Covalent labeling of protein components of the sodium channel with a photoactivatable 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 poly peptide of Mr = 250,000 is specifically labeled in neuroblastoma cells. Labeling is blocked by unlabel ed scorpion toxin and by depolarization. This poly peptide is not labeled in a variant neuroblastoma clone lacking voltage-sensitive sodium channels. Poly peptides of Mr = 350,000 and Mr = 33,000 are specifically labeled in synaptosomes. This labeling is also blocked by unlabeled toxin and by depolarization. These results identify specific poly peptides of Mr = 250,000 and Mr = 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 poly peptides 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 KD 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 photoactivatable 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 quinquestriatus) 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-125I[Sctx]). A photoactivatable 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 (125I[Sctx]) 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 125I[Sctx] or ANB-125I[Sctx] 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 (λmax = 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 (150 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: 125I[Sctx] or 131I[Sctx], mono[125I]iodo or mono[131I]iodo scorpion toxin; ANB-125I[Sctx] or ANB-131I[Sctx], 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 X 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]Scx or ANB-125I Scx 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 X g for 45 min, washed by resuspension and recentrifugations, 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).

**RESULTS**

**Covalent Attachment of Scorpion Toxin.** In previous experiments in which the binding of I₁Scx 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-I₁Scx 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-I₁Scx was blocked by unlabeled toxin or by depolarization compared to 85% for I₁Scx. 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-125I Scx 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 t₁/₂ ≥ 30 min (5). If the cells are depolarized by 135 mM K⁺, dissociation is rapid with t₁/₂ = 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 125I Scx was used, greater than 90% of the specifically bound toxin dissociated with or without UV irradiation (Fig. 1). When ANB-125I Scx 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-125I Scx can be covalently attached to the neuroblastoma cells.

**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-125I Scx 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 125I Scx bound to irradiated cultures minus that remaining on unirradiated controls gave an estimate of the amount of specifically bound ANB-125I Scx that was covalently attached (Fig. 2). Then, in a second incubation, cells were allowed to bind 125I Scx 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-125I Scx 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 125I Scx (Fig. 2). In seven similar experiments with different preparations of ANB-125I Scx, incubation with 5 nM ANB-125I Scx without irradiation had no effect on subsequent binding of 125I Scx [99 ± 1% (SEM) of untreated control], whereas, after irradiation, 125I Scx binding was decreased to 83 ± 2% (SEM) of control.

The data in Figs. 1 and 2 show that 50–70% of specifically bound ANB-I₁Scx 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
covalent bond formation. Because scorpion toxin is a 
macro-molecular 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 mole-
cules 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-131Sctx, 
labelled N18 cell membranes were dissolved in NaDodSO4/2-
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 
Mr \( \approx \) 250,000 was labeled (Fig. 3A, lane 1). No labeling was 
observed if 1Sctx was used rather than ANB-1Sctx, or if UV 
irradiation was omitted (not shown). Labeling was only ob-
served when scorpion toxin was specifically bound. Block of 
specific binding by unlabeled toxin (Fig. 3A, lane 2) or by de-
polarization 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 poly-
peptide is a component of the sodium channel.

We have previously described [24] a specific selection pro-
cedure 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

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**Fig. 2.** Irreversible inhibition of scorpion toxin binding by 
ANB-131Sctx. N18 cells were incubated for 60 min at 36°C with 
the concentrations of ANB-131Sctx indicated on the abscissa. The 
cells were washed to remove unbound toxin and either irradiated for 
15 min at room temperature or irradiated 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 131Sctx 
and washed, and bound 131Sctx and 125I-Sctx were measured. The 
difference between 131I bound by irradiated and unirradiated cultures 
was a measure of specific, covalently bound ANB-131I-Sctx (O). The 
difference between 125I bound by irradiated cultures (●) and unir-
radiated controls (O) represents the scorpion toxin receptor sites 
that were irreversibly blocked by reaction with ANB-131I-Sctx. Control 
experiments showed that irradiation per se had no effect on scorpion 
toxin binding.

**Fig. 3.** Analysis of covalently labeled polypeptides of neuro-
blastoma cells by NaDodSO4 gel electrophoresis. (A) N18 cells were 
covalently labeled by using 2.9 nM ANB-131Sctx in the absence (lane 
1) or presence (lane 2) of 200 nM unlabeled toxin. Membranes were 
prepared, and 300 μg of membrane protein was applied to each lane 
of the gel. This amount of protein contained 0.93 fmol of 1Sctx in the 
absence (lane 1) or 0.45 fmol in the presence (lane 2) of 200 nM un-
labeled toxin. (B) N18 cells (lane 1) or LV9 cells (lane 2) were coval-
ently labeled with 5.0 nM ANB-131Sctx and 240 μg of membrane 
protein was applied to each lane of the gel. This amount of protein 
contained 6.9 fmol of 1Sctx for N18 and 1.4 fmol of 1Sctx for LV9. 
An example of a Coomassie blue-stained gel track is presented on the 
left.

The conclusion that the protein of Mr \( \approx \) 250,000 labeled by 
ANB-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 lyzed, and then the membranes 
are washed again before treatment with NaDodSO4/2-mer-
captoethanol for gel electrophoresis. These treatments 
are sufficient to remove noncovalently bound scorpion toxin from 
the membrane completely. Nevertheless, a large fraction of the 
1Sctx in the labeled membrane migrates at the front of our 
gradient gels or of 7.5% acrylamide gels, as expected for free 
toxin (Mr \( = \) 6700 (25)) or for covalent toxin–receptor complexes 
of Mr 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 syn-
aptosomes by electrophoresis in NaDodSO4 reveals a major 
labelled protein component of Mr \( \approx \) 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 Mr \( \approx \) 39,000 
(Fig. 4, lanes 1 and 2). This toxin conjugate presumably consists 
of 1 mol of scorpion toxin [Mr \( = \) 6700 (25)] and 1 mol of a 
membrane protein of Mr \( \approx \) 32,000.

Covalent labeling of both of these proteins requires specific 
scorpion toxin binding. Incubation in the presence of unlabeled
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-I$_{Sct}$ can be covalently attached to the cells by irradiation. From 25% to 35% of the covalently attached toxin is bound at the scorpion channel receptor site and prevents subsequent binding of scorpion toxin. Analysis of covalently labeled cell membranes by NaDODSO$_4$ 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-I$_{Sct}$ 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$^+$. We believe that 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 $^{125}$I 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-I$_{Sct}$. 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-I$_{Sct}$.

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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