrophoresis as previously pointed out by Gordon

(14). An additionnal remark may be made concerning Fig. 4 : in addition to the 260 KD and 42 KD labeled band, the intensity of the front line, which reflect the labeling of a small component, was decreasing when adding native Css II or Tityus γ to ANPAA[^{125}I]Css II, but not when adding native Lqq V or AaH II to ANPAA[^{125}I]Lqq V.

Furthermore, a 20 KD protein specifically labeled by ANPAA[^{125}I] Css II, but not by ANPAA[^{125}I] Lqq V, was present with variable low labeling intensity.

Predominant labeling of the 35 KD polypeptide by both Css II and Lqq V derivatives suggests either that both receptors are located on the 35 KD component or that the preferential labeling is due to the position of the azido group on the toxin in relation to the subunit components.

The fact that another β -scorpion toxin (toxin γ from Tityus venom) used in radiation inactivation or chemical cross linking experiments, i.e. in technics that are different from the one we used, revealed a 270 KD binding component (15) seem to favor the second hypothesis. Further work is needed both to ascertain the real location of the β -scorpion neurotoxin receptor either on the α- or on the β_1 - subunit or on the both and to understand the real significance of the labeled low molecular weight component.

ACKNOWLEDGEMENTS : We are grateful to Mrs P. Frachon for electrophoresis experiments, to Dr. M.F. Martin for the purification of the scorpion toxins and to Dr. M.J. Seagar for careful reading of the manuscript. This investigation was supported in part by the CNRS (ERA 070617, the ATP "Pharmacologie des récepteurs des neuromédiateurs"), the Fondation pour la Recherche Médicale and the D.R.E.T.

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