0026-895X/91/060988-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:988-994

Photoaffinity Labeling of the Brevetoxin Receptor on Sodium Channels in Rat Brain Synaptosomes

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Received June 12, 1991; Accepted August 23, 1991

SUMMARY

Brevetoxin, a neurotoxin isolated from the marine dinoflagellate *Ptychodiscus brevis*, has been derivatized into a photoaffinity probe by carbodiimide linkage to *p*-azidobenzoic acid. Rosenthal analysis of a tritiated *p*-azidobenzoate brevetoxin derivative indicates that specific binding of the toxin occurs at two distinct and separate sites, with K_d and B_{max} values of 0.21 nM and 2.12 pmol/mg of protein for the high affinity site and 50.7 nM and 91.5 pmol/mg of protein for the low affinity site, respectively. Binding of tritiated photoaffinity probe to the high affinity/low capacity site can be displaced in a competitive manner by native brevetoxin (K_d = 1.9 nM), demonstrating a specific competitive interaction with the receptor site. Rat brain synaptosomes, covalently labeled with the brevetoxin photoaffinity probe, were subjected to detergent solubilization. The covalently labeled membrane protein was estimated to have a Stokes radius of 55 ± 3 Å. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed specific labeling of a 260-kDa protein. Treatment with 2-mercaptoethanol and neuraminidase resulted in retention of brevetoxin binding to this high molecular weight protein. The affinity-purified membrane protein-brevetoxin photoaffinity probe complex was specifically recognized by a sodium channel antibody directed against the intracellular side of transmembrane segment IS6. The sodium channel α subunit is implicated as the specific site of brevetoxin interaction.

Brevetoxins, produced by the marine dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*), are lipid-soluble polyether marine neurotoxins of unique structure and pharmacological function. They are active *in vivo* in the nanomolar to picomolar concentration range and *in vitro* in isolated neuromuscular or giant axon preparations and in single-cell or subcellular model systems. Their effect is excitatory, mediated by the enhancement of cellular Na^+ influx (1, 2). Brevetoxins occupy a unique role in the further development of allosteric mechanisms that explain the function of voltage-sensitive sodium channels, specifically as models for polyether ligands, which interact with site 5 (1, 3, 4). Site 5 is shared with ciguatoxin and is allosterically linked to sites 2, 4, 6, and 7.

Direct measurement of the binding of ^{3}H-labeled PbTx-3 to sodium channels in rat brain synaptosomes revealed a single class of high affinity binding sites. In competitive binding studies using representative neurotoxins that act upon the other previously defined Na^+ channel sites, it was demonstrated, by the failure of these toxins to displace tritiated PbTx-3 from rat brain synaptosome preparations, that brevetoxins bind to a unique site (5). This receptor site is shared by ciguatoxin, which displaces brevetoxin from its specific binding site (6).[2]

Isolation and biochemical characterization of specific neurotoxin receptor sites can be achieved using covalent toxin-receptor complexes. One means by which covalent conjugates can be produced is through use of neurotoxins modified into photoactivable derivatives (7). A variety of photoreactive, ^{125}I-labeled, α-scorpion toxin derivatives have been used to label the Na^+ channel α subunit site 3 in synaptosome preparations (8–10), neuroblastoma cells (8, 10), and reconstituted phospholipid vesicles (11–14). Other sites of neurotoxin interaction with the Na^+ channel have been studied using photoactivable derivatives of both β-scorpion toxin (site 4) (10, 15) and tetro-

This work was supported by the United States Army Medical Research and Development Command under Contracts DAMD17-87-C-7001 and DAMD17-88-C-8148 (D.G.B.) and DAMD17-90-0115 (W.A.C.), by a postdoctoral research fellowship (W.J.T.) from the National Institutes of Health, and by the Florida High Technology Council (V.L.T.). Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Army. In conducting research using animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (National Institutes of Health Publication 86-23; revised 1985).

[1]Work was in partial fulfillment of the requirements for the degree of Ph.D. in Biochemistry and Molecular Biology.

[2]M. Poli, personal communication.

ABBREVIATIONS: PbTx, *Ptychodiscus brevis* toxin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; *p*-AB, *p*-azidobenzoate (-oyl); BSA, bovine serum albumin; HPLC, high performance liquid chromatography.

dotoxin (site 1) (7, 11, 16). All studies of photoactivable neurotoxin binding to the Na^+ channel have made use of aryl azide derivatives, which upon absorption of light are converted into highly reactive nucleophilic nitrenes. These nitrenes covalently bind to a channel region neighboring the specific neurotoxin receptor site.

The recent synthesis of a brevetoxin photoaffinity label now has made possible the isolation of the brevetoxin binding site. Because brevetoxins specifically modify the Na^+ channel activation process, the location of their receptor site within the primary structure of this membrane glycoprotein will aid in the understanding of channel gating. In this study, the specific binding of a *p*-AB [^{3}H]brevetoxin probe to synaptosomal preparations is optimized, and its specific covalent interaction with the Na^+ channel α subunit is demonstrated.

Experimental Procedures

Materials

Sephacryl S-300, Sephadex A-25, and wheat germ agglutinin-Sepharose were obtained from Pharmacia, and Soluene 350 tissue solubilizer was obtained from Packard Instrument Co. All electrophoresis chemicals and 2-mercaptoethanol were purchased from Fisher Scientific Co. Organic counting scintillant, standard high molecular weight rainbow protein markers, and sodium borotritiide were from Amersham Corp. Aquasol was purchased from New England Nuclear. All remaining chemicals were reagent grade or better and were purchased from Sigma. All enzymes were used without further purification.

Toxin Purification

Brevetoxins PbTx-2 and PbTx-3 (Fig. 1) were purified from stationary-phase laboratory cultures of *P. brevis* by a combination of chloroform/methanol extraction, thin layer chromatography, and reverse phase HPLC (5, 17). Both [^{3}H]PbTx-3 (Fig. 1) and unlabeled PbTx-3 were synthesized by reduction of the PbTx-2 aldehyde, using sodium borotritiide or sodium borohydride, respectively (5). Specific activity of the radioactive preparation was determined by HPLC mass quantification, at 215 nm, with toxin standards and scintillation counting with [^{3}H]methanol standards. Tritiated PbTx-3 used for synthesis of the photoaffinity probe had a specific radioactivity in the range of 10–13 Ci/mmol.

Synthesis of *p*-AB

The method described by Rao and Venkataraman (18) was used.

Synthesis of *p*-AB-Linked Brevetoxin

Synthesis was carried out in the dark. Carbonyldiimidazole (10 μmol) was mixed with an equimolar amount of *p*-AB in 10 ml of dry benzene and was allowed to react for 3 hr in the dark at room temperature. One micromole of reacted *p*-AB (1 ml) was added to 1 mg of dry [^{3}H]PbTx-3, sealed in a thick walled glass vial, and heated in a mineral oil bath for 24 hr at 70°. The reaction product was dried under a stream of nitrogen, resuspended in acetone, and chromatographed on silica gel in petroleum ether/acetone (70:30). The desired fraction was visualized as a yellow band, by exposing a small portion of the plate to UV light. The photoaffinity-labeled brevetoxin (Fig. 1) appeared as a clear UV-fluorescent band at $R_F = 0.40$, with other minor products remaining at the plate origin. One-centimeter fractions of the developed and irradiated thin layer chromatography plate were scraped and assayed for ^{3}H by scintillation counting. The fraction showing radioactivity, UV fluorescence, and a yellow color development upon UV irradiation was the brevetoxin photoaffinity derivative. The remaining unirradiated photoaffinity probe was scraped from the silica gel chromatography plate, eluted in acetone, flash-evaporated, and purified by HPLC, with

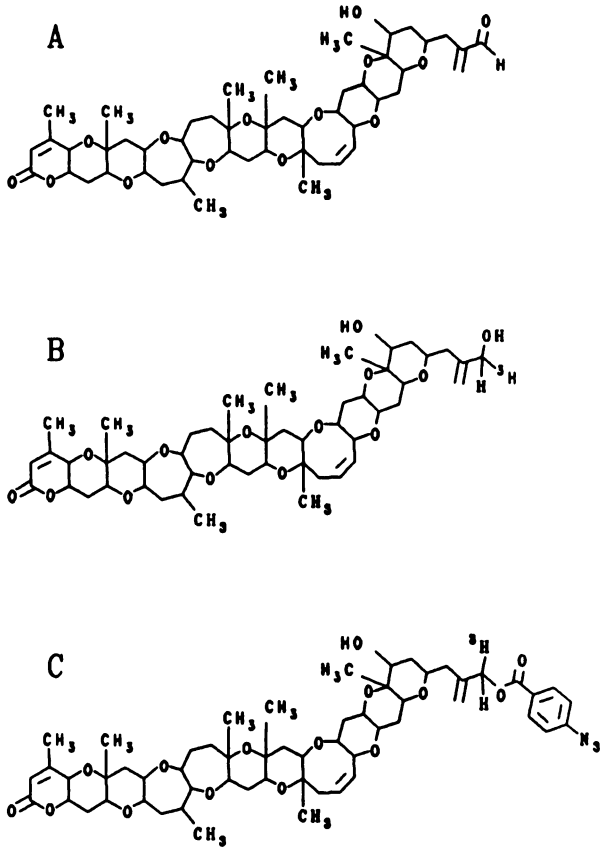

Fig. 1. Structures of the brevetoxins PbTx-2, PbTx-3, and *p*-AB brevetoxin. A, PbTx-2. B, [^{3}H]PbTx-3 has a nonexchangeable tritium on carbon 42. Tritium is replaced by hydrogen in the nonradioactive form of PbTx-3. C, *p*-AB brevetoxin, used in both tritiated (shown here) and nontritiated forms.

monitoring at 215 nm, in 85% isocratic aqueous methanol. Subsequent NMR spectroscopy of the purified photoaffinity probe confirmed its identity (data not shown).

Preparation of Synaptosomes

The method described by Dodd *et al.* (19) was used for preparation of synaptosomes from the brains of male Sprague-Dawley rats (approximately 200–250 g). Synaptosomes were found to retain brevetoxin-binding activity for several months, when stored at −70°.

Binding Experiments and Covalent Modification

Binding of *p*-AB [^{3}H]PbTx-3 was determined using a rapid centrifugation technique, as described (5). Synaptosomes, at a final protein concentration of 100 μg/ml, were added to reaction vials containing *p*-AB [^{3}H]PbTx-3 (total binding), and samples were incubated for 1 hr at 0°. Based upon the known time to equilibrium binding of native brevetoxin of 20–30 min (5), this incubation time exceeded that necessary for equilibrium binding of the photoaffinity probe with synaptosomal tissue. The concentration of *p*-AB [^{3}H]PbTx-3 used for binding studies and purification protocols was 0.2 nM. Concentrations used for inverse reciprocal plots ranged from 0.06 to 1 nM for *p*-AB [^{3}H]PbTx-3 and 0 to 5 nM for PbTx-3. Nonspecific binding was assayed by addition of 10 μM unlabeled *p*-AB PbTx-3. Experiments were carried out in subdued room light. After a 1-hr incubation of *p*-AB [^{3}H]PbTx-3 with synaptosomes at 0°, samples to be used in Rosenthal analyses and inverse reciprocal plots were centrifuged at 15,000 × *g* and assayed for radioactivity. Samples to be solubilized and subjected to gel filtration chromatography or further purification were treated in the following manner. After a 1-hr incubation at 0°, samples were washed three times, by dilution and centrifugation, with binding medium containing

1% BSA. Samples were then resuspended in binding medium containing 1% BSA, transferred to Petri dishes, exposed to UV irradiation (model UVG-11; UVG Inc.) (254 nm maximum, 18 W, at a distance of 1 cm) for 5 min with gentle agitation, and centrifuged for 3.5 min at 15,000 × *g* before being assayed for radioactivity. Data from Rosenthal analysis were analyzed using LIGAND (20).

Solubilization and Gel Filtration

Solubilization of sodium channels covalently linked to the brevetoxin photoaffinity probe using Triton X-100 and gel filtration using a Sephacryl S-300 column were performed as described by Hartshorne *et al.* (21).

SDS-PAGE

Irradiated samples were centrifuged for 2.5 min at 15,000 × *g*, and pellets were prepared for SDS-PAGE by resuspension in nonreducing sample buffer (22) and were incubated at 100° for 2 min. Samples were electrophoresed on 1.5-mm-thick SDS-acrylamide gels (23).

Chemical and Enzymatic Cleavage of the Na^+ Channel

2-Mercaptoethanol treatment. One milliliter of solubilized synaptosomes (both total and nonspecific preparations were treated in an identical manner) was incubated for 4 min at 100° with 15 mM 2-mercaptoethanol. Iodoacetamide in 0.1 M Tris·HCl (pH 8) was added to yield a final concentration of 45 mM, and the mixture was incubated at 100° for 1 min (8). After cooling to 4°, this solution was loaded onto the Sephacryl column.

Neuraminidase treatment. Control enzyme activity was assayed as described by Warren (24). Neuraminidase treatment followed the protocol described by Messner and Catterall (25). Both total and nonspecific column eluents (2-ml aliquots) of the putative α subunit were recovered from the 2-mercaptoethanol treatment and incubated with 0.1 unit of neuraminidase at 37° for 5 hr. A 0.5-ml aliquot of each digest was added to the Sephacryl column.

Ion Exchange and Affinity Chromatography

Ion exchange chromatography using DEAE Sephadex A-25 and wheat germ agglutinin-Sepharose chromatography were performed as previously described (26), except that 1 M KCl was used to elute the brevetoxin-linked membrane protein from the ion exchange column instead of 0.25 M KCl.

Sodium Channel Antibody

A synthetic peptide to residues 427–445 of domain I of the sodium channel α subunit (designated as SP1) was prepared using the method described by Gordon *et al.* (27). A standard glutaraldehyde linkage of the purified peptide to BSA was performed using the method of Orth (28). The antibody to SP1 was developed in rabbits using the immunization protocol described by Gordon *et al.* (29).

Measurement of Protein Concentration

Protein concentration was measured spectrophotometrically at 540 nm in enzyme-linked immunosorbent assay plates using the bicinchonic acid assay (Pierce Chemical Co.) or at 595 nm using the Bradford method (30) (Bio-Rad Laboratories). BSA dissolved in mobile phase was used as a standard in both assays.

Results

Photoaffinity Probe Characterization

Photoaffinity probe stability. Analysis of the *p*-AB [^{3}H]PbTx-3 by reverse phase HPLC (Microsorb, 5 μm, C18, 25-cm bed) was used to determine its purity and specific activity. Underivatized PbTx-3 showed a retention time of 8 min in 85% isocratic methanol (1.4 ml/min), whereas the *p*-AB brevetoxin demonstrated a retention time of 48.5 min. The unlinked *p*-AB eluted with a retention time of 2 min (breakthrough volume). Carbodiimide coupling of *p*-AB to [^{3}H]PbTx-3 was quantitative with respect to PbTx-3, resulting in a photoaffinity derivative with a specific activity of 10–13 Ci/mmol. The photoreactive derivative has been shown by HPLC and binding activity to be stable for at least 1 year when stored in 100% ethanol at −20°.

Irradiation optimization. Through spectrophotometric analysis of UV-irradiated *p*-AB, it was determined that a 30-sec exposure to a wavelength of 254 nm was effective in completely activating the pure compound in a solution not containing synaptosomes. Photoaffinity probe binding to synaptosomes, with no prior BSA incubation or washes, was used to determine the optimal time needed for maximum total binding. Based upon both spectrophotometric analysis of activated *p*-AB and information gained from the time course of photoaffinity probe binding to synaptosomes, 5 min was chosen as the irradiation time needed for optimization of specific binding activity.

Binding Studies

In the absence of irradiation, *p*-AB [^{3}H]PbTx-3 was displaced, in a competitive manner, by PbTx-3 from its specific site of binding in synaptosomes (Fig. 2), illustrating a common binding site for native and derivatized brevetoxin. Half-maximal equilibrium binding of the derivatized brevetoxin was observed at a concentration of 0.2 nM. Underivatized brevetoxin exhibits half-maximal binding at 1.9 nM ligand concentration.

The time necessary to dissociate one half the total unirradiated [^{3}H]PbTx-3 photoaffinity probe from its receptor ($t_{1/2}$) was determined by performing a binding experiment, as described in Experimental Procedures. After this initial incubation, samples were centrifuged and resuspended in standard binding medium. The loss of total radioactivity at various time

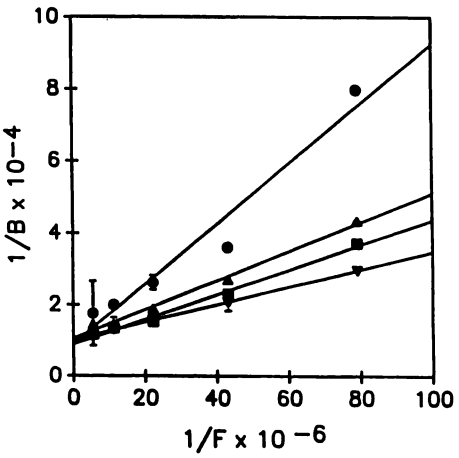

Fig. 2. Inhibition of [^{3}H]PbTx-3 binding by *p*-AB brevetoxin photoaffinity probe. The tritiated photoaffinity derivative (100% pure by HPLC analysis) was displaced by PbTx-3 from its specific site of binding in rat brain synaptosomes. Experiments were carried out as described in Experimental Procedures, at toxin concentrations that surrounded the photoaffinity probe K_d. The concentrations of *p*-AB [^{3}H]PbTx-3 were 1.0, 0.5, 0.25, 0.13, and 0.06 nM. Unlabeled PbTx-3 concentrations were 5 (●), 0.5 (▲), 0.05 (■), and 0 (▼) nM. Bound radioactivity (*B*) was measured as the difference between total and nonspecific values. Free radioactivity (*F*) was determined by assay of supernatant solutions. *Lines* are computer-generated first-order regressions. *Error bars* span the range of duplicate determinations (when not visible, *error bars* are smaller than *symbol size*).

intervals was compared with a control incubation (time zero). The $t_{1/2}$ of the photoaffinity probe was approximately 30 min, a value comparable to the 20-min $t_{1/2}$ determined for native PbTx-3. These results indicate that there is sufficient time for the photoaffinity-labeled sodium channel to be washed, in order to reduce nonspecific binding, without substantial loss of specifically bound photoaffinity probe.

A Rosenthal analysis (31) of photoaffinity probe binding to synaptosomes resulted in a nonlinear plot, suggesting the presence of two binding sites for the brevetoxin molecule in synaptosomes (Fig. 3). Binding of the photoaffinity label to the high affinity site resulted in a LIGAND-derived half-maximal binding concentration (K_d) of 0.21 nM and maximal binding capacity (B_{max}) of 2.12 pmol/mg of protein. Low affinity site binding was characterized by a K_d of 50 nM and B_{max} of 92 pmol/mg of protein. The B_{max} for the high affinity site was similar to the concentration of sodium channels in synaptosomes, whereas the B_{max} for the low affinity site greatly exceeded the concentration of sodium channels. Evidently, only the high affinity site represents binding to sodium channels. Fig. 3, *inset*, shows the LIGAND-derived saturation binding data for both high and low affinity brevetoxin binding sites. At a concentration of 0.2 nM, corresponding to the K_d for the high affinity site, >75% of the total radioactivity was bound to the high affinity site. Because the high affinity/low capacity site was the focus of this study, all receptor purification protocols were performed at concentrations of 0.2 nM *p*-AB [^{3}H]PbTx-3.

Irradiation of synaptosomes at a 254-nm wavelength for 5 min, followed by incubation with 0.2 nM *p*-AB [^{3}H]PbTx-3 for 1 hr at 4°, centrifugation, and assay for tritium activity, indicated that approximately 11% of binding capacity was lost upon UV exposure, compared with unirradiated synaptosomes.

Receptor Purification

Gel filtration. Gel filtration studies were performed under reducing and nonreducing conditions. Under nonreducing conditions, a single peak of protein and specifically bound radioactivity (Fig. 4A), which corresponded to a Stokes radius of 55 ± 3 Å (five experiments), was eluted from the Sephacryl S-300 column. Cleavage of the α subunit from the disulfide-linked β2 subunit with 2-mercaptoethanol resulted in retention of binding at the high molecular weight protein band and gave better resolution of the solubilized proteins (Fig. 4B) (32). These results strongly suggest that *p*-AB [^{3}H]PbTx-3 binds to the α subunit of the α-β2 Na^+ channel subunit complex. Further treatment of column fractions showed that cleavage of terminal sialic acids using neuraminidase (data not shown) resulted in no change in elution characteristics of the brevetoxin-binding protein, indicating that the brevetoxin is not covalently attached to sialic acid residues.

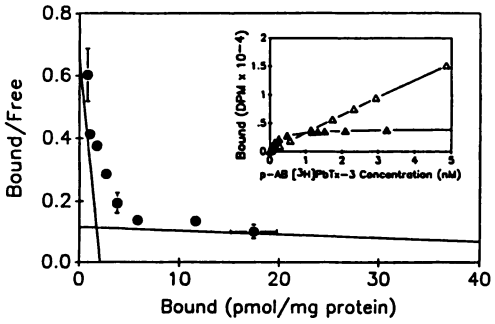

Fig. 3. Rosenthal analysis of [^{3}H]PbTx3 photoaffinity probe binding to crude synaptosomal preparations. Concentrations of photoaffinity probe ranged from 0 to 100 nM. *Inset*, LIGAND-derived saturation binding data for both high affinity/low capacity (▲) and low affinity/high capacity (△) brevetoxin binding sites. Binding experiments were carried out as described in Experimental Procedures. High affinity binding saturates at approximately 0.4 nM, and low affinity binding saturates at approximately 150 nM (off-scale). *Error bars* span the range of duplicate values, and *points* represent the mean (when not visible, *error bars* are smaller than *symbol size*).

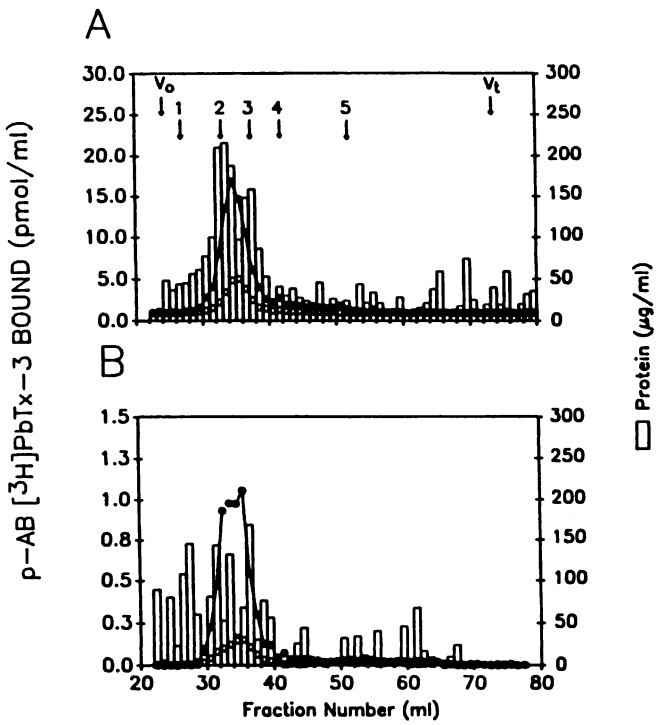

Fig. 4. Gel filtration of the brevetoxin receptor. Measurements of total binding (●), nonspecific binding in the presence of 10 μM nonradioactive *p*-AB PbTx-3 (○), and protein (□) were made. Fraction volume was 1 ml. Molecular mass standards are *1*, thyroglobulin (669 kDa); *2*, ferritin (440 kDa); *3*, catalase (232 kDa); *4*, aldolase (158 kDa); and *5*, ovalbumin (43 kDa). Gel filtration chromatography was performed in both the absence (A) and presence (B) of 15 mM 2-mercaptoethanol.

SDS-PAGE. SDS-PAGE analysis of *p*-AB [^{3}H]PbTx-3 covalently bound to synaptosomes was performed using a 5–20% gradient gel as well as 5% gels. The gradient gels were used to resolve potential binding of the photoaffinity probe to the α subunit (260 kDa), the β2 subunit (33 kDa), and the β1 subunit (36 kDa). Specific binding was determined as the difference between total and nonspecific components, which were electrophoresed in parallel lanes under reducing conditions. A protein band with a molecular mass slightly greater than the 200-kDa standard marker protein, at about 260 kDa, was determined to contain the most specifically bound radioactivity (Fig. 5A). Because this band was located in close proximity to the stacking gel, which contained radiolabeled proteins unable to enter the resolving gel, a 5% gel was used to separate these two components and to provide further evidence of α subunit interaction with the radiolabeled photoaffinity probe (Fig. 5B). SDS-PAGE indicated that approximately 20% of the specific binding component was covalently incorporated into a single 260-kDa protein after photolysis.

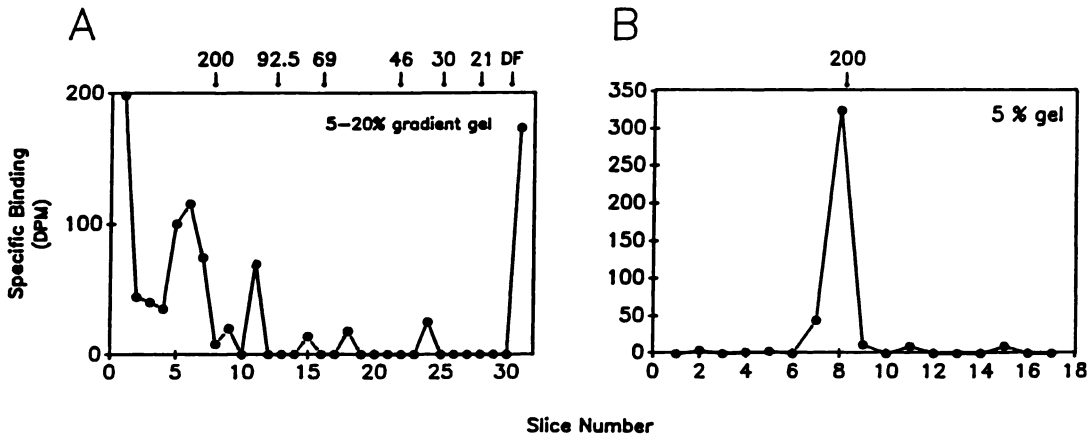

Fig. 5. SDS-PAGE analysis of the covalently labeled brevetoxin receptor. Specific binding was calculated as the difference between total and nonspecific components, which were electrophoresed in different lanes. SDS-PAGE was performed under reducing conditions, as described in Experimental Procedures. Molecular masses, designated in kDa on the *upper abscissa*, were determined by comparison with molecular mass markers in adjacent lanes. Gel slices were 4 mm thick. Slices were dissolved in 0.5 ml of Soluene and 0.1 ml of H_2O_2 by heating at 60° for 3 hr. After addition of 6 ml of organic counting scintillant, samples were placed in the dark overnight and then assayed for radioactivity. The number of slices per gel varied due to differing amounts of agarose (used as a plug to allow acrylamide to polymerize), which entered the bottom of the electrophoresis chamber by capillary action. Analyses on a 5–20% gradient gel (A) and a 5% gel (B) were performed. Stacking gel and dye front (*DF*) in B were not assayed for radioactivity.

Immunoprecipitation. The antibody raised to the SP1 region of the sodium channel α subunit was incubated with PbTx photoaffinity probe-linked sodium channel purified through affinity chromatography on wheat germ agglutinin-Sepharose. This antibody showed significant ($p < 0.05$) concentration-dependent recognition of the brevetoxin-linked Na^+ channel protein covalent conjugate, compared with the preimmune serum control (Fig. 6). These results further confirm that the 260-kDa protein labeled by *p*-AB [^{3}H]PbTx-3 is the α subunit of the sodium channel.

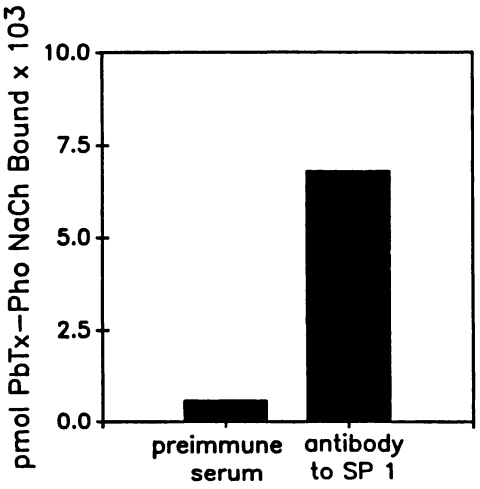

Fig. 6. Immunoprecipitation of the covalently labeled brevetoxin receptor. [^{3}H]PbTx photoaffinity probe-labeled sodium channel was solubilized from synaptosomes, purified through ion exchange and wheat germ affinity chromatography, and incubated with either preimmune serum or antibody raised to the SP1 portion of the sodium channel α subunit domain I. Protein concentrations, determined by absorbance at 280 nm, were 0.3 mg (preimmune serum) and 0.1 mg (antibody to SP1); 40 pmol of sodium channel/tube were used. The experiment was repeated, and similar results were obtained. An increase of antibody protein to 1 mg/ml resulted in 30% greater binding to [^{3}H]PbTx photoaffinity probe-labeled sodium channel than the 0.1 mg/ml value (not shown). Samples were counted for 20 min, resulting in a 95% confidence level of ±2% of the mean.

Discussion

A necessary component of successful receptor isolation is the ability to assay toxin-binding activity at all stages of purification. Na^+ channels that have been solubilized from neuronal tissue with nonionic detergents retain neurotoxin-binding activity at receptor site 1 but not at sites 2 and 3 (33). Similarly, preliminary work indicated that specific binding could not be effectively monitored by incubation of tritiated brevetoxin with detergent-solubilized membrane preparations; the hydrophobic interactions of the toxin with its solubilized receptor appear to be largely nonspecific (34). Thus, it was necessary to develop a radiolabeled photoaffinity probe that could be covalently linked to the receptor site in synaptosomes before solubilization, allowing for assay of the receptor throughout the purification protocol.

Characterization of Brevetoxin Photoaffinity Probe

Reduction of nonspecific binding. Elevated nonspecific binding is a common problem encountered when working with photoaffinity labels, due to the presence of a highly reactive photosensitive group free in solution. Nonspecific interaction was reduced by the use of BSA, a scavenger protein that provided the photoaffinity derivative with numerous sites for nonspecific attachment without interfering with specific site derivatization. Three washes by dilution and centrifugation before photolysis, using a solution containing 1% BSA, resulted in a decrease in nonspecific binding of the brevetoxin photoaffinity probe of approximately 85%, without loss of specific binding (data not shown).

Binding characteristics. Additional experiments were performed to confirm that the binding characteristics of the derivatized compound and the native ligand are similar. The lower K_d of the photoaffinity derivative (0.2 nM), compared with that of native brevetoxin (1.9 nM), may be due to the added hydrophobicity of the benzene ring, which is clearly evident in the substantially increased retention time on C18 reverse phase HPLC columns. Binding studies utilizing PbTx-3 to displace unirradiated [^{3}H]PbTx3 photoaffinity probe from its mem-

brane receptor demonstrated displacement patterns indicative of competitive inhibition. These results show that PbTx-3 and its *p*-AB derivative bind to the same receptor site on sodium channels.

Rosenthal analysis of the *p*-AB brevetoxin binding demonstrated the presence of two distinct binding sites. Previous work documented a single brevetoxin binding site in experiments that utilized radioligand concentrations up to 20 nM (5). Evidence for two-site binding has been seen in Rosenthal analyses of native brevetoxin that used radiolabeled toxin concentrations up to 175 nM,[3] as well as in competition binding studies with underivatized brevetoxins (35).

In both types of experiment, low affinity binding became evident above 25 nM, a concentration of brevetoxin well beyond that at which the half-maximal biological effect was observed (1.6 nM) (2). In a study in which the same synaptosomal preparation was incubated with either [^{3}H]saxitoxin or [^{3}H]PbTx-3, the B_{max} of saxitoxin and high affinity brevetoxin were found to be identical ($p < 0.01$). This indicates that saxitoxin and high affinity site brevetoxin binding occur in equivalent ratios and suggests that low concentrations of brevetoxin bind in a 1:1 stoichiometry with the sodium channel. In view of these results indicating a high affinity interaction of brevetoxin with the sodium channel, the high affinity low capacity site was the focus of the present study. Further work is in progress to confirm that the low affinity brevetoxin binding occurs on a separate protein component of synaptosomal preparations, as expected from its high B_{max} value.

Receptor Isolation and Characterization

The size estimate of the tritiated, photoaffinity-labeled, Na^+ channel eluted from a Sephacryl S-300 column (55 Å) is an indication that the photoaffinity label is incorporated into a large protein/detergent complex of similar size as the sodium channel (32). Separation of the covalently bound α and $\beta 2$ subunits with 2-mercaptoethanol resulted in no significant change in elution profile, indicating that the brevetoxin photoaffinity probe preferentially binds to the α subunit. Due to the low resolution capability of the gel, a shift in molecular weight was not observed upon treatment with either 2-mercaptoethanol or neuraminidase; therefore, this method was used only to give a molecular weight approximation.

The Na^+ channel α subunit from rat brain contains more than 20% (w/w) carbohydrate (25), approximately 12% of which is negatively charged sialic acids. More than 100 sialic acid residues are believed to surround the Na^+ channel pore, in an approximate spherical volume of 75 Å. It has been suggested that sialic acids may play an important role in channel activation through their electrostatic influence on membrane potential, because removal of sialic acids alters the voltage dependence and subconductance states of purified and reconstituted sodium channels (36). The potential reactivity of the *p*-AB brevetoxin with these sugar molecules was tested by specific cleavage of sialic acid residues with neuraminidase. Treatment of PbTx photoaffinity probe-labeled sodium channel with this enzyme resulted in no change in elution pattern of the tritiated complex by gel filtration, demonstrating that terminal sialic acids do not bind to the photoreactive compound.

Immunoprecipitation of the covalently linked brevetoxin-sodium channel complex with an antipeptide antibody directed against a segment of the α subunit conclusively demonstrated covalent incorporation of this neurotoxin to the Na^+ channel α subunit. The location of the brevetoxin receptor site on the α subunit correlates well with previous findings, which demonstrate the importance of this subunit in channel-gating and voltage-sensing mechanisms. It has also been shown to contain binding sites for other neurotoxins, such as tetrodotoxin, saxitoxin, and α-scorpion toxins, that are known to alter normal Na^+ channel function specifically.

The tritiated *p*-AB brevetoxin photoaffinity derivative, which forms a covalent bond with its membrane receptor upon UV irradiation, will be useful for assay of the ligand-receptor complex through all stages of purification. Because the binding site of [^{3}H]PbTx3 photoaffinity probe has been shown to be identical to the receptor with which native brevetoxin interacts, the mapping of this covalently bound ligand to its receptor sequence may ultimately result in precise localization of Na^+ channel receptor site 5. Knowledge of the position of this binding site may increase our understanding of the molecular mechanism of channel activation, a process altered by interaction of brevetoxin with a specific portion of the Na^+ channel α subunit.

Acknowledgments

We thank Dr. T. J. Mende and L. Schulman for the initial purification of the photoaffinity probe and L. Roszell for excellent technical assistance.

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