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AFFINITY LABELLING OF THE TETRODOTOXIN-BINDING COMPONENT OF THE Na^+ CHANNEL

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SUMMARY : Tetrodotoxin-binding sites were covalently labelled with a highly tritiated derivative of tetrodotoxin. Cross-linking experiments, using dissucinimidyl suberate, on partially purified tetrodotoxin-binding component from electroplax of Electrophorus electricus, revealed covalent labelling of a single polypeptide chain of MW 270,000.

INTRODUCTION : The voltage-sensitive Na^+ channel is one of the most important ionic channels involved in the generation of electrical signals by nerve, muscle and cardiac cells. This channel is the target of a large number of specific neurotoxins (1, 2). The best known of these neurotoxins are tetrodotoxin (TTX) and saxitoxin (STX). Both toxins bind to the same site and block the passage of Na^+ through the Na^+ channel (3). Recently several attempts have been made to purify the TTX/STX receptor from various excitable tissues. The TTX/STX receptor is believed to be a large glycoprotein of MW 260,000 in the electric organ of Electrophorus electricus (4, 5), a combination of polypeptide chains of MW 270,000, 39,000 and 37,000 in rat brain (6), and a combination of polypeptide chains of MW 140-220,000, 48,000 and a doublet at 37,000-38,000 in rat skeletal muscle (7).

This paper reports on the use of a highly radioactive TTX derivative, prepared by coupling one molecule of TTX with one molecule of [^{3}H]ethylenedia-

The abbreviations used are ; TTX, tetrodotoxin; STX, saxitoxin; [^{3}H]en-TTX$_{II}$, tritiated ethylenediamine tetrodotoxin derivative II; DSS, disuccinimidyl suberate; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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mine, to affinity label the polypeptide chain containing the TTX receptor in the Na^+ channel structure of the electric organ of Electrophorus electricus.

MATERIALS AND METHODS :

Chemicals. TTX was obtained citrate-free from the Sankyo Chemical Co. (Japan). A tritiated ethylenediamine TTX derivative ([^{3}H]en-TTX$_{II}$, 26 Ci/mmol) was prepared as previously described (8, 9). It corresponds to the following structure : TTX C_{11}-NH-CH_2-CH_2-NH_2. Disuccinimidyl suberate (DSS) was supplied by Pierce Chemical Co. (USA). Sephadex G50 and DEAE-Sephadex A25 were obtained from Pharmacia, France SA. All other chemicals were of the highest grade commercially available.

Preparation of fractions enriched in the TTX-binding component associated with the Na^+ channel of the electric organ of Electrophorus electricus. Packed membranes were prepared from electric organ as reported by Ellisman et al. (10) and stored in liquid nitrogen. Protease inhibitors (1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-o-phenanthroline, 1 mM iodoacetamide, 1 µM pepstatin A) were present during the membrane preparation and during subsequent purifications to minimize peptide degradation. The Na^+ channel was partially purified as follows : 10 ml of packed membranes were thawed, made 1% (wt/vol) with deionised Lubrol PX, gently homogenized with a Potter-Elvehjem homogenizer and left at 0°C for 30 min. The mixture was then centrifuged at 150,000 g for 30 min, and the supernatant was adjusted to contain 150 mM KCl. This supernatant was added to 4 ml of DEAE-Sephadex A25 preequilibrated in 150 mM KCl and 50 mM Hepes at pH 7.4 with 0.1% Lubrol PX and 0.02% phosphatidylcholine in a 20 ml syringe. After 30 min of gentle agitation at 0°C, the supernatant was filtered and the gel was washed in the syringe with 25 ml of the same buffer. The gel was rapidly equilibrated at high ionic strength by the passage of 4 ml of the buffer made 0.5 M in KCl. Elution of the TTX receptor was obtained by passage of a further 4 ml of the latter buffer at 0.5 M KCl. This fraction which will be called F-DEAE, was used for covalent cross-linking of [^{3}H]en-TTX$_{II}$ to its binding site. The specific activity of this fraction was 270 pmoles of [^{3}H]en-TTX binding sites per mg of protein, corresponding to a purity of 6-8% of the TTX receptor (11).

Covalent cross-linking of [^{3}H]en-TTX$_{II}$ to its binding site. The F-DEAE fraction was adjusted to a pH of 8.5 with 1 M KOH and was diluted in an equal volume of 50 mM potassium phosphate at pH 8.5 containing 0.1% Lubrol PX and 0.02% phosphatidylcholine. Incubation with 8 nM [^{3}H]en-TTX$_{II}$ was carried out for 15 min at 4°C either in the absence or in the presence of 10 µM unlabelled TTX. DSS, a classical cross-linking agent (12) was dissolved in dry dimethyl sulfoxide (0.1-30 mM) and was added to the incubation medium to reach final concentrations between 1 and 150 µM. After 15 min of incubation at 4°C, the cross-linking reaction was stopped by addition of a solution of 1 M Tris-Cl (10 mM final). The pH was then lowered to 7.4 with HCl 1 M to avoid degradation of the TTX receptor. Non-specific binding was measured by performing the same experiments in parallel in the presence of 10 µM TTX.

Binding assays. Binding experiments with [^{3}H]en-TTX$_{II}$ on solubilized Na^+ channels were performed as described (13). Fifty microlitres of the incubation media were first diluted to 500 µl with 50 mM potassium phosphate buffer at pH 7.4, 0.1% Lubrol PX and 0.02% phosphatidylcholine. Two aliquots of 200 µl were then taken and rapidly centrifuged through Sephadex G50 fine minicolumns. The eluate was counted for radioactivity with 7 ml Aquassure (NEN).

Polyacrylamide gel electrophoresis. Cross-linked samples were reductively denaturated (5% β-mercaptoethanol) and analyzed by 4-12% polyacrylamide gel electrophoresis according to Laemmli (14). Molecular weight calibrations were obtained using thyroglobulin (MW 330,000), myosin (MW 200,000), RNA polymerase (MW 165,000 and 155,000), the α-subunit from Electrophorus electricus Na^+,K^+-ATPase (MW 94,000), bovine serum albumin (MW 68,000), ovalbumin (MW 45,000) and cytochrome C (MW 12,400). After fixation and coloration the different lanes of the slab gel were sliced (2 mm) and treated to extract the radioactivity as described by Mahin and Lofberg (15). Seven millilitres of Aquassure (NEN) were added

to each extract and the samples were counted with a Packard 2450 scintillation spectrometer. Protein concentration was determined by the method of Peterson (16) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION : [^{3}H]en-TTX$_{II}$ is a TTX derivative containing a free amino group which can be obtained with a high specific radioactivity and which has a high affinity for Na^+ channels (8, 9). The paper describes cross-linking experiments with disuccinimidyl suberate to covalently associate [^{3}H]en-TTX$_{II}$ via its amino group to its specific receptor. Fig. 1 shows the analysis of a partially purified Na^+ channel fraction extracted from the electric organ of Electrophorus electricus cross-linked with [^{3}H]en-TTX$_{II}$ using 1 µM DSS. In this typical experiment, [^{3}H]en-TTX$_{II}$ was covalently linked to a single polypeptide chain with a MW of 270,000. This covalent labelling was totally prevented when the incubation of [^{3}H]en-TTX$_{II}$ with the partially purified Na^+ channel preparation and DSS was performed in the presence of a 1000-fold excess of unlabelled TTX. Under these conditions the excess of unlabelled TTX

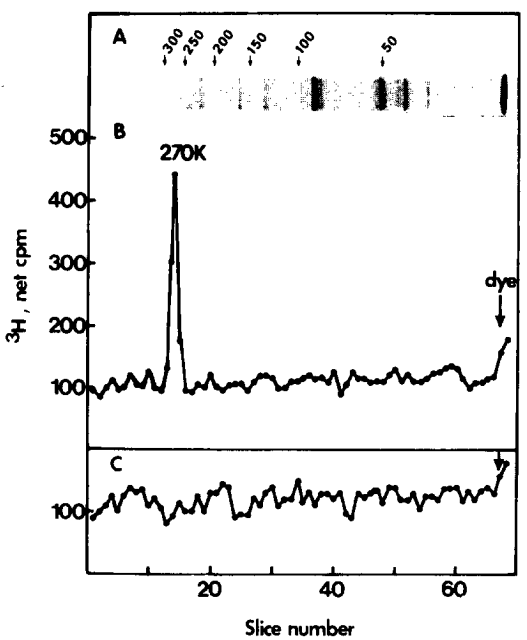

Fig. 1. NaDodSO$_4$ polyacrylamide gel electrophoresis of proteins from the F-DEAE fraction prepared from Electrophorus electricus electroplax membranes incubated in 8 nM [^{3}H]en-TTX$_{II}$ and cross-linked with 1 µM DSS. The direction of migration was left to right. (A) Coomassie blue staining profile. Gels of the same fraction incubated with [^{3}H]en-TTX$_{II}$ in the absence (B) or presence (C) of 10 µM TTX were sliced and the amount of radioactivity was determined in each 2 mm slice.

prevented the specific binding of [^{3}H]en-TTX$_{II}$ to the Na^+ channel and, consequently the grafting of [^{3}H]en-TTX$_{II}$ to its specific receptor in the presence of DSS. Profiles identical to the one presented in Fig. 1 were obtained with DSS concentrations between 1 and 5 μM. Greater DSS concentrations greatly changed the polypeptide profile seen in polyacrylamide gels coloured with Coomassie blue producing a disappearance of bands corresponding to polypeptide chains with higher MW (>340,000). Under these conditions, DSS still provoked specific covalent incorporation of [^{3}H]en-TTX$_{II}$ but the labelled material could not penetrate into the running gel indicating extensive cross-linking of the MW 270,000 polypeptide either with itself or with other polypeptide chains in its neighbourhood.

The experiments presented in Fig. 1 clearly show that the TTX binding component of the Na^+ channel extracted from Electrophorus electricus electroplax is strongly localized in a protein of MW 270,000. This conclusion is consistent with (i) the observation that antibodies prepared against a MW 250,000 polypeptide, obtained in the most extensively purified preparations of the Na^+ channel from Electrophorus electricus, immunoprecipitate the TTX/STX binding activity (17) and (ii) the MW determination of 230-260,000 obtained for the TTX receptor by radiation inactivation (18, 19). It eliminates the possibility that the TTX/STX binding activity is correlated with lower MW polypeptide chains in Electrophorus electricus electric organ.

Peptide mapping of the MW 270,000 polypeptide cross-linked with [^{3}H]en-TTX$_{II}$ will probably allow the localization of the TTX/STX receptor site associated with ion selectivity on this large polypeptide chain. This information will be useful since the same polypeptide chain is also known to contain binding sites for polypeptide toxins extracted from scorpion venom which unlike TTX and STX are known to alter preferentially the gating system of the Na^+ channel (20, 21).

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