

Covalent labeling of protein components of the sodium channel with a photoactivable derivative of scorpion toxin

(electrical excitability/sodium transport/photoaffinity labeling)

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ABSTRACT Azidonitrobenzoyl mono[¹²⁵I]iodo scorpion toxin can be covalently attached to its receptor site in electrically excitable neuroblastoma cells or synaptosomes by photolysis. A polypeptide of $M_r \approx 250,000$ is specifically labeled in neuroblastoma cells. Labeling is blocked by unlabeled scorpion toxin and by depolarization. This polypeptide is not labeled in a variant neuroblastoma clone lacking voltage-sensitive sodium channels. Polypeptides of $M_r \approx 250,000$ and $M_r \approx 32,000$ are specifically labeled in synaptosomes. This labeling is also blocked by unlabeled toxin and by depolarization. These results identify specific polypeptides of $M_r \approx 250,000$ and $M_r \approx 32,000$ that are components of the sodium channel.

Voltage-sensitive sodium channels have three separate receptor sites for neurotoxins (1, 2). Site 1 binds the inhibitors tetrodotoxin and saxitoxin (reviewed in ref. 3). Site 2 binds the lipid-soluble toxins batrachotoxin, veratridine, aconitine, and grayanotoxin (1, 4). These toxins cause persistent activation of sodium channels by an allosteric mechanism. Site 3 binds the polypeptides scorpion toxin and sea anemone toxin II (1, 2, 5). These toxins enhance activation of sodium channels by the lipid-soluble toxins (1, 2, 4, 6). Specific binding of ¹²⁵I-labeled derivatives to receptor site 3 can be measured in neuroblastoma cells (5, 7) and synaptosomes (8, 9). The K_d for binding is increased by depolarization (5, 8). The voltage dependence of binding is closely correlated with activation of the sodium channel (10), suggesting that receptor site 3 is located on a voltage-sensitive component of the sodium channel that undergoes a conformational change leading to activation. Scorpion toxin provides a valuable probe for studies of this component of the sodium channel.

Although receptor site 1 can be solubilized with retention of toxin binding activity (11-14), all scorpion toxin binding activity is lost under these conditions (14). Therefore, a method to covalently attach scorpion toxin to its receptor site would be valuable. Ligands have been specifically attached to cell surface receptors for acetylcholine (15), epidermal growth factor (16), concanavalin A (17), cyclic AMP (18), and others. Photoaffinity labeling techniques are particularly advantageous because nonspecifically bound ligand can be removed by washing in the dark before photoactivation of the reactive group (19). In this report, we describe the use of a photoactivable derivative of ¹²⁵I-labeled scorpion toxin to covalently label components of its receptor site in neuroblastoma cells and rat brain synaptosomes. A preliminary report of these results was presented at the Taniguchi International Symposium in Tokyo, November, 1979.

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EXPERIMENTAL PROCEDURES

Materials. Scorpion venom (*Leiurus quinquestratus*) was obtained from Sigma. Scorpion toxin was purified and radioactively labeled by lactoperoxidase-catalyzed iodination as described (5). Batrachotoxin was generously provided by John Daly and Bernhard Witkop (National Institute of Arthritis, Metabolism, and Digestive Disease, National Institutes of Health, Bethesda, MD 20205). Succinimidyl-5-azido-2-nitrobenzoate was synthesized from 5-amino-2-nitrobenzoic acid (K & K Chemicals) as described by Lewis *et al.* (20).

Preparation of 5-Azido-2-nitrobenzoyl Mono[¹²⁵I]iodo Scorpion Toxin (ANB-¹²⁵I₁Sctx). A photoactivable derivative of scorpion toxin was prepared by using the approach described by Lewis *et al.* (20) in their study of the oligomeric structure of cobra venom phospholipase A₂. All manipulations were carried out in red light in low actinic red glassware. Mono[¹²⁵I]iodo scorpion toxin (¹²⁵I₁Sctx) in 0.1 M Na₂CO₃, pH 9.0/1 mg of bovine serum albumin per ml was treated with 0.1 vol of succinimidyl-5-azido-2-nitrobenzoate in dioxane. The concentration of reagents was selected to give a one-to-one molar ratio of succinimidyl-5-azido-2-nitrobenzoate to total reactive amino groups (scorpion toxin plus bovine serum albumin). This procedure was chosen because it is difficult to handle low concentrations of ¹²⁵I-labeled scorpion toxin without a carrier protein. The reaction mixture was incubated at room temperature for 4 hr. The reaction was stopped by addition of standard binding medium. The quenched reaction mixture was used immediately in experiments.

Covalent Labeling of Neuroblastoma Cells. Clone N18 neuroblastoma cells were cultured as described (1). For binding experiments, confluent cells in multiwell plates were incubated with ¹²⁵I₁Sctx or ANB-¹²⁵I₁Sctx under red light in standard binding medium consisting of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM MgSO₄, 5.4 mM KCl, and bovine serum albumin at 1 mg/ml as described (5). Unbound toxin was removed by washing as described (5). Specific and nonspecific binding were determined as before (5). Then, the cells were either irradiated for 15 min at 20°C with a Sylvania Blacklite blue fluorescent bulb ($\lambda_{max} = 356$ nm) at a distance of 3 cm or kept in the dark at 20°C. After irradiation, cells were incubated for 70 min under depolarized conditions in high K⁺ binding medium (130 mM KCl replacing 130 mM choline chloride) to allow dissociation of reversibly bound toxin. The cells were washed and gently scraped from the multiwells into phosphate-buffered saline with a rubber policeman.

Abbreviations: ¹²⁵I₁Sctx or ¹³¹I₁Sctx, mono[¹²⁵I]iodo or mono[¹³¹I]iodo scorpion toxin; ANB-¹²⁵I₁Sctx or ANB-¹³¹I₁Sctx, 5-azido-2-nitrobenzoyl mono[¹²⁵I]iodo or mono[¹³¹I]iodo scorpion toxin.

A partially purified surface membrane preparation was made by allowing the cells to swell in 1.5 mM CaCl₂/10 mM NaCl/10 mM Tris-HCl, pH 7.5 for 30 min and then homogenizing in a Teflon/glass homogenizer. After addition of 1/7 vol of 2 M sucrose/34 mM EDTA/50 mM Tris-HCl, pH 7.5, the membranes were sedimented at 40,000 × *g* for 45 min and resuspended in 10 mM Tris-HCl, pH 7.4. These samples were used for gel electrophoresis.

Covalent Labeling of Synaptosomes. Synaptosomes were prepared from rat brain by a modification of the method of Gray and Whittaker (21) as described (8). Synaptosomes (0.4 mg/ml) were incubated with ¹²⁵I-Sctx or ANB-¹²⁵I-Sctx in standard binding medium for 30 min at 36°C (8). The entire binding reaction was then irradiated at 36°C as described for N18 cells. Samples were then centrifuged at 40,000 × *g* for 45 min, washed by resuspension and recentrifugation, and finally suspended at 5 mg/ml in 10 mM Tris-HCl, pH 7.4. These samples were used for gel electrophoresis.

NaDodSO₄ Gel Electrophoresis. Polyacrylamide gel electrophoresis in NaDodSO₄/2-mercaptoethanol was performed essentially as described by Maizel *et al.* (22). Protein samples were depolymerized in 3% NaDodSO₄/5% sucrose/30 mM Tris-HCl, pH 7.4/2 mM EDTA/1% 2-mercaptoethanol by incubation at 100°C for 2 min. Samples were then applied to slab gels with a linear gradient of acrylamide from 4.5% to 10% in the separating gel. Other conditions were as described (22). Molecular weights of protein bands were estimated by comparison with the migration of the following proteins of known molecular weight: thyroglobulin, 330,000; β-galactosidase, 117,000; bovine serum albumin, 68,000; catalase, 60,000; and glyceraldehyde-3-phosphate dehydrogenase, 36,000.

RESULTS

Covalent Attachment of Scorpion Toxin. In previous experiments in which the binding of I₁Sctx to neuroblastoma cells was studied, it was demonstrated that toxin binding that was blocked by 200 nM unlabeled scorpion toxin and by depolarization of the cells with 135 mM K⁺ represented specific binding to receptor sites associated with sodium channels (5). Preliminary experiments showed that ANB-I₁Sctx retained the ability to bind specifically to sodium channels as defined by these criteria. At a concentration of 0.2 nM, 60% of the binding of ANB-I₁Sctx was blocked by unlabeled toxin or by depolarization compared to 85% for I₁Sctx. In the results presented below, the nonspecific component of binding has been measured in the presence of 200 nM unlabeled scorpion toxin and subtracted from all results.

In order to test the reversibility of the specific component of ANB-¹²⁵I₁Sctx binding before and after irradiation, rates of dissociation of the toxin-receptor complex were measured (Fig. 1). At the resting membrane potential of N18 cells, the scorpion toxin-receptor complex dissociates slowly with *t*_{1/2} ≥ 30 min (5). If the cells are depolarized by 135 mM K⁺, dissociation is rapid with *t*_{1/2} = 4 min (5). In order to measure covalent attachment of scorpion toxin, cells were incubated at 36°C for 60 min with 0.2 nM toxin derivative under red light. Unbound toxin was washed away and half of the cultures were irradiated with long-wavelength UV light. The rate of dissociation of bound toxin was then measured under depolarized conditions in 135 mM K⁺. When native ¹²⁵I₁Sctx was used, greater than 90% of the specifically bound toxin dissociated with or without UV irradiation (Fig. 1). When ANB-¹²⁵I₁Sctx was used, greater than 90% of the bound toxin dissociated without irradiation (Fig. 1), whereas only 40% of the bound toxin dissociated after irradiation (Fig. 1). These results indicate that 50% of the specifically bound ANB-¹²⁵I₁Sctx can be covalently attached to the neuroblastoma cells.

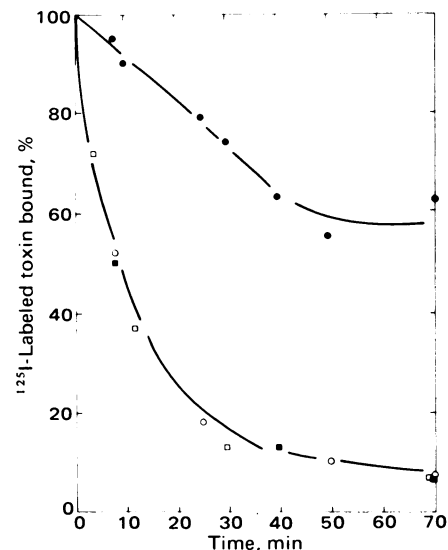


FIG. 1. Dissociation of covalent and noncovalent scorpion toxin-receptor complexes. N18 cells were incubated with 0.2 nM I₁Sctx (□, ■) or ANB-I₁Sctx (○, ●) for 60 min at 36°C. The cells were washed and then either irradiated for 15 min at 0°C (closed symbols) or incubated in the dark for 15 min at 0°C (open symbols). Finally, high K⁺ binding medium was added, the cells were incubated at 36°C for the periods of time indicated on the abscissa and washed to remove unbound toxin, and the remaining bound toxin was measured. In the experiment illustrated, total binding of ANB-I₁Sctx was 1.13 fmol/mg and nonspecific binding was 0.47 fmol/mg.

Specificity of Covalent Attachment. In some cases, apparently specific photoaffinity labeling has been observed at sites distant from the receptor site of interest (for example, ref. 23). We devised a double label experiment in order to estimate the fraction of the covalently bound scorpion toxin that occupies the toxin receptor site and blocks further toxin binding. N18 cells were incubated with ANB-¹³¹I₁Sctx and washed. Half of the cultures was irradiated with long-wavelength UV light and the other half was kept in the dark. Reversibly bound toxin was removed from both sets of cultures by incubation in 135 mM K⁺. Measurement of ¹³¹I cpm bound to irradiated cultures minus that remaining on unirradiated controls gave an estimate of the amount of specifically bound ANB-¹³¹I₁Sctx that was covalently attached (Fig. 2). Then, in a second incubation, cells were allowed to bind ¹²⁵I₁Sctx and the fraction of scorpion toxin receptor sites remaining capable of toxin binding was measured (Fig. 2). The results of Fig. 2 show that, as the concentration of ANB-¹³¹I₁Sctx is increased to 5 nM, toxin specifically bound to 70% (17.8 fmol/mg) of the receptor sites is covalently attached upon irradiation (Fig. 2). Under these conditions, up to 18% (3.2 fmol/mg) of the receptor sites were prevented from subsequent binding of ¹²⁵I₁Sctx (Fig. 2). In seven similar experiments with different preparations of ANB-¹³¹I₁Sctx, incubation with 5 nM ANB-¹³¹I₁Sctx without irradiation had no effect on subsequent binding of ¹²⁵I₁Sctx [99 ± 1% (SEM) of untreated control], whereas, after irradiation, ¹²⁵I₁Sctx binding was decreased to 83 ± 2% (SEM) of control.

The data in Figs. 1 and 2 show that 50–70% of specifically bound ANB-I₁Sctx can be covalently attached to N18 cells. The data of Fig. 2 and other similar experiments show that this amount of covalently attached toxin blocks 17% of the toxin receptor sites. Thus, 25%–35% of the specific covalently bound scorpion toxin is bound to component(s) of the sodium channel at the scorpion toxin receptor site. This estimate may be a lower limit because it measures only those covalently attached toxin molecules that actually remain bound to the receptor site after

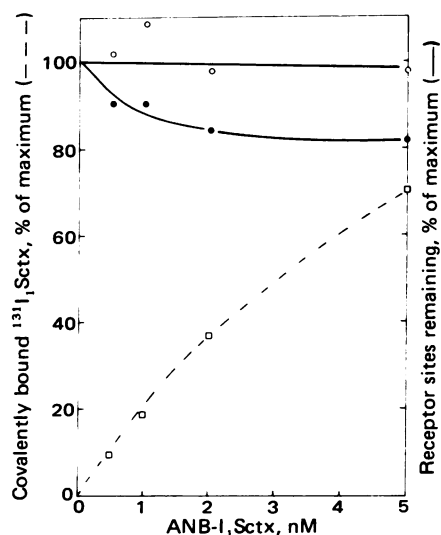


FIG. 2. Irreversible inhibition of scorpion toxin binding by ANB- $^{131}\text{I}_1\text{Sctx}$. N18 cells were incubated for 60 min at 36°C with the concentrations of ANB- $^{131}\text{I}_1\text{Sctx}$ indicated on the abscissa. The cells were washed to remove unbound toxin and either irradiated for 15 min at room temperature or incubated for 15 min at room temperature in the dark. Reversibly bound toxin was allowed to dissociate for 70 min at 36°C in high K^+ binding medium and the cells were washed. The cultures were then incubated for 60 min at 36°C with $^{125}\text{I}_1\text{Sctx}$ and washed, and bound $^{131}\text{I}_1\text{Sctx}$ and $^{125}\text{I}_1\text{Sctx}$ were measured. The difference between ^{131}I bound by irradiated and unirradiated cultures was a measure of specific, covalently bound ANB- $^{131}\text{I}_1\text{Sctx}$ (\square). The difference between ^{125}I bound by irradiated cultures (\bullet) and unirradiated controls (\circ) represents the scorpion toxin receptor sites that were irreversibly blocked by reaction *per se* had no effect on scorpion toxin binding.

covalent bond formation. Because scorpion toxin is a macromolecular ligand, it is likely that some ANB groups, located at points on the toxin molecule distant from the receptor binding region, could covalently attach to components of the sodium channel distant from the toxin receptor site. Such toxin molecules would label specific sodium channel components, but would not block the toxin receptor site.

Identification of Specifically Labeled Sodium Channel Components in Neuroblastoma Cells. In order to identify the membrane components covalently labeled by ANB- I_1Sctx , labeled N18 cell membranes were dissolved in $\text{NaDodSO}_4/2$ -mercaptoethanol solution and subjected to electrophoresis in slab gels with an acrylamide concentration gradient from 4.5% to 10%. After electrophoresis, the protein bands were visualized by Coomassie blue staining, the gels were dried onto filter paper, and radioactively labeled protein bands were visualized by autoradiography. In N18 cells, only a single protein band of $M_r \approx 250,000$ was labeled (Fig. 3A, lane 1). No labeling was observed if I_1Sctx was used rather than ANB- I_1Sctx , or if UV irradiation was omitted (not shown). Labeling was only observed when scorpion toxin was specifically bound. Block of specific binding by unlabeled toxin (Fig. 3A, lane 2) or by depolarization with 135 mM K^+ (not shown) completely prevents covalent labeling of this specific protein band. These results, together with those of Fig. 2, indicate that the labeled polypeptide is a component of the sodium channel.

We have previously described (24) a specific selection procedure that allows isolation of toxin-resistant variant clones of neuroblastoma cells lacking sodium channels. Fig. 3B (lanes 1 and 2) shows that clone LV9, a variant subclone of N18 that lacks sodium channels (24), does not have a specific covalently labeled protein band. These results provide further support for

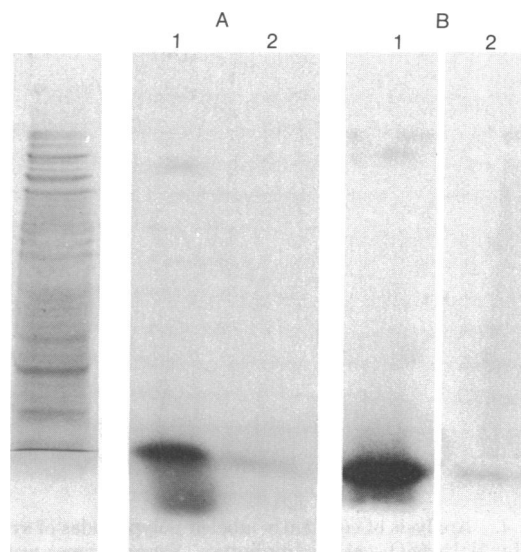


FIG. 3. Analysis of covalently labeled polypeptides of neuroblastoma cells by NaDodSO_4 gel electrophoresis. (A) N18 cells were covalently labeled by using $2.9\text{ nM ANB-}^{125}\text{I}_1\text{Sctx}$ in the absence (lane 1) or presence (lane 2) of 200 nM unlabeled toxin. Membranes were prepared, and $300\text{ }\mu\text{g}$ of membrane protein was applied to each lane of the gel. This amount of protein contained 0.93 fmol of I_1Sctx in the absence (lane 1) or 0.45 fmol in the presence (lane 2) of 200 nM unlabeled toxin. (B) N18 cells (lane 1) or LV9 cells (lane 2) were covalently labeled with $5.0\text{ nM ANB-}^{125}\text{I}_1\text{Sctx}$ and $240\text{ }\mu\text{g}$ of membrane protein was applied to each lane of the gel. This amount of protein contained 6.9 fmol of I_1Sctx for N18 and 1.4 fmol of I_1Sctx for LV9. An example of a Coomassie blue-stained gel track is presented on the left.

the conclusion that the protein of $M_r \approx 250,000$ labeled by ANB- I_1Sctx in N18 cells is a component of the sodium channel.

In our labeling experiments, N18 cells are incubated in 135 mM K^+ for 70 min, washed, and lysed, and then the membranes are washed again before treatment with $\text{NaDodSO}_4/2$ -mercaptoethanol for gel electrophoresis. These treatments are sufficient to remove noncovalently bound scorpion toxin from the membrane completely. Nevertheless, a large fraction of the I_1Sctx in the labeled membrane migrates at the front of our gradient gels or of 7.5% acrylamide gels, as expected for free toxin [$M_r = 6700$ (25)] or for covalent toxin-receptor complexes of M_r , 10,000 or less. It is likely that this toxin has been covalently attached to low molecular weight membrane components such as lipids or small peptides located at or near the scorpion toxin receptor site. Further work is required to establish the nature of the low molecular weight membrane components that react with the toxin derivative and to determine whether they are specific components of the sodium channel.

Identification of Specifically Labeled Sodium Channel Components in Synaptosomes. Synaptosomal preparations from rat brain have a much higher binding capacity for scorpion toxin [1500 fmol/mg (8)] than N18 cells [47 fmol/mg (5)]. We have, therefore, conducted covalent labeling experiments with synaptosomes also. Analysis of the covalently labeled synaptosomes by electrophoresis in NaDodSO_4 reveals a major labeled protein component of $M_r \approx 250,000$, as in N18 cells (Fig. 4, lanes 1 and 2). In addition, in synaptosomes we observe a second specifically labeled protein component of $M_r \approx 39,000$ (Fig. 4, lanes 1 and 2). This toxin conjugate presumably consists of 1 mol of scorpion toxin [$M_r = 6700$ (25)] and 1 mol of a membrane protein of $M_r \approx 32,000$.

Covalent labeling of both of these proteins requires specific scorpion toxin binding. Incubation in the presence of unlabeled

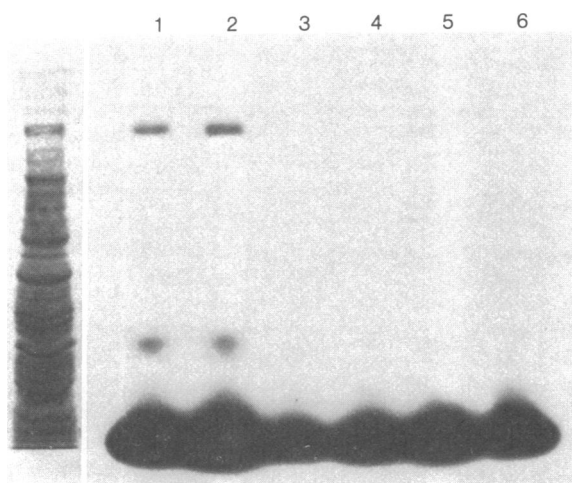


FIG. 4. Analysis of covalently labeled polypeptides of synaptosomes by NaDodSO₄ gel electrophoresis. Synaptosomes were prepared and covalently labeled by using 2 nM ANB-¹²⁵I₁Sctx in standard binding medium containing 1 μM tetrodotoxin (lane 1), 1 μM tetrodotoxin plus 1 μM batrachotoxin (lane 2), 1 μM tetrodotoxin plus 200 nM unlabeled scorpion toxin (lane 3), or 1 μM tetrodotoxin, 1 μM batrachotoxin, plus 200 nM unlabeled scorpion toxin (lane 4), or in high K⁺ binding medium containing 1 μM tetrodotoxin plus 1 μM batrachotoxin (lane 5), or 1 μM tetrodotoxin, 1 μM batrachotoxin, plus 200 nM unlabeled scorpion toxin (lane 6). The labeled synaptosomes were washed and 50 μg of synaptosomal protein was applied to each gel track. An example of a Coomassie blue-stained gel track is presented on the left.

scorpion toxin completely blocks covalent labeling (Fig. 4, lanes 3 and 4). Incubation in the presence of 135 mM K⁺ markedly reduces covalent labeling of both proteins (Fig. 4, lanes 5 and 6), consistent with the 5-fold increase in K_d for scorpion toxin caused by depolarization in the presence of batrachotoxin (8). These data show that the covalent labeling of both of these proteins is specific and indicate that they are both components of the sodium channel in synaptosomes.

Proteolytic degradation of proteins during membrane preparation, incubation, and processing for gel electrophoresis is a potential source of artifact in covalent labeling experiments. In order to test the possibility that the protein of $M_r \approx 32,000$ was produced by proteolytic degradation of the larger component, synaptosomes were prepared and all subsequent manipulations were carried out in the presence of a freshly prepared cocktail of protease inhibitors consisting of 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM pepstatin A, and 1 mM *o*-phenanthroline. No change in covalent labeling was observed. Although these results do not exclude the possibility of proteolysis, they provide support for the conclusion that both labeled proteins are components of the sodium channel.

DISCUSSION

Our results with neuroblastoma cells show that 50%–70% of the specifically bound ANB-I₁Sctx can be covalently attached to the cells by irradiation. From 25% to 35% of the covalently attached toxin is bound to the scorpion toxin receptor site and prevents subsequent binding of scorpion toxin. Analysis of covalently labeled cell membranes by NaDodSO₄ polyacrylamide gel electrophoresis reveals a single labeled protein component of $M_r \approx 250,000$. Covalent labeling of this protein is prevented when specific binding of ANB-I₁Sctx is blocked by excess unlabeled scorpion toxin or by depolarization with 135 mM K⁺. No covalent labeling of this protein is observed in clone LV9 neuroblastoma cells, a variant subclone that lacks sodium

channels (24). These results provide strong evidence that the specifically labeled polypeptide of $M_r \approx 250,000$ is a component of the voltage-sensitive sodium channel in neuroblastoma cells.

Our experiments with synaptosomes provide further evidence for identification of the $M_r \approx 250,000$ polypeptide as a component of the sodium channel. Labeling of this protein in synaptosomes is blocked by unlabeled scorpion toxin and by depolarization with 135 mM K⁺ as in neuroblastoma cells. Thus, we have identified a polypeptide of $M_r \approx 250,000$ that is a component of the voltage-sensitive sodium channel in both neuroblastoma cells and synaptosomes.

In addition, our experiments with synaptosomes detect a second polypeptide of $M_r \approx 32,000$ which also is specifically labeled. The labeling is blocked by unlabeled scorpion toxin and by depolarization with 135 mM K⁺. Although we cannot exclude the possibility that this polypeptide is a proteolytic fragment of the larger polypeptide of $M_r \approx 250,000$, we believe it is most likely that this polypeptide is a separate component of the sodium channel. It is unexpected that this polypeptide is labeled in synaptosomes but not in neuroblastoma cells. In experiments in which similar numbers of ¹²⁵I cpm from labeled N18 cells and labeled synaptosomes were analyzed in the same gel and autoradiographed together, the ratio of intensities in the two bands in the synaptosomal membranes was approximately 1.0, whereas no band of $M_r \approx 32,000$ was detectable in the neuroblastoma cells. These experiments indicate that the $M_r \approx 32,000$ protein is labeled with approximately equal efficiency to the $M_r \approx 250,000$ protein in synaptosomes, but with less than 10% of the efficiency of the $M_r \approx 250,000$ protein in neuroblastoma cells. This different labeling pattern could reflect a different subunit composition of the sodium channel in these two membranes or a more subtle difference in the availability of suitable reactive groups on the $M_r \approx 32,000$ component in the proximity of the aryl azide moiety of ANB-I₁Sctx. Further experiments are required to distinguish between these two alternatives.

Sea anemone toxin II and scorpion toxin bind to a common receptor site in neuroblastoma cells and synaptosomes (2, 7–9). A preliminary description of photoaffinity labeling of the sea anemone toxin I receptor site in crayfish walking leg nerve has recently appeared (26). A protein of $M_r \approx 53,000$ was labeled. We do not observe specific labeling of such a protein band in either neuroblastoma cells or synaptosomes. However, our results do not exclude the presence of such a protein at or near the scorpion toxin receptor site located in a position that is not appropriate for reaction with the aryl azide moiety of ANB-I₁Sctx.

The saxitoxin/tetrodotoxin binding component of the sodium channel has been solubilized from garfish olfactory nerve, eel electroplax, and rat brain (11–14) and the solubilized receptor from electric eel has been partially purified (13). The most highly purified preparations from electric eel are estimated to be 25–50% pure and contain major polypeptides of $M_r \approx 300,000$, 59,000 and 46,000 along with minor components (13). Because molecular weight estimates of large polypeptides by gel electrophoresis are not very precise, it is possible that the $M_r \approx 250,000$ polypeptide we have labeled corresponds to the $M_r \approx 300,000$ polypeptide in the partially purified receptor preparations of Agnew *et al.* (13). It will be of interest to determine whether the component of $M_r \approx 250,000$ labeled in our experiments with scorpion toxin derivatives is associated with the solubilized saxitoxin/tetrodotoxin receptor site from rat brain (14).

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