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Structure and action of heteronemertine polypeptide toxins. Specific cross-linking of *Cerebratulus lacteus* toxin B-IV to lobster axon membrane vesicles

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The binding of the crustacean-selective protein neurotoxin, toxin B-IV, from the heteronemertine *Cerebratulus lacteus*, to lobster axonal and muscle membranes has been studied. Synthesis of a radioactive bifunctional cross-linking reagent, ^{125}I-azidosalicylic acid (ASA) B-IV, has allowed these studies as well as experiments that show cross-linking of toxin B-IV to its receptor in axonal membranes. In the absence of photolysis ^{125}I-ASA-B-IV binds to vesicles with an apparent K_d of 30 nM and maximal binding of 7.5 pmol per mg membrane protein. Photolysis of the toxin-receptor complex at 366 nm greatly diminishes the rate of dissociation of bound toxin B-IV. Photolysis also results in the specific cross-linking to axonal proteins of molecular masses 38 and 40 kDa. This cross-linking is not observed in the presence of micromolar unlabeled toxin, in the absence of photolysis or in the presence of 150 mM K^+. There is no evidence of cross-linking to proteins of higher molecular weight. The radiolabeled toxin B-IV was also found to bind to lobster muscle membranes with a dissociation constant of 500 nM and a maximum binding of approx. 4.50 pmol per mg membrane protein.

Introduction

Neurotoxins have been used as molecular probes of the Na channel to identify its components and serve as markers during its purification and reconstitution. Four classes of sites on the sodium channel have been identified [1–3]. Receptor site 1 binds saxitoxin and tetrodotoxin, which inhibit ion flux. Site 2 binds batrachotoxin, veratridine, aconitine and grayanotoxin, whose effects lead to persistent activation [4]. Neurotoxin site 3 binds polypeptide neurotoxins from *Leiurus, Androctonus* and sea anemone in a voltage-dependent manner causing inhibition of inactivation of the sodium channel [5]. Site 4 binds the polypeptide β-scorpion toxins, which alter the voltage dependence of channel activation.

The saxitoxin receptor from eel electroplax [6–9], rat skeletal muscle [10–12] and rat brain [13,14] has been solubilized and purified to homogeneity [15]. The solubilized channel has a molecular weight of 316 000 and is composed of subunits of molecular weights of 250 000–270 000 (α), 39 000 (β_1) and 37 000 (β_2). The purified system can be reconstituted into phospholipid vesicles that exhibit neurotoxin-activated sodium flux [16].

Lobster axonal vesicles also have been shown to

Abbreviations: NHS-ASA, *N*-hydroxysuccinimide ester of azidosalicylic acid; ASA, azidosalicylic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF: phenylmethanesulfonyl fluoride.

exhibit Na^+ fluxes that are activated by veratridine and batrachotoxin and blocked by tetrodotoxin [17,18]. Binding of polypeptide neurotoxins to crustacean nerve has been shown with sea anemone toxin [19] and with *Cerebratulus* toxin B-IV [20]. Toxin B-IV is the most abundant of the B-toxins, a class of structurally homologous, crustacean-selective polypeptide neurotoxins produced by the heteronemertine *Cerebratulus lacteus*. These toxins (B-toxins) are highly basic small proteins (6 kDa) of established structure [21] that are known to contain essential tyrosine [22] and tryptophan residues [22] and affect action potential generation in crab and lobster walking leg nerve [24]. Recent studies in our laboratory have been directed toward identification of the toxin-B IV binding site in crustacean nerve. Binding of Bolton-Hunter labeled B-IV to lobster axonal membranes exhibits a K_d of about 10 nM and has a maximum binding capacity of 7.0 pmol per mg membrane protein. In addition, there is no competition of B-IV binding by either *Leiurus* or *Centruroides sculpturatus* Ewing I toxins, representatives of the α and β types.

In this paper we present the synthesis of a bifunctional cross-linking photo-affinity derivative of toxin B-IV (^{125}I-ASA-B-IV), determine its binding to and dissociation from axonal membranes in the absence of photolysis and, in the presence of photolysis show cross-linking of toxin B-IV to its receptor. Finally we show binding of this derivative to lobster muscle membranes.

Experimental procedures

Materials. Live *Cerebratulus lacteus* were obtained from Northeast Marine Specimen Company and toxin B-IV was purified as described by Kem [24]. NHS-ASA and silica TLC sheets were obtained from Pierce. $Na^{125}I$ (carrier-free) was purchased from ICN Pharmaceuticals. Lobsters were purchased locally. The proteinase inhibitors pepstatin, PMSF and 1,10-phenanthroline were purchased from Sigma and iodoacetamide from Calbiochem. All other reagents were of the purest grade commercially available.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. [24].

Radioiodination of N-hydroxysuccinimide 4-azidosalicylic acid (NHS-ASA). ^{125}I-NHS-ASA was prepared according to the method of Ji and Ji [26] with minor modifications. NHS-ASA (3.0 mg per ml), chloramine T (4.0 mg per ml) and $NaHSO_3$ (1.0 mg per ml) were prepared fresh in dry acetone. The reactions were carried out in red light. NHS-ASA (6.8 μl) was added to 1.5 mCi $Na^{125}I$ (in 2 μl NaOH) followed by 15 μl of the chloramine T solution. After thorough mixing (20 s) another 15 μl chloramine T were added, and 30 s later 10 μl of $NaHSO_3$ were added to stop the reaction. The reaction mixture was applied to a silica gel F 254 analytical TLC plate and chromatographed in benzene/ethyl acetate/chloroform 1 : 1 : 1, v/v). The ^{125}I-NHS-ASA ($R_F = 0.67$) was visualized by autoradiography, scraped and eluted with 2 ml $CHCl_3$. Depending on the age of the NHS-ASA, varying amounts of iodinated hydrolysis products are obtained; since NHS-ASA appears to be quite labile, even when stored dessicated, we never use material that is more than 6 months old. After elution of the desired band the $CHCl_3$ is evaporated with a stream of N_2 and the product is used immediately in the next step. We usually obtain 10% labeling.

Preparation of ^{125}I-ASA-B-IV. In a typical experiment, the dried ^{125}I-NHS-ASA ($2.0 \cdot 10^8$ cpm, 74 nmol) was dissolved in 5 μl absolute ethanol. To this were added 25 μl of 100 mM sodium borate, pH 7.0, containing 25 μg toxin B IV (4.2 nmol). After 1 h at room temperature the mixture was desalted on a 2 ml G-25 column eluted with 100 mM ammonium acetate, pH 5.0, containing 200 μg per ml bovine serum albumin. The B-IV peak was pooled and frozen. Specific activities were usually in the range of 10^6 cpm per nmol protein.

Preparation of lobster axonal membrane vesicles. The method used was that of Balerna et al. [27] with the following minor changes; Hepes buffer was substituted for Tes, and nerves from walking leg and claws of the lobster *Homarus americanus* were used.

Preparation of lobster muscle membranes. The method followed was basically that of Moczydlowski et al. [28] with modifications as specified below. All preparative procedures were carried out at 4°C. Tail muscle (105 g) obtained from two lob-

sters was put through a meat grinder and then combined with 3 vol. of 10 mM Hepes (adjusted to pH 7.4 with NaOH), containing 0.3 M sucrose, 0.02% sodium azide, 0.2 mM EDTA and 0.4 ml of 0.2 M PMSF (final concentration 2.7 mM). This was homogenized for 2 × 30 s in a Waring blender. After centrifugation at 4000 × *g* for 10 min, the supernatant was filtered through cheesecloth. The pellets were combined with another 3 vol. of buffer, homogenized and centrifuged as above. The supernatants were combined and solid KCl was added to 0.6 M. The suspension was centrifuged at 8500 × *g* for 10 min, the small pellets were discarded, and the membranes were pelleted by centrifugation at 100 000 × *g* for 60 min. The supernatant was carefully removed with a pasteur pipette and the pellets resuspended in 100 ml of the homogenization buffer (no KCl) and homogenized in a Dounce homogenizer. Following centrifugation at 6000 × *g* for 10 min pellets were discarded and the supernatant was centrifuged at 100 000 × *g* for 1 h. The pellets were resuspended in 15 ml of homogenization buffer, homogenized in a Dounce and layered onto gradients consisting of 10 ml 33% (w/w) sucrose and 20 ml 28% (w/w) sucrose in 10 mM Hepes-Na (pH 7.4), 0.02% NaN_3, 0.2 mM EDTA. The gradients were centrifuged in an SW 27 rotor at 80 000 × *g* for 17 h. The plasma membrane (SL) fraction appears at 20–26% sucrose and the sarcoplasmic reticulum bands at a density of 30% sucrose. With rigorous homogenizations one can obtain 100 mg SL from 100 g muscle tissue.

Measurement of ^{125}I-ASA-IV binding. Binding of ^{125}I-ASA-B-IV was measured using a rapid filtration assay described by Ray et al. [29]. Assays and incubations were done at 4°C. Lobster axonal vesicles or lobster muscle plasma membranes were suspended in binding medium consisting of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with 1 M Tris base), 5.4 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$ and 1 mg per ml bovine serum albumin to give 0.5–1.0 mg membrane protein per ml. After this point all manipulations were carried out in red light only. Binding reactions were initiated by addition of 350 μl of ^{125}I-ASA-B-IV containing varying amounts of native toxin B-IV to 350 μl vesicles to give final concentrations from 0 to 1000 nM unlabeled B-IV and 29 nM ^{125}I-ASA-B-IV. Nonspecific binding was determined with the inclusion of 2 μM native B-IV. The samples were mixed and incubated for 15 min at 4°C. Aliquots (200 μl) were collected on glass-fiber filters (Whatman GF/C), that had been preequilibrated with 1 ml 7.4 μM native B-IV in the above binding medium and washed with 10 ml ice-cold washing medium, 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with 1 M Tris base), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg per ml bovine serum albumin. After application of the membranes the filters were washed with 20 ml of the wash medium and counted. Each concentration was done in triplicate.

Time-course of dissociation of ^{125}I-ASA-B-IV from lobster axonal vesicles. Dissociation was measured via the rapid filtration technique described above. All manipulations were done under red light at 4°C. Freshly isolated vesicles, 1.0–2.0 mg per ml membrane protein, were suspended in binding medium containing 30 nM ^{125}I-ASA-B-IV and allowed to bind for 30 min at 4°C. One half of the suspension was then irradiated at long-wavelength (366 nm) with a Blak · Ray UVL-56 lamp for 5 min. The membranes from both the photolyzed and nonphotolyzed samples were centrifuged at 40 000 × *g* for 30 min to remove unbound ^{125}I-ASA-B-IV. Dissociation was initiated by resuspension of pellets (0.5–1.0 mg per ml membrane protein) in binding medium containing 5 μM unlabeled B-IV. Aliquots (200 μl, triplicate) were removed at various times from 0.5 to 80 min and applied to the glass-fiber filters, which were washed and counted.

Cross-linking of ^{125}I-ASA-B-IV to lobster axonal vesicles. Lobster axonal vesicles (0.5–1.0 mg per ml) were suspended in 130 mM choline chloride, 50 mM Hepes, 5.4 mM KCl, 5.4 mM glucose, 0.8 mM $MgSO_4$, 0.1 mM PMSF, 1 mM 1,10-phenanthroline, 0.1 mM pepstatin and 1 mM iodoacetamide, adjusted to pH 7.4 with 1 M Tris base. At this point, all procedures were carried out at 4°C under red light. Four sets of samples containing 0.1–0.2 mg membrane protein and 50 nM ^{125}I-ASA-B-IV were incubated for 15 min at 4°C in the dark, then spun in a Beckman airfuge for 1 min to remove unbound toxin B-IV. The pellets were resuspended in 200 μl of the above buffer and were photolyzed at 366 nm, with the exception

of an unphotolyzed control. Other controls contained either 5 μM unlabeled B-IV or 150 mM K^+. Vesicles were reisolated by centrifugation in the airfuge for 1 min and the supernatants were removed and counted. The pellets were washed twice with binding medium containing 1 mg per ml bovine serum albumin and the supernatants were counted again. Finally, the pellets were solubilized in Laemmli running buffer [30] containing 1 M urea, 40 mM dithiothreitol and 1% SDS and heated overnight at 37°C. An 8% polyacrylamide gel as described by Laemmli with bovine serum albumin, concanavalin A and RNA-ase as molecular weight standards was run, dried and autoradiographed. In some studies, electrophoresis was done on a 5% gel as described by Fairbanks et al. [31] using erythrocyte membrane proteins as molecular weight markers.

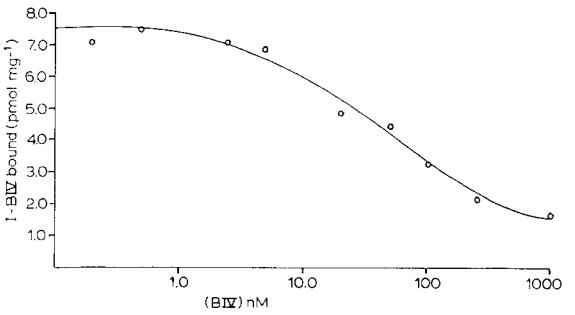

Fig. 1. Binding of ^{125}I-ASA-B-IV to lobster axon vesicles. Lobster axon membrane vesicles were incubated with ^{125}I-ASA-B-IV (28 nM, in the dark and at 4°C), and increasing concentrations of native toxin B-IV as described in Experimental procedures. Aliquots (200 μl) were filtered on Millipore or Whatman GF/C filters and counted; each point was performed in triplicate. The amount of ^{125}I-ASA-B-IV bound (pmol per mg) is plotted on a semi-logarithmic plot versus concentration of native toxin B-IV.

Results

Modification of toxin B-IV with ^{125}I-NHS-ASA. NHS-ASA was first radioiodinated according to the procedure of Ji and Ji [26]. This was found to be a sufficiently efficient process that we were consistently able to iodinate material to a specific activity of $(1.0–3.0) \cdot 10^9$ cpm per μmol.

After purification by thin-layer chromatography the ^{125}I-NHS-ASA was dissolved in an organic solvent (ethanol or acetone) and reacted with toxin B-IV in 100 mM sodium borate, pH 7.0. The reaction was desalted on a G-25 column and the B-IV peak was pooled. The yield from this reaction was generally 1% or less, as judged by percent incorporation of ^{125}I.

Binding of ^{125}I-ASA-B-IV to axonal membrane vesicles. The vesicles were prepared from the Lazdunski [27] 'heavy' band. The binding of ^{125}I-ASA-B-IV to lobster axonal membranes was competed by unlabeled toxin B-IV, as shown in Fig. 1. Vesicles (0.5–1.0 mg per ml) were incubated with 28 nM ^{125}I-ASA-B-IV and increasing concentrations of native B-IV ranging from 0.2 to 1000 nM. The midpoint of the curve in Fig. 1 gives an apparent dissociation constant for the toxin-receptor complex of 30 nM, in fairly good agreement with earlier binding data obtained using Bolton-Hunter labeled toxin B-IV [20]. The B_{max} for binding is 7.5 pmol per mg membrane protein.

Dissociation of ^{125}I-ASA-B-IV lobster axonal membrane vesicles. If ASA-B-IV is capable of covalent attachment to its receptor, then photolysis should decrease the rate of dissociation of preformed, specific toxin-receptor complexes. Samples containing ^{125}I-ASA-B-IV (30 nM) were incubated in the dark with axonal vesicles for 15 min at 0°C. One sample was then placed in a quartz cell and irradiated with long-wave ultraviolet radiation (366 nm); the second sample was maintained in the dark. Both sets were then centrifuged to pellet the membranes and to remove the unbound ^{125}I-ASA-B-IV from solution.

Dissociation was initiated by addition of buffer containing 5 μM unlabeled toxin B-IV. Aliquots were taken at various time points and filtered to detect loss of toxin binding. The earliest point obtainable was at 30 s, thus zero-time is extrapolated (using a least-squares program).

The nonirradiated set exhibits a residual binding of 60% at 80 min (Fig. 2) whereas irradiated samples show residual binding of 77% at 80 min. In addition, the nonirradiated material dissociates very rapidly in the first 5 min to 75% residual activity, while the photolyzed sample still retains 95% of its initial radioactivity. It is clear that photolysis results in a significant inhibition of toxin-receptor dissociation. It should be noted that previous studies [20] have shown toxin B-IV dissociation to be slow and nonlinear.

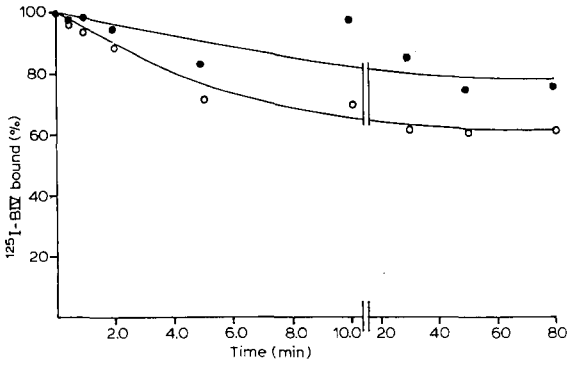

Fig. 2. Dissociation of ^{125}I-ASA-B-IV from lobster axon vesicles. Lobster axon vesicles were incubated with ^{125}I-ASA-B-IV (30 nM, in the dark at 4°C), spun down to remove unbound ^{125}I-ASA-B-IV, then kept in the dark (○) or irradiated at 366 nM (●). Both sets were resuspended in 5 μM native B-IV and aliquots were taken and assayed as described in Experimental procedures. The percent of total B-IV bound (that bound at time divided by that bound at t_0) is plotted versus time.

Cross-linking of ^{125}I-ASA-B-IV to lobster axonal membrane. The Na channel from mammalian brain and muscle has been purified by Catterall et al. [15] and Barchi et al. [12], respectively. Analysis of these proteins by SDS-electrophoresis reveals major bands of 260, 39 and 37 kDa in brain [13], and 260, 43 and 38 kDa in muscle [32]. It has been suggested that the binding site for α-scorpion toxins lies at the α-β interface, while that for β-toxins is confined to β_1. These same proteins can be labeled by photoactivatable derivatives of the scorpion toxin ligands [33].

Cross-linking of B-IV to its binding site could help to resolve the physiological function of this membrane protein. The results of a typical cross-linking experiment are shown in Fig. 3. Lane 1 shows that, upon photolysis of derivatized B-IV-receptor complexes, two proteins of apparent molecular masses 46 and 44 kDa become labeled. Correction for the molecular mass B-IV [6 kDa) gives true molecular masses of 40 and 38 kDa, very similar to the β_1 and β_2 subunits of mammalian Na-channels. No labeling is observed in the absence of photolysis (lane 3) or in the presence of 5 μM unlabeled B-IV (lane 2), indicating that cross-linking is specific for the B-IV binding site. Labeling is also prevented when the binding step is carried out in buffer containing 150 mM K^+ (lane 4). Although the significance of this inhibition is not clear, it should be noted that binding of B-IV is blocked about 60% under these conditions [20].

It should be stressed that the gel in Fig. 3 shows

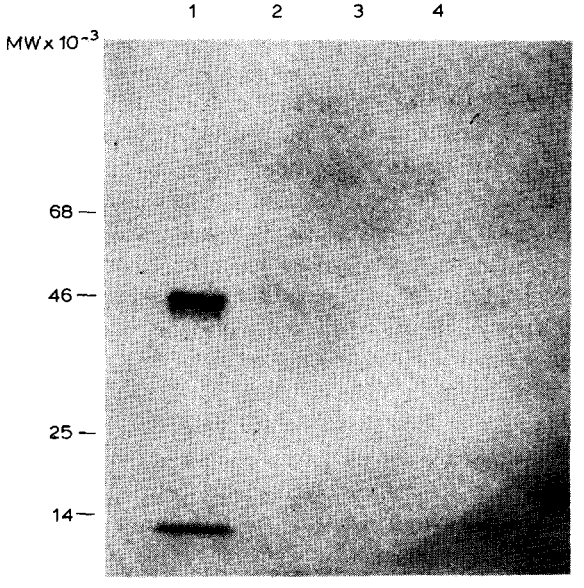

Fig. 3. Cross-linking of ^{125}I-ASA-B-IV to its receptor on lobster axon membranes. Cross-linking of ^{125}I-ASA-B-IV to lobster axon membranes was carried out as described in Experimental procedures. Lane 1: ^{125}I-ASA-B-IV cross-linked to lobster axon membranes by photolysis at 366 nM; lane 2: in the presence of excess native B-IV; lane 3: no photolysis; lane 4: in the presence of 150 mM K^+.

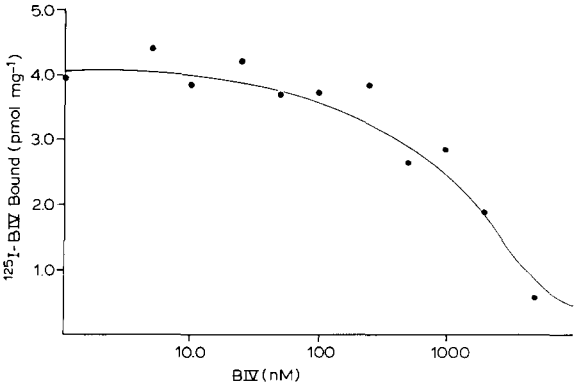

Fig. 4. Binding of ^{125}I-ASA-B-IV to lobster muscle membranes. Lobster muscle membranes were incubated with ^{125}I-ASA-B-IV (28 nM, in the dark at 0°C) and increasing concentrations of native B-IV and then assayed as described in Experimental procedures. The amount of ^{125}I-ASA-B-IV (pmol per mg) is plotted on a semi-logarithmic plot versus concentration of native B-IV.

absolutely no labeling of proteins of higher molecular weight. When labeled membrane preparations are analysed on 5% gels (data not shown), a single, broad band migrating between bovine serum albumin and the tracking dye is observed, again with no evidence of labeling of higher molecular weight components.

Binding of ^{125}I-ASA-B-IV to lobster muscle membrane vesicles. Since a definitive identification of the toxin B-IV binding site would be facilitated by use of a more abundant source of membrane protein, it was decided to examine lobster muscle membranes for specific B-IV binding sites.

The binding competition experiment was performed in an fashion identical to that for axonal vesicles except that it was important to use a lower protein concentration (0.25 mg per ml). This is necessary because of the high amount of nonspecific binding with muscle tissue. Fig. 4 illustrates that there is detectable binding with a maximal site density of 4.5 pmol per mg protein and an apparent K_d of 500 nM. The detection of this binding suggests the feasibility of purification of the B-IV receptor from lobster muscle.

Discussion

In this paper, we have shown binding and cross-linking of *Cerebratulus* toxin B-IV to its receptor on vesicles from lobster axonal membranes. In addition, we have shown binding to vesicles from lobster muscle. The dissociation constant for axonal vesicles, 30 nM, is in good agreement with the findings of Toth and Blumenthal [20].

Although the binding of toxin B-IV (labeled with iodinated Bolton-Hunter reagent) has been described previously, we felt it necessary to repeat these studies, since both our probe and our vesicle preparation were different from those of Toth. Our reasoning for using ^{125}I-ASA-B-IV was based on attempts in our laboratory (unpublished results) to utilize the *N*-hydroxysuccinimide ester of *N*-4-azido-2-nitrophenyl-β-alanine (NAP-β-Ala-NHS), in a one-to-one labeling of toxin B IV and to trace label B-IV with ^{125}I-labeled Bolton-Hunter reagent (*N*-succinimidyl-4-hydroxyphenyl propionate), to give a radiolabeled toxin molecule which upon photolysis would be capable of covalently cross-linking B-IV to its receptor. Attempts at cross-linking with this system were not successful. This may be because not all the toxin molecules are derivatized with both probes, due to their similar reactivities, or to diminished binding of doubly labeled B-IV molecules, or both. Thus, cross-linking might have occurred but would never have been detected if no iodine label was present in the cross-linking protein. It was for these reasons that ^{125}I-ASA-B-IV was synthesized; the ^{125}I could be put directly on the cross-linking agent and we were able to obtain a much higher specific activity.

Our system was not without problems. NHS-ASA is extremely labile and should be kept very dry and not used after 6 months. Iodination of NHS-ASA was generally an efficient process, yielding material with a specific activity of $(1.0–3.0) \cdot 10^9$ cpm per μmol. The reaction of ^{125}I-NHS-ASA with B-IV is a much less satisfactory process. We found that the best yields of active toxin were obtained using a sodium borate buffer (100 mM, pH 7.0), for 1 h at room temperature. It was essential to dissolve the ^{125}I-NHS-ASA in an organic solvent (ethanol or acetone), prior to addition of the B-IV or the reaction was notably less efficient. NHS-ASA hydrolyzes very rapidly in the presence of water, so there is a competition between water and B-IV for the NHS-ASA; thus it was necessary to keep the amount of buffer to a minimum. We typically ran the reaction in less than 100 μl buffer. Nevertheless, the yield of this reaction was generally 1% or less (based on amount of radioactivity incorporated). A specific activity of approx. $1 \cdot 10^6$ cpm per nmol was required to carry out the cross-linking experiments.

Binding of ^{125}I-ASA-B-IV to axonal vesicles (Fig. 1) reaches a maximum at about 7.5 pmol bound per mg membrane protein and has a K_d of 30 nM, both in good agreement with binding of Bolton-Hunter B-IV ($B_{max} = 7.0$ pmol per mg membrane protein, $K_d = 10$ nM). Dissociation of ^{125}I-ASA-B-IV was carried out both before and after photolysis (Fig. 2). The results of this experiment show that in the absence of photolysis there is a rapid release of radioactivity to 75% of the original amount within 5 min, followed by a slower rate, reaching a plateau at 60% in 50 min. On the other hand, dissociation of photolyzed material

occurs at a much slower rate (still 95% at 5 min) and plateaus with 77% of the original radioactivity bound. This experiment strongly suggests covalent binding of ^{125}I-ASA-B-IV to axon membrane vesicles upon photolysis.

Photolysis of ^{125}I-ASA-B-IV in the presence of axon membrane vesicles causes cross-linking to two proteins of molecular mass 38 and 40 kDa (Fig. 3, lane 1, molecular mass of B-IV subtracted). When photolysis is performed with excess unlabeled B-IV, the cross-linking reaction is prevented (lane 2, Fig. 3). Cross-linking is not seen in the absence of photolysis, (lane 3, Fig. 3) and is also abolished by high potassium (lane 4, Fig. 3). It should be noted that the 46 kDa band is far more intense than that at 44 kDa; this is consistent with other studies [33,35] wherein β_1 is more highly labeled than β_2. The sodium channel from mammalian brain and muscle consists of an α subunit of 270 kDa and two β subunits, β_1 of 39 kDa and β_2 of 37 kDa. Neurotoxins have been used to label and identify these proteins as well as to provide information concerning their relationship with one another. The α-toxin from *Leiurus quinquestriatus* labels the α and β_1 subunits [14,34]; the β-toxin CssII also labels α and β_1 [35]. It has recently been shown [33] that purified derivatives of *Leiurus* toxin can preferentially label the α subunit or the β_1 subunit depending on the site of attachment of the arylazide moiety within the toxin sequence. The β-scorpion toxin from *Tityus serrulatus* labels only a protein of 260 kDa in electroplax [36]. The similarity of the molecular weights of the β subunits of the mammalian sodium channel and the binding site of toxin B-IV identified here suggests that B-IV binds to these components of the Na channel in lobster nerve. If so, this would represent the first report of β-subunits in nonvertebrate Na-channels.

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