Site of Covalent Labeling by a Photoactive Batrachotoxin Derivative near Transmembrane Segment IS6 of the Sodium Channel α Subunit*

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The binding site for batrachotoxin, a lipid-soluble neurotoxin acting at Na⁺ channel receptor site 2, was localized using a photoactive, radiolabeled batrachotoxin derivative to covalently label purified and reconstituted rat brain Na⁺ channels. In the presence of the brevetoxin 1 from Ptychodiscus brevis and the pyrethroid RU51049, positive allosteric enhancers of batrachotoxin binding, a protein with an apparent molecular mass of 240 kDa corresponding to the Na⁺ channel α subunit was specifically covalently labeled. The region of the α subunit specifically photolabeled by the photoactive batrachotoxin derivative was identified by antibody mapping of proteolytic fragments. Even after extensive trypsinization, an anti-peptide antibody mapping of proteolytic fragments. Even after extensive trypsinization, an anti-peptide antibody recognizing an amino acid sequence adjacent to Na⁺ channel transmembrane segment IS6 was able to immunoprecipitate up to 70% of the labeled peptides. Analysis of a more complete digestion with trypsin or V8 protease indicated that the batrachotoxin receptor site is formed in part by a portion of domain I. The identification of a specifically immunoprecipitated photolabeled 7.3-kDa peptide containing transmembrane segment S6 from domain I restricted the site of labeling to residues Asn-388 to Glu-429 if V8 protease digestion was complete or Leu-388 to Glu-429 if digestion was incomplete. These results implicate the S6 transmembrane region of domain I of the Na⁺ channel α subunit as an important component of the batrachotoxin receptor site.

Batrachotoxin (BTX) is a steroidal alkaloid toxin from skin secretions of South American frogs, Phyllobates aurotaenia and Phyllobates terribilus, which are used by the native Indians of Colombia to make poison blowdarts and arrows (1). It is one of the most toxic nonproteinaceous substances known and is capable of inducing membrane depolarization at concentrations in the low nanomolar range. The voltage-gated Na⁺ channels of excitable membranes are the molecular targets of BTX, and all aspects of Na⁺ channel function are altered upon exposure to BTX: inactivation is blocked, single channel conductance is decreased, voltage dependence of activation is shifted to more negative potentials, and selectivity for Na⁺ is impaired (reviewed in Refs. 2 and 3).

Competitive binding studies with radiolabeled neurotoxin analogues have distinguished five distinct receptor sites for neurotoxins on the Na⁺ channel, including neurotoxin receptor site 2 which is occupied by the full agonist BTX and the partial agonists veratridine, aconitine, and grayanotoxin which modulate Na⁺ channel gating (2, 3). The binding of BTX at neurotoxin receptor site 2 results in stabilization of an open conformation of the channel. At least four of the five receptor sites have been shown to be located on the 260-kDa α subunit, which is composed of four homologous domains (I–IV) containing six putative transmembrane segments (S1–S6; Refs. 4, 5). Site-directed mutagenesis experiments have identified amino acid residues of the α subunit that are required for high affinity binding of tetrodotoxin and saxitoxin at neurotoxin receptor site 1 (6–8). Peptide segments from neurotoxin receptor site 3, which binds α-scorpion toxins and sea anemone toxins, and neurotoxin receptor site 5, which binds brevetoxins, have been identified by photoaffinity labeling and peptide mapping with sequence-specific antibodies (9–11), and individual amino acid residues that are required for high affinity binding of α-scorpion toxins and sea anemone toxins to receptor site 3 have been identified by site-directed mutagenesis (12).

BTX binding to neurotoxin receptor site 2 is allosterically modulated by other Na⁺ channel neurotoxins. The α-polypeptide toxins from scorpion and sea anemone (13), the α-cyanopregyrophoroid insecticides (3, 14, 15), and brevetoxin (14, 15) all enhance BTX binding. Other compounds such as tetrodotoxin and saxitoxin (16), local anesthetics (17, 18), and the anticonvulsants diphenylhydantoin and carbamazepine (19) decrease BTX-binding affinity. These results indicate that the BTX-binding site is particularly sensitive to conformational alterations induced at distinct sites.

Neurotoxin receptor site 2 is present on Na⁺ channels expressed from α subunit cDNA alone (20, 21). Mapping of the peptide segments of the α subunit that form receptor site 2 by photoaffinity labeling has not been feasible because the ligands binding at that site have relatively low affinity and are hydrophobic, resulting in low specific binding values. These difficulties can be circumvented by taking advantage of the allosteric enhancement of BTX binding by pyrethrroids and the brevetoxin analogue, PbtX-1. This combination of effectors can enhance BTX binding in a purified and reconstituted Na⁺ channel preparation up to 1000-fold, reducing the Kₚ to a value below 1 nM (15). In this study, this combination of toxins is used to enhance high affinity binding and covalent incorporation of a photoactive, radiolabeled BTX derivative, [³H]BTX-OAB,
into purified Na⁺ channels, and the major site of incorporation is identified by peptide mapping with anti-peptide antibodies.

**EXPERIMENTAL PROCEDURES**

Materials—Eculome was from ICN Biomedicals, Inc. and Soluene was from DuPont NEN. Phosphatidylcholine and phosphatidylethanolamine were used for sodium channel reconstitution as supplied from Avanti Polar Lipids and l-1-tyosylamide-2-phénylethyl chloride were from Sigma. Phosphatidylcholine/phosphatidylethanolamine vesicles (65:35, v/v) were prepared by rapid filtration technique (29). Purified samples containing 250–350 pmol of Na⁺ channel (approximately 10,000 cpm of [³H]BTX-OAB per channel) were used to determine the molecular mass of the Na⁺ channel peptides.

Sequence-directed Antibodies—Polyclonal antisera were raised in rabbits against synthetic Na⁺ channel peptides (SP) corresponding to residues 110–112 of the rat brain type II alpha subunit (4, 5) as described previously (22) from [³H]anthranilic acid (47 Ci/mmol) by selective esterification of BTX-A.

[³H]BTX-OAB Binding to Reconstituted Na⁺ Channels—Binding reactions were initiated by a 4-fold dilution of 50 µl of purified and reconstituted Na⁺ channel (5–10 pmol) in standard binding medium (23). Reconstituted channels were incubated at 25 °C for 16 h with [³H]BTX-OAB and effectors as shown in the figure legends. Binding reactions were stopped by addition of choline wash medium, and samples were filtered through GF/F filters as described previously (24). Nonspecific binding was determined in the presence of 300 µM veratridine.

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Preparation of Antibodies Bound to Protein A-Sepharose—Protein A-Sepharose was swollen for 20 min at 25 °C in 0.1 M sodium phosphate, pH 8.1, to give a final concentration of 100 mg/ml. One ml of serum was added per 0.75 ml of swollen protein A-Sepharose and mixed by rotation at 25 °C for 30 min or at 4 °C overnight. Supernatants were removed, and the pellets were washed two times with 5 ml of buffer S (10 mM Tris, adjusted to pH 7.4 with HCl, 150 mM NaCl). The proteins were solubilized from the pellet by incubation with 100 µl of 1% (w/v) SDS in 50 mM ammonium bicarbonate buffer adjusted to pH 8.1, and the pellets were washed two times with 5 volumes of buffer S. For analysis of [³H]BTX-OAB bound to immunoprecipitated Na⁺ channel peptides, a variety of gel systems were used as described below. Prestained molecular mass standards were used to determine the molecular mass of the Na⁺ channel peptides. To determine protein-bound radioactivity, individual gel lanes were manually cut into 1-3-mm slices, and radioactivity was eluted in 5% (v/v) Soluene in Ecolourne according to the manufacturer’s instructions.

**RESULTS**

Characterization of [³H]BTX-OAB Binding to Purified and Reconstituted Sodium Channels—Previous studies (15) have demonstrated specific, high affinity binding of [³H]BTX to neurotoxin site 2 on sodium channels purified from rat brain and reconstituted in phospholipid vesicles. In order to demonstrate that the radiolabeled, photoactive derivative of BTX, [³H]BTX-OAB (Fig. 1), has the same binding characteristics as [³H]BTX-B, we examined the binding of [³H]BTX-OAB and [³H]BTX-OAB to the same reconstituted Na⁺ channel preparation. Specific binding of both [³H]BTX-B and [³H]BTX-OAB is inhibited by saturation of receptor site 2 by unlabeled veratridine with half-maximal inhibition at approximately 8 µM (Fig. 2, solid symbols), in good agreement with the value of 7 µM observed for syntaxotoxin (13). Using a concentration of 300 µM unlabeled veratridine to measure nonspecific binding, specific binding was determined to be 92–94% of total binding using either radiolabeled BTX derivative. No saturable component of [³H]BTX-B binding was observed in parallel experiments with sodium channels that had been heat-inactivated by incubation at 60 °C for 2 min (Fig. 2, open circles) or with phospholipid vesicles without added protein (Fig. 2, open squares). The loss of binding at an elevated temperature provides evidence that conformational integrity of the purified Na⁺ channel is a requirement for high affinity interaction with BTX, as previously observed for brevetoxins (11), saxitoxin, and α-scorpion toxin (24).

Synergistic Effects of Pyrethroids and Brevetoxins on BTX Binding—[³H]BTX-B binding to purified and reconstituted rat brain Na⁺ channel is enhanced by pyrethroids (15). In that study, specific [³H]BTX-B binding was 99% of total binding in the presence of 10 µM RU39568, the highest level of specific BTX binding yet demonstrated. In the present study, half-maximal enhancement of [³H]BTX-B binding by RU39568 was observed at approximately 2 µM (Fig. 3A, solid circles), consistent with previous studies (15), and the half-maximal effect of RU51049 was achieved at about 600 nM (Fig. 3A, solid squares). The maximal enhancement of BTX binding in the presence of 100 µM RU51049 is approximately 30% greater than that ob-
served with RU39568. Binding of the pyrethroids, RU51049 and RU39568, and the brevetoxin, PbTx-1, to their receptor sites in purified Na\textsuperscript{+} channel preparations reconstituted into phospholipid vesicles leads to a marked increase in specific binding of BTX at receptor site 2 (Fig. 3A). PbTx-1 increases the level of specific binding observed in the presence of a saturating concentration of either pyrethroid. No specific binding of \(^{3}\text{H}\)BTX-B was observed with purified and solubilized Na\textsuperscript{+} channel preparations that had not been reconstituted into vesicles, which is consistent with previous studies (29).

Specific Photolabeling of Reconstituted Sodium Channels by \(^{3}\text{H}\)BTX-OAB in the Presence of RU51049 and PbTx-1—Purified and reconstituted Na\textsuperscript{+} channels were specifically photolabeled by \(^{3}\text{H}\)BTX-OAB in the presence of 20 \(\mu\text{M}\) RU51049 and 100 \(\text{nM}\) PbTx-1. Analysis of specifically labeled, reconstituted Na\textsuperscript{+} channels by SDS-PAGE, gel slicing, and scintillation counting revealed a single peak of covalently incorporated \(^{3}\text{H}\)BTX-OAB with an apparent molecular mass of 240 kDa, consistent with covalent labeling of the \(\alpha\) subunit (Fig. 4, solid circles). Samples labeled in the presence of an excess of unlabeled veratridine did not contain any \(^{3}\text{H}\)BTX-OAB covalently attached to proteins (Fig. 4, open circles). Some degradation of the specifically labeled protein was observed as a band of radioactivity at approximately 50 kDa, which is expected since a 4-h incubation at 25 °C was used in this experiment. Consistent with this result, previous studies have indicated that the Na\textsuperscript{+} channel \(\alpha\) subunit contains a relatively protease-resistant domain of 50–70 kDa (9).

Isolation and Characterization of Smaller Proteolytic Fragments Labeled with \(^{3}\text{H}\)BTX-OAB—To localize the position of the covalently attached photolabel, the reconstituted Na\textsuperscript{+} channel preparations were incubated in the presence of \(^{3}\text{H}\)BTX-OAB and then treated with proteases. The resulting proteolytic fragments were resolved by two-dimensional (2D) gel electrophoresis and visualized by autoradiography. The results indicated that the \(^{3}\text{H}\)BTX-OAB label was covalently attached to a protein with a molecular mass of approximately 100 kDa, consistent with the location of the \(\alpha\) subunit in the Na\textsuperscript{+} channel.

**Fig. 1.** Structure of BTX and its derivatives. The positions of \(^{3}\text{H}\) labels are shown by asterisks. Batrachotoxinin-A (BTX-A) is the non-toxic natural precursor of batrachotoxin from which both radiolabeled derivatives (bottom two structures) were synthesized.

**Fig. 2.** Competitive displacement of \(^{3}\text{H}\)BTX-B bound to reconstituted Na\textsuperscript{+} channels by unlabeled veratridine. Binding of \(^{3}\text{H}\)BTX-B (17 \(\text{nM}\), solid circles) and \(^{3}\text{H}\)BTX-OAB (10 \(\text{nM}\), solid squares) to reconstituted Na\textsuperscript{+} channels was measured as described under "Experimental Procedures" in the presence of the indicated concentrations of veratridine. Control binding of \(^{3}\text{H}\)BTX-B to heat-inactivated and reconstituted Na\textsuperscript{+} channels (open circles) and reconstituted phospholipids (open squares) was also determined.

**Fig. 3.** Enhancement of specific \(^{3}\text{H}\)BTX-B binding by RU39568, RU51049, and PbTx-1. A, \(^{3}\text{H}\)BTX-B binding to reconstituted Na\textsuperscript{+} channels was measured as described under "Experimental Procedures" in the presence of 5 \(\text{nM}\) \(^{3}\text{H}\)BTX-B and increasing concentrations of RU39568 (solid circles) or RU51049 (solid squares). B, \(^{3}\text{H}\)BTX-B binding was measured in the presence of 0.5 \(\text{nM}\) \(^{3}\text{H}\)BTX-B and 10 \(\mu\text{M}\) RU pyrethroid with and without 100 \(\text{nM}\) PbTx-1 in the presence (stippled bars) or absence (solid bars) of 300 \(\mu\text{M}\) veratridine for determination of nonspecific and total binding, respectively. Binding of \(^{3}\text{H}\)BTX-B to purified soluble Na\textsuperscript{+} channel was measured in the presence of 10 \(\mu\text{M}\) RU51049 and 100 \(\text{nM}\) PbTx-1. Samples were incubated at 25 °C for 17 h for determination of maximum allosteric interaction.
channel labeled with $[^3H]$BTX-OAB was digested at lysine and arginine residues with TPCK-trypsin. The digested, photolabeled fragments were then probed by immunoprecipitation with a series of sequence-directed antibodies recognizing regions of the $\alpha$ subunit within or bordering each of the four homologous domains as illustrated in Fig. 5A. All of the antibodies specifically immunoprecipitated the intact $\alpha$ subunit. The amount of radioactivity bound by all of the Na$^+$ channel antibodies progressively decreased with increasing trypsin concentrations, suggesting that the antibody binding sites were separated from the $[^3H]$BTX-OAB incorporation site upon proteolysis (Fig. 5B). After cleavage of the $[^3H]$BTX-OAB-labeled $\alpha$ subunit with increasing concentrations of trypsin, anti-SP1, directed against a sequence immediately following transmembrane segment IS6, immunoprecipitated over 70% of the total specifically photolabeled channel at the highest concentration of trypsin tested (Fig. 5B). The antibodies within or bordering domains II, III, and IV recognized only a small fraction of the radioactivity (less than 20%) after extensive trypsinization. These results indicate that a substantial fraction of covalently incorporated $[^3H]$BTX-OAB is located within domain I.

The site of BTX covalent labeling of the Na$^+$ channel $\alpha$ subunit was more precisely localized by more extensive proteolytic cleavage at lysine and arginine residues with trypsin and at glutamic acid and aspartic acid residues with V8 protease from S. aureus. Under the conditions for proteolysis described in Fig. 6A, approximately 50% of the total radioactivity was precipitable by anti-SP11 antibody and 70% by anti-SP1 antibody after cleavage with 10 $\mu$g/ml trypsin for 1 h. These antibodies, directed against peptides within the first 60 amino acids at the intracellular side of transmembrane segment IS6, completely lost their ability to recognize $\alpha$ subunit-bound $[^3H]$BTX-OAB upon digestion with 100 $\mu$g/ml V8 protease for 1 h (Fig. 6B). However, anti-SP31 and anti-SP28, antibodies directed against peptides within the first 50 amino acids extracellular to transmembrane segment IVS6, were able to precipitate 30–60% of radioactivity after cleavage with trypsin (Fig. 6A) and over 50% of the total specific radioactivity after cleavage with V8 protease (Fig. 6B). Anti-SP15, anti-SP14, and anti-SP19, which are directed against sequences farther toward the carboxyl terminus, do not immunoprecipitate a significant amount of photolabel (Fig. 6, A and B). These results indicate that the site of $[^3H]$BTX-OAB covalent labeling is located to the amino-terminal side of the anti-SP1 recognition peptide.

Photolabeling of a 7-kDa Peptide by $[^3H]$BTX-OAB—A complete tryptic digest was obtained by covalently labeling the purified and reconstituted Na$^+$ channel with $[^3H]$BTX-OAB and subjecting the labeled protein to digestion with 100 $\mu$g/ml trypsin overnight at 37°C and then with V8 protease under the same conditions overnight. This extensively digested preparation was used to restrict further the sites of labeling by $[^3H]$BTX-OAB to small Na$^+$ channel peptide fragments from proteolytic cleavage by trypsin. Photolabeled reconstituted Na$^+$ channels were digested with increasing concentrations of TPCK-trypsin, and the resulting peptide fragments were probed with the indicated antibodies as described under “Experimental Procedures.” TPCK-trypsin concentrations for each antibody treatment were as follows (from left to right): 0.3, 1, 10, 100 $\mu$g/ml. Values are expressed as the percentage of total cpm immunoprecipitated without trypsin treatment.

**Fig. 5.** Immunoprecipitation of peptides covalently labeled by $[^3H]$BTX-OAB. A, recognition sites of anti-peptide antibodies. Antibodies directed against synthetic peptides corresponding to different sequences of the $\alpha$ subunit of the type IIA sodium channel were prepared as described (Gordon et al., 25, 26). Antibodies were directed against synthetic sodium channel peptides corresponding to the amino acid sequences 355–372 (SP31), 382–400 (SP28), 427–445 (SP1), 468–504 (SP11), 531–547 (SP32), 708–722 (SP15), 1145–1164 (SP20), 1480–1498 (SP14), 1541–1561 (SP19), 1736–1753 (SP29), 1789–1798 (SP13). B, immunoprecipitation of $[^3H]$BTX-OAB-labeled Na$^+$ channel peptide fragments from proteolytic cleavage by trypsin. Photolabeled reconstituted Na$^+$ channels were digested with increasing concentrations of TPCK-trypsin, and the resulting peptide fragments were probed with the indicated antibodies as described under “Experimental Procedures.” TPCK-trypsin concentrations for each antibody treatment were as follows (from left to right): 0.3, 1, 10, 100 $\mu$g/ml. Values are expressed as the percentage of total cpm immunoprecipitated without trypsin treatment.
precipitated a photolabeled peptide with a molecular mass of approximately 6 kDa (Fig. 7A, solid triangles). Some reversibly bound photolabel, which was not removed by washing prior to electrophoresis, was observed at the gel dye front. Immunoprecipitation of the 6-kDa peptide by anti-SP28 was specific, because neither preimmune IgG nor anti-SP28 recognize this small labeled peptide.

Experiments like the one illustrated in Fig. 7 were carried out with several preparations of purified and reconstituted Na\(^+\) channels, two different preparations of \([3H]\)BTX-OAB, and three different SDS-PAGE separation systems with similar results. Although the small 6–7-kDa peptide, which can be precipitated by anti-SP28, migrates close to the dye front in all of the SDS-PAGE systems used, the estimation of size is consistent in all three gel systems (Fig. 7B). These results indicate that \([3H]\)BTX-OAB is covalently incorporated into a peptide of about 7.3 kDa containing the anti-SP28 antibody recognition site.

Examination of the amino acid sequence of the \(a\) subunit near the SP28 peptide (Fig. 8) allows identification of the photolabeled fragment obtained from extensive cleavage with trypsin and V8 protease. In domain I, the peptide which includes all of the anti-SP28 recognition sequence from the trypsin cleavage site, Leu-380, to the V8 protease cleavage site, Glu-429, has a calculated molecular mass of 7.3 kDa (Fig. 8). Trypsin cleavage within peptide SP28 was previously found to occur only under extreme conditions (9), so cleavage at Arg-395 or Lys-399 is unlikely. More complete cleavage by V8 protease at Glu-387 within the SP28 sequence might yield the peptide from Asn-388 to Glu-429 with a calculated molecular mass of 6.0 kDa, which contains all but 6 residues of the sequence of peptide SP28. Because peptides are linked through their amino termini to bovine serum albumin for immunization, the first few amino acid residues of the peptide sequence may not be essential for antibody recognition of the peptide. Thus, the possible \([3H]\)BTX-OAB-binding peptides containing the SP28 sequence begin 13 or 21 amino acid residues on the extracellular side of transmembrane segment IVS6, contain the entire region of the peptide which can be precipitated by anti-SP28.
transmembrane segment IVS6, and 3 amino acids on the intracellular side of segment IVS6.

Less extensive cleavage with trypsin alone yields peptides that contain the recognition sites for anti-SP1 and anti-SP11 on the intracellular, carboxyl-terminal side of transmembrane segment IS6 (Fig. 6A). These peptides must contain sequences extending beyond Ser-504 (Fig. 8). In contrast, cleavage with V8 protease removes the recognition site for these two antibodies (Fig. 6B) by cleavage at one of the numerous Glu residues in the SP1 sequence (Fig. 8). Cleavage of the SP1 peptide from transmembrane segment IS6 by treatment with V8 protease and resistance of the SP28 peptide within the α subunit to cleavage by trypsin as observed here are consistent with previous work on peptide mapping of the α-scorpion toxin receptor site (9, 34).

**DISCUSSION**

High Affinity Binding of a Photoreactive BTX Derivative to Neurotoxin Receptor Site 2 on Purified and Reconstituted Sodium Channels—The synthesis of a photoreactive, radiolabeled derivative of BTX, [3H]BTX-OAB, has made possible the biochemical localization of a component of the BTX receptor site. In the presence of α-scorpion toxin, this ligand binds to the BTX site of Na⁺ channels in rat brain synaptoneurosomes with a KD of 23 nM (22). In the present study, the Na⁺ channel activators, RU51049 and PbtX-1, are used to allosterically enhance [3H]BTX-OAB binding, resulting in a KD of less than 1 nM and a specific binding value of greater than 90%.

We have found that in the presence of the pyrethroid, RU51049, and the brevetoxin, PbtX-1, both [3H]BTX-B and [3H]BTX-OAB bound to purified and reconstituted sodium channels with similar high affinity. No high affinity binding of [3H]BTX-B was present in heat-inactivated sodium channel preparations or to phospholipid alone. These results establish that high affinity binding of BTX derivatives requires the native conformation of the purified sodium channel and that neurotoxin receptor site 2 is present in active form on the solubilized, purified, and reconstituted sodium channel. Covalent labeling of purified and reconstituted Na⁺ channel with [3H]BTX-OAB provided the initial material used for biochemical localization of a peptide sequence that contributes to formation of neurotoxin receptor site 2.

Location of the BTX Receptor Site Near Transmembrane Segment IS6 in Domain I—The photoreactive derivative of BTX, [3H]BTX-OAB, is incorporated specifically into the rat brain Na⁺ channel by photoactivation of the ligand after equilibrium binding is complete. The specific reaction of this derivative with a 240-kDa protein implicates the α subunit in BTX binding. Mapping of the peptide segment that is covalently labeled by [3H]BTX-OAB using sequence-directed antibodies identifies one transmembrane segment as a component of the BTX receptor site. After proteolytic cleavage, the amount of radioactivity bound by each sequence-directed antibody progressively decreased as antibody-binding sequences were separated from the [3H]BTX-OAB incorporation site by proteolysis. However, even after extensive trypsinization, the anti-peptide antibody recognizing an amino acid sequence adjacent to transmembrane segment IS6 was able to immunoprecipitate up to 70% of the labeled peptides. In contrast, antibodies recognizing sequences within or adjacent to domains II, III, and IV do not immunoprecipitate labeled peptides. These results implicate a segment in domain I in formation of the BTX receptor site.

Further localization of the site of covalent labeling by [3H]BTX-OAB was achieved by analysis of immunoprecipitated fragments with antibodies recognizing peptide sequences within or near domain I after more extensive proteolytic cleavage with TPCK-trypsin and V8 protease. After trypsinization, two anti-peptide antibodies recognizing the amino acid sequence on the intracellular side of transmembrane segment IS6 were able to immunoprecipitate at least 50% of the [3H]BTX-OAB-labeled peptides, and two anti-peptide antibodies recognizing the amino acid sequence on the extracellular side of this same transmembrane region were able to immunoprecipitate 30–60% of the labeled peptides. In contrast, after treatment with V8 protease, the ability of the two antibodies recognizing the intracellular amino acid sequences to precipitate the labeled peptide was completely lost, whereas the antibodies recognizing the sequences extracellular to IS6 retained their ability to precipitate the labeled peptide. These results indicate that the BTX receptor site is formed by a portion of domain I and that the site of photolabeling does not include the amino acids to the carboxyl-terminal side of transmembrane region IS6.

A more precise biochemical localization of the site of covalent labeling by [3H]BTX-OAB was achieved by SDS-PAGE analysis of cleavage products from proteolytic digestions of labeled Na⁺ channels with both TPCK-trypsin and V8 protease. The identification of a specifically immunoprecipitated 7-kDa peptide from domain I restricts the labeled peptide fragment to residues Asn-388 to Glu-429 (calculated molecular mass, 6.0 kDa, Fig. 8) or to the region from Leu-380 to Glu-429 (7.3 kDa) if V8 protease cleavage is incomplete. When estimations of molecular mass from three different gel systems are averaged together, the value for the radiolabeled, immunoprecipitated peptide is 7.3 kDa, in excellent agreement with the size of the predicted cleavage product from Leu-380 to Glu-429.

The photoreactive azide of [3H]BTX-OAB is positioned on an aromatic side chain known to be important in conferring toxicity to BTX because the precursor BTX-A, which lacks this aromatic moiety, is nontoxic (3). Therefore, the photoreactive side chain most likely interacts with an integral part of the receptor site for BTX on the Na⁺ channel α subunit. Thus, our results implicate transmembrane segment IS6 in direct interaction with bound BTX in receptor site 2.

Analysis of Covalent Labeling by Antibody Mapping of Labeled Peptides—Photoaffinity labeling of ion channels and other minor membrane proteins is an effective method for identification of their functional components. However, the low level of incorporation of most photolabels and the low abundance and hydrophobicity of the labeled proteins often prevent high resolution analysis of the labeled products by complete purification of the labeled peptides and amino acid sequence determination. Identification of labeled peptides by mapping with sequence-directed anti-peptide antibodies has proven to be a powerful and reliable method of peptide identification.

This approach has been used to identify covalently labeled receptor sites for dihydropyridines (35, 36) and phenylalkylamines (37) on L-type Ca²⁺ channels and for α-scorpion toxin (34), brevetoxins (11), and BTX (this paper) on Na⁺ channels. Subsequent analysis by site-directed mutagenesis has identified individual amino acid residues within or near the site of photoaffinity labeling that are required for binding of dihydropyridines (38) and phenylalkylamines (39) to L-type Ca²⁺ channels and α-scorpion toxin to Na⁺ channels (12). The photoaffactive derivatives of small organic ligands have closely mapped the receptor site residues (35–39); however, the site of α-scorpion toxin binding has only been approximated by photolabeling due to the relatively large size of the ligand (12).

Mutagenesis studies have confirmed the accuracy of the photoaffinity labeling and antibody mapping approaches and provided a higher resolution view of the components of these receptor sites. Analysis of the region of transmembrane seg-
membrane by site-directed mutagenesis should yield additional insight into the molecular requirements for high affinity binding of BTX.

Allosteryic Interactions with the BTX Receptor Siter–A model for allosteric interactions with the BTX receptor site can be proposed by taking into consideration the lipophilic nature of the toxin, its physiological effects, and the location of the photolabeled peptide. BTX can gain access to its binding site from either side of the membrane, since BTX-induced membrane depolarization is observed in squid giant axon when the toxin is applied either internally or externally to the perfusion medium (40). The BTX molecule is approximately 12 Å long, much smaller than the width of the phospholipid bilayer. Therefore, the BTX-binding site may be located within a transmembrane region of the α subunit, in a relatively inaccessible position in the resting Na+ channel conformation. Activation of the Na+ channel by repetitive depolarization dramatically accelerates BTX binding (41). In addition, several chemical activators, including the pyrethrins, RU39568 and RU51049, and the brevetoxin, PbTx-1, induce a conformational change which results in the greater accessibility and higher affinity of BTX to its receptor site. The site of brevetoxin binding has been localized to a transmembrane region at the interface of domains I and IV that includes transmembrane segment 156 (11), to which BTX also binds. Thus, it is possible that multiple sites for hydrophobic toxin binding include determinants within this transmembrane segment and allow discrete changes in three-dimensional structure of the Na+ channel caused by binding of one toxin to affect the binding of others through allostERIC interactions.

BTX binding is also affected by binding of toxins to more distant regions of the Na+ channel. Site-directed mutagenesis studies have identified the amino acid residues lining the mouth of the channel pore between transmembrane segments 5 and 6 in each of the four domains as critical for binding of tetrodotoxin and saxitoxin at neurotoxin receptor site 1 (6–8). The binding of these toxins to site 1 has been shown to allosterically alter the affinity of BTX binding (41). In addition, several chemical activators, including the pyrethrins, RU39568 and RU51049, and the brevetoxin, PbTx-1, induce a conformational change which results in the greater accessibility and higher affinity of BTX to its receptor site. The site of brevetoxin binding has been localized to a transmembrane region at the interface of domains I and IV that includes transmembrane segment 156 (11), to which BTX also binds. Thus, it is possible that multiple sites for hydrophobic toxin binding include determinants within this transmembrane segment and allow discrete changes in three-dimensional structure of the Na+ channel caused by binding of one toxin to affect the binding of others through allostERIC interactions.

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J. Biol. Chem. 1996, 271:11261-11267.
doi: 10.1074/jbc.271.19.11261

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