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Site of covalent attachment of α-scorpion toxin derivatives in domain I of the sodium channel α subunit

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ABSTRACT **Purified and reconstituted sodium channels from rat brain have been photoaffinity labeled with a photoactivable derivative of the α-scorpion toxin V from *Leiurus quinquestriatus* (LqTx). A battery of sequence-specific antibodies has been used to determine which of the peptides produced by chemical and enzymatic cleavage of the photolabeled sodium-channel α subunit contain covalently attached LqTx. Nearly all of the covalently attached LqTx is found within homologous domain I. Two site-directed antisera, which recognize residues 317 to 335 and residues 382 to 400, respectively, specifically immunoprecipitate a 14-kDa peptide produced by CNBr digestion to which LqTx is covalently attached. It is proposed that a portion of the receptor site for α-scorpion toxins is formed by peptide segment(s) between amino acid residues 335 and 378 which is located in an extracellular loop between transmembrane helices S5 and S6 of homologous domain I of the sodium channel α subunit.**

Voltage-sensitive sodium channels are the molecular structures responsible for the increase in sodium permeability during the initial phase of the action potential in most excitable cells (1). The principal α subunit of the sodium channel was first identified by photoaffinity labeling with α-scorpion toxin derivatives (2). Sodium channels have been purified in functional form from eel electroplax (3), mammalian brain (4, 5), and mammalian skeletal muscle (6). The purified sodium channel from rat brain is a complex of three glycoprotein subunits, α (260 kDa), β1 (36 kDa), and β2 (33 kDa), in a 1:1:1 stoichiometry (4, 5). The β2 subunit is covalently attached to the α subunit by disulfide bond(s), whereas the β1 subunit is noncovalently associated. The amino acid sequences of three sodium channel α subunits from rat brain have been inferred from the nucleotide sequences of cDNA clones (7, 8).

Five receptor sites for distinct families of neurotoxins have been shown to be present on voltage-sensitive sodium channels by ligand binding studies (9–13). The α-scorpion toxins constitute a family of structurally and functionally related polypeptide neurotoxins, each containing 60–70 amino acids crosslinked by four disulfide bonds (for review, see refs. 14 and 15). These toxins bind to neurotoxin receptor site 3 on the sodium channel in a voltage-dependent manner (16–18) and modify the activity of the channel by slowing inactivation and altering its voltage-dependence (9, 19, 20). Voltage-dependent binding of α-scorpion toxins to purified sodium channels can be restored by reconstitution into phospholipid vesicles of appropriate lipid composition (21, 22). Photoreactive derivatives of α-scorpion toxins have been successfully used for affinity labeling of the α subunit of sodium channels in synaptosomes (2, 23, 24), neurons in cell culture (2, 25), and reconstituted phospholipid vesicles (26, 27). Since α-scorpion toxins are specific modifiers of the inactivation process of the sodium channel, determination of the location of their receptor site within the primary structure of the Na^+ channel α subunit will help to define the molecular mechanisms of ion-channel gating. In this report, we describe experiments that localize a site of covalent attachment of α-scorpion toxin derivatives by using chemical and proteolytic cleavage methods and sequence-specific antibodies.

EXPERIMENTAL PROCEDURES

Materials. The *Leiurus quinquestriatus* toxin V (LqTx) was purified from the venom of the scorpion *Leiurus quinquestriatus*, radioactively labeled with $Na^{125}I$ by lactoperoxidase, and repurified to give the monoiodo derivative (16). Antisera directed against defined peptide segments (SP_{x-y}, the sodium channel peptide extending from amino acid residues x to y) of the R_{II} sodium channel from rat brain were prepared as described (28, 29) and IgG fractions were isolated by precipitation with 50% saturated $(NH_4)_2SO_4$ at 4°C. Anti-$SP_{317-335}$ antibodies were affinity-purified by adsorption to immobilized sodium channels (30). In all cases, antibody volumes refer to the equivalent volume of original antiserum.

Purified sodium channels from rat brain (31) at concentrations of 300–400 nM in a solution of 25 mM Hepes·Tris (pH 7.4), 100 mM Na_2SO_4, 0.4 mM $MgSO_4$, 157 mM *N*-acetylglucosamine, 4 μM tetrodotoxin, 1.65% Triton X-100, 0.19% phosphatidylcholine and 0.12% phosphatidylethanolamine were reconstituted in phospholipid vesicles by removing the detergent from the solution with Bio-Beads SM-2 (21, 22). The channel reconstituted in this way was immediately used for photolabeling studies. The concentration of purified sodium channels was measured by determination of specific binding of [^{3}H]saxitoxin by using a rapid gel-filtration assay for solubilized channels (31) or filtration through glass fiber filters for reconstituted vesicles (21).

Synthesis of ^{125}I-Labeled-Azidonitrobenzoyl(ANB)-LqTx. All manipulations were carried out in the dark or under a red light. Twenty microliters of 0.2 M triethanolamine hydrochloride (pH 9.0) was added to 200 μl of 100–150 nM ^{125}I-labeled LqTx in a solution of 25 mM sodium phosphate (pH 7.4), 50 mM NaCl, and bovine serum albumin (BSA) at 0.4 mg/ml to give a final pH of 8.3. The coupling reaction was initiated by addition of 1.6 μl of a concentrated solution of *N*-(5-azido-2-nitrobenzoyloxy)succinimide in dioxane containing 0.75 mol of reagent per mol of lysine residues. After 90 min at room temperature, the addition was repeated and the incubation continued for 90 min. The reaction was stopped by adding 1 M Tris·HCl to a final concentration of 40 mM and a pH of 7.4. This solution was stored at 4°C and used for photoaffinity labeling experiments within 48 hr.

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Abbreviations: ANB, 5-azido-2-nitrobenzoyl; BSA, bovine serum albumin; LqTx, toxin V from *Leiurus quinquestriatus*; SP_{x-y}, sodium channel peptide extending from amino acid residues x to y; WGA, wheat germ agglutinin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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Photoaffinity Labeling of Sodium Channel. Purified and reconstituted sodium channels (200 μl of 25 nM) were desalted by rapid gel filtration on 2-ml Sephadex G-50 columns equilibrated with a solution of 10 mM Hepes·Tris (pH 7.4), isotonic sucrose, and BSA at 4 mg/ml (23). The desalted vesicles were diluted with 1 vol of the same buffer containing 20 nM ^{125}I-labeled ANB-LqTx, 2 μM batrachotoxin, and 1 μM tetrodotoxin. The solution was incubated at 37°C for 10 min and irradiated at 0°C for 10 min by using a dual Sylvania blacklite blue fluorescent lamp (λ_{max} = 356 nm; 15 W per bulb) that was placed 5 cm from the sample. LqTx (1 μM) was present in some samples to determine nonspecific photolabeling.

Isolation of Photolabeled Sodium Channels. Photolabeled reconstituted sodium channel (1 ml) was solubilized by incubation at 4°C for 20 min in a solution of 25 mM sodium phosphate (pH 7.5), 100 mM NaCl, 5 mM EDTA, and 1.5% Triton X-100 containing phenylmethylsulfonyl fluoride at 16 μg/ml, 1 μM pepstatin A, and 0.5 mM iodoacetamide as protease inhibitors. Then 250 μl of a 10% (wt/vol) wheat germ agglutinin (WGA)-Sepharose suspension was added and the sample was mixed by rotation at 4°C for 30 min. The pellet was first extensively washed at 4°C with buffer S (10 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM EDTA) containing 0.5% Triton X-100 and BSA at 4 mg/ml, and then twice with the same solution without BSA. The proteins bound to WGA-Sepharose were eluted by washing three times for 5 min each at 0°C with 250 μl of 0.2 M *N*-acetylglucosamine/0.1% Triton X-100 in buffer S. This solution was stored at −80°C and used within 2 weeks for proteolysis experiments.

Immunoprecipitation of Photolabeled Sodium Channel. Antibodies were incubated with photolabeled sodium channel preparations, or fragments prepared from them, overnight at 0°C in buffer S containing 1% Triton X-100 and BSA at 1 mg/ml. One milligram of protein A-Sepharose per μl of serum was added from a 10% (wt/vol) suspension prepared in the same solution. The samples were mixed by rotation at 4°C for 30 min. Supernatants were removed and the pellets were washed twice with 10 vol of buffer S containing BSA at 4 mg/ml and once with 5 vol of the same solution without BSA. The proteins were finally solubilized from the pellet with NaDodSO$_4$/PAGE loading solution by incubation at 100°C for 2 min, and the samples were analyzed by NaDodSO$_4$/PAGE and autoradiography.

Electrophoresis Methods. Three gel electrophoresis systems have been used. System A was a standard NaDodSO$_4$/PAGE method with 9% polyacrylamide gels (32). System B consisted of a 5–20% linear gradient polyacrylamide gel prepared with a 30:0.8 weight ratio of acrylamide to *N,N'*-methylenebisacrylamide (32). System C was a modification of the method of Kyte and Rodriguez (33) with a 3.5% polyacrylamide stacking gel and a 10% polyacrylamide separation gel. A good resolution of peptides ranging from 2 to 45 kDa is obtained with system C.

RESULTS AND DISCUSSION

Isolation of a 48-kDa Glycoprotein Fragment of the Sodium Channel α Subunit Containing Covalently Attached LqTx. Purified rat brain sodium channels were reconstituted in phosphatidylcholine/phosphatidylethanolamine vesicles under conditions (22) that give efficient recovery of high-affinity binding of the α-scorpion toxin LqTx (14). LqTx binding to sodium channels reconstituted in vesicles under these conditions is modulated by membrane potential and by allosteric interactions with neurotoxins binding at sites 1 and 2 as for sodium channels in intact membranes (26, 27). The α subunits of the sodium channels in these reconstituted vesicles were specifically labeled with ^{125}I-labeled ANB-LqTx (Fig. 1*A*, lanes 1 and 2) and isolated by affinity chromatography on WGA-Sepharose. This one-step purification yields a single

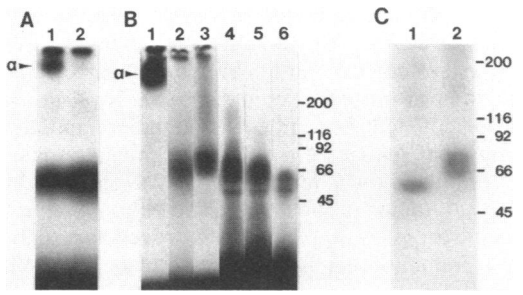

FIG. 1. Isolation of a glycosylated 48-kDa α-subunit fragment containing covalently attached LqTx. (*A*) Twenty microliters of a solution containing purified and reconstituted sodium channels was photolabeled in the absence (lane 1) or presence (lane 2) of 1 μM LqTx and analyzed by NaDodSO$_4$/PAGE by gel system B. Radiolabeled protein bands were visualized by autoradiography. (*B*) Thirty microliters of a solution containing photoaffinity-labeled sodium channel purified by WGA-Sepharose affinity chromatography was incubated at 37°C for 20 min with no protease treatment (lane 1), α-chymotrypsin at 30 μg/ml (lane 2), *S. aureus* protease V8 at 150 μg/ml (lane 3), elastase at 5 μg/ml (lane 4), TPCK-trypsin at 5 μg/ml (lane 5), and thermolysin at 50 μg/ml (lane 6) in a final volume of 100 μl of buffer S containing 0.5% Triton X-100. The reaction was stopped by addition of concentrated NaDodSO$_4$/PAGE sample loading solution and immediate incubation at 100°C for 2 min. The samples were analyzed by NaDodSO$_4$/PAGE in gel system B. (*C*) One hundred-microliter samples containing photolabeled sodium channels digested by protease V8 (*B*, lane 5) was mixed with 7 μl of 10% (wt/vol) NaDodSO$_4$ and incubated at 100°C for 2 min. The concentration of Triton X-100 was increased to 1.5%. After incubation at 0°C for 1 hr, samples were desalted by rapid gel filtration with Sephadex G-50 columns equilibrated in buffer S containing 0.5% Triton X-100 and BSA at 0.1 mg/ml. The samples were finally incubated at 0°C for 1 hr in the presence (lane 1) or absence (lane 2) of 0.5 unit of neuraminidase. Samples were analyzed by NaDodSO$_4$/PAGE in gel system A and the photolabeled protein bands were visualized by autoradiography.

labeled protein of ≈300 kDa (Fig. 1*B*, lane 1) that was identified as the disulfide-linked complex of the α and β2 subunits of the sodium channel by immunoprecipitation with specific anti-α and anti-β2 antibodies (26, 27).

In preliminary experiments, proteases were screened to find experimental conditions under which the α subunit could be cleaved while the LqTx label remained intact. Chymotrypsin, *Staphylococcus aureus* protease V8, elastase, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin, and thermolysin all cleave the α subunit without cleaving LqTx when disulfide bonds are not reduced, probably because LqTx has a compact, highly disulfide-bonded structure (14, 15). Proteolysis of the photolabeled αβ2 complex under nonreducing conditions with these four proteases yields a single major labeled fragment of 55–72 kDa in each case (Fig. 1*B*). Since these proteases cleave at different amino acid residues, these results suggest that most of the covalently attached LqTx is located within a structurally compact, relatively protease-resistant domain of 55–72 kDa within the α subunit. This region is rich in carbohydrate chains since digestion of the 72-kDa photolabeled fragment produced by protease V8 with neuraminidase yields a desialylated polypeptide of 55 kDa (Fig. 1*C*). Since the molecular mass of LqTx is 6.7 kDa, these results imply that the molecular mass of the desialylated glycoprotein produced by treatment with protease V8 and neuraminidase is ≈48 kDa.

Covalent Attachment of α-Scorpion Toxin to a Site in Domain I of the α Subunit. Since photoactivable derivatives of LqTx covalently label only 2–5% of the α subunits to which they are specifically bound (R. Sharkey and W.A.C., unpublished results), isolation of a sufficient quantity of labeled peptide fragments for determination of their amino acid

sequence is difficult. Therefore, we have used a battery of antibodies directed against synthetic peptides whose amino acid sequences correspond to defined segments (Fig. 2A) of the α subunit of rat brain sodium channel R_{II} (7) to identify the site of covalent attachment. Immunoprecipitation with antibodies that recognize α subunit segments ranging from the N terminus to the C terminus (Fig. 2A) showed that only anti-$SP_{317-335}$ (data not shown) and anti-$SP_{382-400}$ (Fig. 3, lane 2), which recognize a proposed extracellular segment in domain I, immunoprecipitated the photolabeled 72-kDa glycopeptide produced by proteolysis with V8 protease. Immunoprecipitation by both antibodies was blocked by prior incubation with the corresponding peptide, demonstrating its specificity (Fig. 3, lane 3). This experiment approximately localizes the labeled region within the first of the four homologous domains of the α subunit (7), since antibodies directed against SP_{31-47}, located in the N-terminal region of the proposed intracellular segment, and $SP_{427-445}$, located immediately on the C-terminal side of the transmembrane segment IS6, did not immunoprecipitate the photolabeled 72-kDa V8 glycopeptide fragment.

To locate the photolabeled region more precisely, we carried out partial proteolytic digestions followed by immunoprecipitation with anti-$SP_{317-335}$, anti-$SP_{382-400}$, or anti-$SP_{427-445}$. As shown in Fig. 4A, all three antibodies are able to recognize 105-kDa and 82-kDa photolabeled fragments produced by protease V8, but anti-$SP_{427-445}$ failed to recognize the final 72-kDa glycopeptide in agreement with the results of Fig. 3. This experiment shows that the final cleavage by V8 protease, which is highly specific for cleavage at glutamate or aspartate residues, eliminates the immunoreactivity of the photolabeled fragment toward anti-$SP_{427-445}$. There are no glutamate or aspartate residues between Glu-429, which is in $SP_{427-445}$, and Glu-387, which must remain in the photolabeled fragment since it is recognized by anti-$SP_{382-400}$ (Fig. 2B). Therefore, this final cleavage by V8 protease must take place at one of the six glutamate residues within peptide $SP_{427-445}$, and the C-terminal region of the 48-kDa glycopeptide segment must be located between Glu-429 and Glu-443. The size of this fragment (48 kDa) indicates that it may contain as many as 420 amino acids, depending upon the contribution of the desialylated core of the carbohydrate chains to its apparent size. Thus, this fragment contains most or all of homologous domain I, which includes residues 125–427.

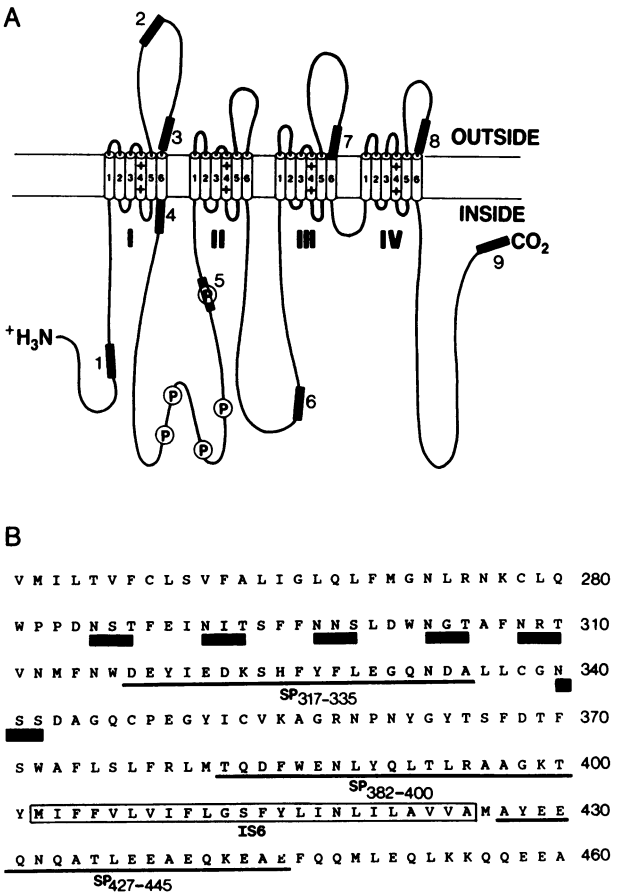

FIG. 2. Structure of the α subunit of the R_{II} sodium channel. (A) Transmembrane folding model of the α subunit of the rat brain sodium channel (6) showing the approximate location of the segments of the amino acid sequence (positions: 1, SP_{31-47}; 2, $SP_{317-335}$; 3, $SP_{382-400}$; 4, $SP_{427-445}$; 5, $SP_{676-690}$; 6, $SP_{1106-1125}$; 7, $SP_{1430-1448}$; 8, $SP_{1729-1748}$; and 9, $SP_{1988-2005}$) that were used to raise anti-peptide antibodies and the sites of *in vivo* phosphorylation by cAMP-dependent protein kinase (34). (B) Detail of the primary structure of the region surrounding the site of covalent attachment of LqTx. Solid boxes underline potential N-linked glycosylation sites; bold lines underline sites of binding of three anti-peptide antibodies; open box encloses the proposed transmembrane segment S6. The single-letter amino acid code is used.

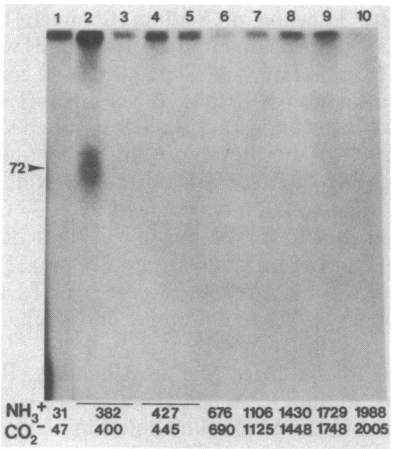

FIG. 3. Immunoprecipitation of proteolytic fragments of photolabeled $\alpha\beta 2$ with sequence-specific antibodies. Samples (150 μl) of photolabeled $\alpha\beta 2$ were cleaved with protease V8 as described in Fig. 1C and incubated overnight at 4°C with 15 μl of various sequence-specific antibodies. The numbers under each lane correspond to the N (NH_3^+) and C (CO_2^-) termini of the α subunit segment against which the antibody was raised. Immunoprecipitated samples were analyzed by $NaDodSO_4$/PAGE in gel system A, and radiolabeled protein bands were visualized by autoradiography. In the samples in lanes 3 and 5, 5 nmol of the corresponding peptide was added with the antibody to block specific immunoprecipitation.

The experiment illustrated in Fig. 4B eliminates an additional segment at the C-terminal end of domain I. The purified photolabeled $\alpha\beta 2$ complex was desialylated by treatment with neuraminidase and cleaved with TPCK-trypsin at 1, 10, or 100 μg/ml. The highest concentration of trypsin produced two photolabeled cleavage products of 46 kDa and 37 kDa, corresponding to desialylated α-subunit glycopeptide masses of 39 kDa and 30 kDa. Anti-$SP_{317-335}$, anti-$SP_{382-400}$, and anti-$SP_{427-445}$ all precipitated the 39-kDa desialoglycopeptide, but neither anti-$SP_{382-400}$ nor anti-$SP_{427-445}$ immunoprecipitated the 30-kDa desialoglycopeptide. Since TPCK-trypsin is highly specific for cleavage at lysine and arginine residues, the last cleavage that removes the immunoreactivity toward anti-$SP_{382-400}$ must take place at Lys-355, Arg-358, or Arg-378, the only lysine or arginine residues between $SP_{317-335}$ and $SP_{382-400}$ (Fig. 2B). The size of this small fragment (30 kDa) indicates that it may contain up to 260 amino acids, depending upon the contribution of the desialylated core of the N-linked carbohydrate chains to its apparent mass.

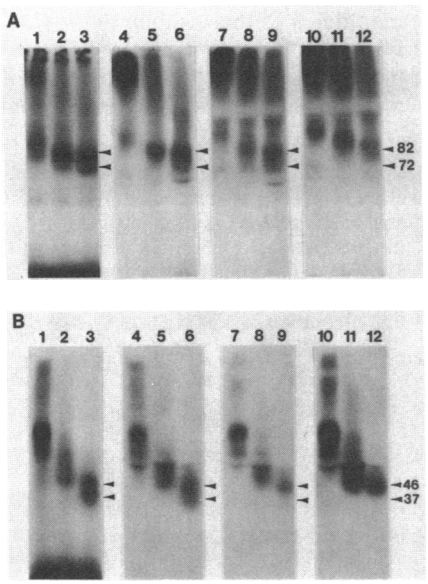

FIG. 4. Immunoprecipitation of partial proteolytic fragments of photolabeled $\alpha\beta 2$ with sequence-specific antibodies. (*A*) Samples (100 μl) of photolabeled $\alpha\beta 2$ (Fig. 1*B*, lane 1) in buffer S containing 0.1% Triton X-100 were incubated at 37°C for 25 min in the presence of *S. aureus* V8 protease at 7.5 μg/ml (lanes 1, 4, 7, and 10), 32 μg/ml (lanes 2, 5, 8, and 11), and 150 μg/ml (lanes 3, 6, 9, and 12). After stopping the digestion and desalting as in Fig. 1*C*, the samples were analyzed by $NaDodSO_4$/PAGE in the gel system B (lanes 1–3) or immunoprecipitated with 15 μl of sequence-specific antibodies and analyzed by $NaDodSO_4$/PAGE. Lanes: 4–6, anti-$SP_{317-335}$; 7–9, anti-$SP_{382-400}$; 10–12, anti-$SP_{427-445}$. Photolabeled protein bands were visualized by autoradiography. (*B*) Samples (125 μl) of photolabeled $\alpha\beta 2$ were incubated at 0°C for 2.5 hr with 0.5 unit of neuraminidase and then for 25 min at 37°C with TPCK-trypsin at 1 μg/ml (lanes 1, 4, 7, and 10), 10 μg/ml (lanes 2, 5, 8, and 11), and 100 μg/ml (lanes 3, 6, 9, and 12). After stopping the digestion and desalting as in Fig. 1*C*, the samples were analyzed by immunoprecipitation and $NaDodSO_4$/PAGE as in *A*.

Therefore, the C-terminal region of this 30-kDa fragment is located between residues 355 and 378, and its N-terminal region may extend as far as residue 95. This region of domain I contains transmembrane segments S1, S2, S3, S4, S5, the hydrophilic segments connecting them, and approximately half of the proposed extracellular segment between transmembrane segments S5 and S6.

Identification of a 14-kDa Peptide Fragment of the α Subunit That Is Covalently Labeled by LqTx. To generate smaller fragments of the α subunit that contain covalently attached LqTx, we digested labeled α subunits with trypsin to produce fragments of ≈70 kDa (Fig. 1*B*, lane 5) and then cleaved these polypeptides at methionine residues with CNBr. This approach permitted us to work with denatured and reduced sodium channel preparations without cleaving LqTx, since there are no methionine residues in LqTx (14). After CNBr digestion, most of the radioactivity is recovered in a peptide whose size is similar to LqTx. Lower-intensity photolabeled protein bands of 50 kDa and 21 kDa are also observed. These larger peptides are specifically immunoprecipitated by anti-$SP_{317-335}$ and anti-$SP_{382-400}$ (Fig. 5*A*), but not by anti-$SP_{427-445}$ (data not shown). When the same peptide fragments were prepared from photolabeled α subunits that had been incubated with neuraminidase, the 50-kDa fragment was reduced to 43 kDa, but the size of the 21-kDa photolabeled fragment was unaffected (Fig. 5*B*). The labeled peptide band of 21 kDa corresponds to a 14-kDa sodium channel fragment without the covalently attached LqTx. Since the apparent size of this fragment is not affected by neuraminidase treatment, it is unlikely to contain complex N-linked carbohydrate chains. If it is unglycosylated, its length would be ≈120 amino acids.

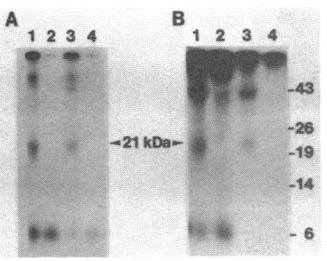

FIG. 5. Immunoprecipitation of CNBr fragments of photolabeled $\alpha\beta 2$ with sequence-specific antibodies. (*A*) Samples (300 μl) of photolabeled $\alpha\beta 2$ in buffer S containing 0.1% Triton X-100 were incubated at 37°C for 25 min in the presence of TPCK-trypsin at 10 μg/ml. The reaction was stopped by addition of $NaDodSO_4$ and 2-mercaptoethanol to final concentrations of 0.7% and 10 mM, respectively; the reaction medium was incubated at 100°C for 2 min; iodoacetamide was added to a final concentration of 15 mM; and the samples were incubated for an additional hour at room temperature and finally desalted by rapid gel filtration on Sephadex G-25 columns equilibrated in 0.2% $NaDodSO_4$. The material obtained in this way was digested at room temperature for 20 hr with 1% CNBr in 70% (vol/vol) formic acid. At the end of the digestion, the solution was evaporated to dryness under vacuum, and the residual solid was resuspended in 300 μl of 0.1 M ammonium acetate, pH 8.5/0.1 M 2-mercaptoethanol and lyophilized. The final solid was dissolved in 160 μl of 75 mM sodium phosphate (pH 7.5). Aliquots (25 μl) were immunoprecipitated with 15 μl of sequence-specific antibodies and analyzed by a $NaDodSO_4$/PAGE in gel system C. Lanes: 1, anti-$SP_{317-335}$; 2, anti-$SP_{317-335}$ previously blocked with peptide; 3, anti-$SP_{382-400}$; 4, anti-$SP_{382-400}$ previously blocked with peptide. Photolabeled protein bands were visualized by autoradiography. (*B*) A similar experiment was carried out with 300 μl of photolabeled $\alpha\beta 2$ that was desialylated by incubation with 1.5 units of neuraminidase at 0°C for 2 hr prior to digestion with trypsin. These samples were then processed as described for *A*.

In contrast to the 50- and 21-kDa CNBr digestion products, none of the available antibodies specifically recognized the small fragment similar in size to free LqTx. This small fragment might represent free LqTx released from covalent attachment to the α subunit by the CNBr treatment or LqTx covalently attached to a fragment of less than 20 amino acids that is not recognized by any of the available antibodies.

Location of the Site of Covalent Attachment of LqTx. Our results sharply restrict the possible locations of the site of covalent attachment of LqTx but do not yet precisely determine the amino acid residues involved. Analysis of cleavage products from limited proteolytic digestions with V8 protease restrict the C-terminal end of the labeled peptide to residue 427 (Fig. 4*A*). Similar experiments with trypsin restrict it further to residue 379 (Fig. 4*B*). These results place the C-terminal limit for the location of the site of covalent attachment of LqTx at Arg-379. The major site of LqTx attachment must be located toward the N-terminal end from this site since most of the LqTx label is immunoprecipitated in a single fragment (Fig. 1) that is recognized by our antibodies (Figs. 3 and 4).

The N-terminal limit for the location of the site of covalent attachment of LqTx is established by analysis of the properties of the CNBr peptide of 14 kDa with covalently attached LqTx. Inspection of the amino acid sequence of the α subunit (Fig. 2*B*) shows that the C-terminal end of this CNBr peptide must be at Met-402 or Met-426, since these are the only two methionine residues between peptides $SP_{382-400}$ and $SP_{427-445}$ where the C-terminal end of this fragment must be located (Fig. 5). Considering the size of this peptide, its N-terminal end could be located at Met-313, Met-270, or Met-252. The minimum mass of the polypeptide portion of this fragment is 10.2 kDa, the mass contributed by amino acid residues 313–402.

Consideration of the contribution of N-linked glycosylation to the size of the peptide fragments we have characterized allows a clear choice among these three possible N

termini. The V8 fragment containing most of domain I is reduced in apparent size from 72 kDa to 55 kDa by treatment with neuraminidase (Fig. 1*C*). Approximately half of the mass of N-linked carbohydrate of the total α subunit is accounted for by sialic acid, as measured by direct chemical analysis or by treatment with glycosidases and $NaDodSO_4$/PAGE analysis (35–37). Thus, as much as 34 kDa of N-linked carbohydrate may be attached to glycosylation sites in domain I. There are seven possible sites of attachment: Asn-212, Asn-285, Asn-291, Asn-297, Asn-303, Asn-308, and Asn-340. Five of these sites lie between Met-270 and Met-313. Since the typical mass of an N-linked carbohydrate chain is 3–4 kDa, it is likely that most, if not all, of these possible glycosylation sites contain N-linked carbohydrate chains. If the 14-kDa fragment to which LqTx is covalently attached included the peptide segment from residues 270 to 313 that contains five sites of glycosylation, it would have a peptide mass of 15 kDa plus 17–34 kDa of N-linked carbohydrate. Since this fragment is only 14 kDa and contains no sialic acid, we conclude that its N-terminal end is likely to be located at Met-313. With this N-terminal end, the size of the 14-kDa fragment can be accounted for in either of two ways. If the C-terminal end is at Met-426, the protein mass of the predicted polypeptide is 13 kDa in close agreement with the experimental observations. If the C-terminal end is at Met-402, the protein mass of 10.2 kDa plus the expected size of an N-linked carbohydrate chain at Asn-340 would give ≈14 kDa. In either case, the N-terminal limit for the location of the site of covalent attachment of LqTx would be at Met-313.

LqTx derivatives prepared by reaction at amino groups consist of a mixture of isomers substituted on Lys-58 or -60 (38), which are located on the periphery of the array of highly conserved amino acid residues that is thought to define the active site for binding to the sodium channel (14, 15, 34). The corresponding lysine residues of some α-scorpion toxins are essential for toxicity (14). We expect, therefore, that the site of covalent attachment of LqTx to the sodium channel is close to the active site for toxin binding. Neither anti-$SP_{317-335}$ nor anti-$SP_{382-400}$ block LqTx binding (unpublished results), indicating that neither of these peptide segments is an essential part of the toxin receptor site on the α subunit. Therefore, it seems most likely that the site of covalent attachment of LqTx derivatives and at least a portion of the site of toxin binding are located between amino acid residues 335 and 378. Since high-affinity LqTx binding activity requires the native conformation of the sodium channel (21, 22), it is likely that multiple polypeptide segments from different regions of the α subunit contribute to formation of the active receptor site. Further analysis of the interactions between LqTx and sodium channel segments in the extracellular region of domain I with synthetic peptides and anti-peptide antibodies may provide a more complete understanding of the multiple polypeptide segments of the sodium channel that form the receptor site for LqTx.

Transmembrane Topology of the α Subunit. The α-scorpion toxins modify sodium channel properties from the extracellular surface of the channel (9, 19, 20). Therefore, our results provide direct evidence that at least a portion of the segment of the α subunit located between amino acid residues 335 and 378 is extracellular as illustrated in Fig. 2. The long hydrophilic segment between domains I and II contains at least four sites of *in vivo* phosphorylation by cAMP-dependent protein kinase (39), defining this segment as intracellular. Considered together, these two lines of evidence confirm the presence of at least one transmembrane segment, IS6, between the intracellular phosphorylation sites and the site of covalent attachment of LqTx. These results are also compatible with the location of three transmembrane segments between these two sites as suggested in two alternative models for the transmembrane folding of the α subunit (40, 41). Additional direct experimental information is required to define the transmembrane folding pattern within the homologous domains of the sodium channel α subunit more completely.

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