Differential Labeling of the α and β1 Subunits of the Sodium Channel by Photoreactive Derivatives of Scorpion Toxin†

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ABSTRACT: The separation of two photoreactive derivatives of the α-scorpion toxin from *Leiurus quinquestriatus* is described. When the two photoreactive derivatives were photolyzed separately in the presence of brain membranes containing voltage-sensitive sodium channels, one labeled the α subunit preferentially while the other labeled β1 more intensely than α. Batrachotoxin enhanced the efficiency of covalent labeling by the photoreactive derivatives of scorpion toxin. In all the labeling experiments, the specific incorporation of radioactive scorpion toxin was eliminated by an excess of nonradioactive scorpion toxin. The α polypeptide labeled in synaptosomes by photoreactive scorpion toxin was demonstrated by immunological techniques to be the same large polypeptide identified in sodium channels purified by their saxitoxin binding activity. The α and β1 subunits were detected by rapid photoaffinity labeling of a freshly prepared brain homogenate in the presence of a mixture of nine protease inhibitors, indicating that they are components of the sodium channel in intact brain tissue. The association of the covalently labeled polypeptides with the membrane was investigated by treatment of labeled synaptosomes with various agents known to remove proteins only indirectly attached to the lipid bilayer via a membrane-bound protein. In all cases, both the α and the β1 polypeptides remained in the membrane fraction following extraction. This confirms earlier proposals that the α polypeptide has a portion of its mass embedded within the lipid bilayer and suggests that the β1 polypeptide does as well.

The voltage-sensitive sodium channel is the membrane-bound glycoprotein complex responsible for the transient influx of sodium ions during an action potential. Sodium channels from mammalian brain (Beneski & Catterall, 1980; Hartshorne & Catterall, 1981, 1984; Hartshorne et al., 1982) or muscle (Barchi, 1983) consist of a complex of a large polypeptide and two smaller polypeptides whereas purified preparations from electric eel electroplax contain only a single large glycoprotein subunit with a molecular weight of 260 000 (Agnew et al., 1980; Nakayama et al., 1982; Miller et al., 1983; Norman et al., 1983). Biochemical characterization of the sodium channel from the rat brain revealed that three polypeptides, designated α, β1, and β2 with molecular weights of 270 000, 39 000, and 37 000, respectively, form the sodium channel complex (Hartshorne et al., 1982; Hartshorne & Catterall, 1984). The β2 polypeptide is covalently attached to the α polypeptide by one or more disulfide linkages, and the β1 polypeptide is noncovalently associated with the complex of α and β2 (Hartshorne et al., 1982). This purified complex is sufficient to mediate ^{22}Na influx when incorporated into phospholipid vesicles (Talvenheimo et al., 1982; Weigele & Barchi, 1982; Tanaka et al., 1983; Tamkun et al., 1984).

A number of plants and animals have evolved toxins which bind specifically to the sodium channel complex and modify its properties (Narahashi, 1974; Catterall, 1980). Sodium channels contain at least four distinct receptor sites for neurotoxins (Ritchie & Rogart, 1977; Catterall, 1980, 1984; Jover et al., 1980). Site 1 binds the inhibitors tetrodotoxin and saxitoxin; site 2 binds the lipid-soluble toxins batrachotoxin, veratridine, aconitine, and grayanotoxin; site 3 binds the polypeptide α-scorpion toxins and sea anemone toxin II; site 4 binds the polypeptide β-scorpion toxins.

Covalent labeling has proven to be a powerful tool in the identification of the polypeptide components of receptors and enzymes and in the localization of specific functional components within their protein structure. The neurotoxins specific for the sodium channel provide potentially useful reagents for the preparation of probes for covalent labeling. The first specific covalent labeling of polypeptide components of the sodium channel utilized an azidonitrobenzoyl derivative of ^{125}I-labeled α-scorpion toxin from *Leiurus quinquestriatus*. Upon absorption of light, the azide was coverted to a highly reactive nitrene, and the prebound photoreactive scorpion toxin was found by gel electrophoresis to be attached covalently to two polypeptides which have the same apparent molecular weights as α and β1 (Beneski & Catterall, 1980; Hartshorne et al., 1982). Labeling of these polypeptides was blocked by inclusion of an excess of nonradioactive α-scorpion toxin, demonstrating the specificity of this reaction. More recently, similar labeling was obtained by using an aryl azide derivative of a β-scorpion toxin (Darbon et al., 1983). A polypeptide with a molecular weight of 260 000 can also be labeled by covalent cross-linking of specifically bound β-scorpion toxin to sodium channels in synaptosomes (Barhanin et al., 1983) or of a derivative of tetrodotoxin to the purified tetrodotoxin binding component from electric eel electroplax (Lombet et al., 1983). These results provide further evidence for identification of the α and β1 polypeptides as components of the voltage-sensitive sodium channel in isolated membranes and purified preparations. In this report, we describe the differential photoaffinity labeling of the α and β1 polypeptides by separated derivatives of *Leiurus* scorpion toxin, examine the relationship among the sodium channel polypeptides in purified preparations, isolated membranes, and freshly homogenized brain tissue, and investigate the nature of the association of the α and β1 subunits of the sodium channel with the synaptosomal plasma membrane.

Experimental Procedures

Materials. Chemicals were obtained from the following sources: scorpion venom (*Leiurus quinquestriatus*) and bovine

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serum albumin (BSA)[1] from Sigma Chemical Co.; Pansorbin (*Staphylococcus aureus* cells) and tetrodotoxin from Calbiochem Behring Corp.; Linbro multiwell plates (16-mm diameter, 2 cm^2) from Flow Laboratories Inc. Amberlite CG-50 from Mallinckrodt was purified by the method of Hirs (1955) before use. Scorpion toxin was purified (Catterall, 1976) and radioactively labeled by lactoperoxidase-catalyzed iodination as described (Catterall, 1977). Batrachotoxin was generously provided by Dr. John Daly (Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases). A stock solution of batrachotoxin was prepared in ethanol at 250 times the desired final concentration and then diluted into standard binding medium immediately prior to use. Succinimdyl 5-azido-2-nitrobenzoate (ANB-NOS) was synthesized from 5-amino-2-nitrobenzoic acid (K and K Chemicals) as described by Lewis et al. (1977) and was stored protected from light in a desiccator under vacuum.

Protein Determinations. Protein concentrations were estimated by the method of Lowry et al. (1951) as modified by Bailey (1967), after an initial precipitation with trichloroacetic acid. A solution of BSA, whose protein concentration was evaluated by quantitative amino acid analysis (Spackman et al., 1958), was used as the standard.

Preparation of the 5-Azido-2-nitrobenzoyl Derivative of Mono-^{125}I-labeled Scorpion Toxin (ANB-$^{125}I_1$-Lqtx). Mono-^{125}I-labeled scorpion toxin (^{125}I-Lqtx) in 0.1 mg/mL BSA and 200 mM ammonium acetate, pH 8.5, was lyophilized to remove the ammonium acetate buffer utilized in the column separation of mono- ^{125}I- and di-^{125}I-labeled toxin and redissolved in 50 mM NaCl + 10 mM sodium phosphate buffer (pH 7.5) to a concentration of 40 nM. All subsequent manipulations were carried out under red light in low actinic red glassware. The $^{125}I_1$-Lqtx sample (3.6 volumes) was mixed with 0.2 M Na_2CO_3, pH 9.0 (4.5 volumes), and then treated with 0.45 volumes of ANB-NOS in dioxane. Unless stated otherwise, the concentration of reagents was selected to give a 1.5:1 molar ratio of ANB-NOS to lysine amino groups of the BSA (lysine amino groups of the scorpion toxin contribute less than 1% of those from the BSA). This procedure was chosen because it is difficult to handle low concentrations of $^{125}I_1$-Lqtx without a carrier protein. The reaction mixture was incubated at room temperature for 1.5 h, and then another 0.45 volume of ANB–NOS in dioxane (molar ratio of 1.5:1) was added and the incubation period was repeated.

Separation of ANB-$^{125}I_1$-Lqtx Derivatives. All manipulations were carried out under red light. Following incubation of the reaction mixture for a total of 3 h, it was diluted with 4.8 volumes of 10 mM ammonium acetate and 0.1 mg/mL BSA, pH 7.5, to reduce the ionic strength for ion-exchange chromatography. The reaction mixture was adsorbed to a column (Kontes, plastic disposable column, 20 × 0.6 cm) of Amberlite CG-50 (NH_4 form) equilibrated with 10 mM ammonium acetate and 0.1 mg/mL BSA, pH 7.5. Adsorbed ANB-$^{125}I_1$-Lqtx was eluted with a 60-mL linear gradient from 30 mM ammonium acetate and 0.1 mg/mL BSA, pH 7.5, to 250 mM ammonium acetate and 0.1 mg/mL BSA, pH 7.5. The column was run at 12 mL/h, and 0.5-mL fractions were collected. Aliquots of fractions were counted in a γ scintillation spectrometer. Selected fractions were pooled, concentrated by lyophilization, and used within 24 h for the covalent labeling experiments.

Covalent Labeling of Synaptosomes. Synaptosomes were prepared from rat brain by a modification of the method of Gray & Whittaker (1962) as described (Tamkun & Catterall, 1981). Unless stated otherwise, synaptosomes (0.57 mg/mL) were incubated in multiwell plates (1 mL/well) with 0.5–1.5 nM ANB-$^{125}I_1$-Lqtx under red light for 15 min at 36 °C in standard binding medium containing 0.1 μM tetrodotoxin and 1 μM batrachotoxin. Standard binding medium consists of 130 mM choline chloride, 50 mM Hepes (adjusted to pH 7.4 with Tris base), 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and BSA at 1 mg/mL. Nonspecific labeling was determined in the presence of 200 nM unlabeled toxin. Following the equilibration period, the reaction mixture was irradiated for 15 min at 36 °C with dual Sylvania Blacklite blue fluorescent bulbs (λ_{max} 356 nm; 15 W per bulb) at a distance of 3 cm. After irradiation, samples were then centrifuged at 30000*g* for 45 min, supernates discarded, and finally the pellets suspended in standard binding medium (without BSA) containing 0.1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 1 mM 1,10-phenanthroline, and 1 mM iodoacetamide.

Preparation of Rat Brain Homogenate. Immediately prior to being labeled by ANB-$^{125}I_1$-Lqtx, the brain of a Sprague-Dawley rat was removed and minced in ice-cold 0.32 M sucrose and 5 mM KH_2PO_4, pH 7.2 (10 mL/g wet weight), containing 0.1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 1 mM iodoacetamide, 1 μg/mL antipain, 1 μg/mL phosphoramidon, 5 mM EDTA, 20 μg/mL soybean trypsin inhibitor, 100 μg/mL bacitracin, and 10 μg/mL leupeptin. The brain fragments were homogenized with 10 strokes of a motor-driven Teflon–glass homogenizer as was performed in the preparation of the synaptosomes. The homogenate was diluted 1:1 (v/v) with ice-cold standard binding medium excluding BSA. Specific binding of $^{125}I_1$-Lqtx represented 75% of the total binding to the brain homogenate as compared to 82% in the synaptosomes.

$NaDodSO_4$ Gel Electrophoresis. Polyacrylamide gel electrophoresis in $NaDodSO_4$ was performed essentially as described by Maizel et al. (1971). Protein samples were dissolved in 3% $NaDodSO_4$, 10% (v/v) glycerol, 12 mM EDTA, 30 mM Tris-HCl, pH 7.4, and 1% 2-mercaptoethanol (unless stated otherwise) and incubated at 100 °C for 2 min. For experiments in which reduced and unreduced samples were compared, the following procedure was used. Protein samples were dissolved in $NaDodSO_4$ sample buffer as described above except 2-mercaptoethanol was omitted. Tris base was added to all the samples to raise the Tris concentration an additional 45 mM. The samples to be reduced were made 15 mM in 2-mercaptoethanol and incubated at 100 °C for 4 min. All samples were made 45 mM in iodoacetamide and incubated at 100 °C for 1 min. In all the experiments described in this report, samples were applied to slab gels with a stacking gel of 3% acrylamide and a linear gradient of acrylamide from 4.5% to 10% in the separation gel. Unless stated otherwise, 200 μg of synaptosomal protein was applied to each gel lane. Following the electrophoresis step, the separating gel was stained with Coomassie brilliant blue R-250 and destained with acetic acid. Autoradiograms were made by drying the gels onto a piece of blotting paper and then exposing Kodak X-Omat film at −75 °C using a Du Pont Lighting Plus intensifier screen and a single preflash.

Immunoprecipitation of Labeled Sodium Channel Polypeptides. Antiserum was prepared against purified, native

[1] Abbreviations: BSA, bovine serum albumin; ANB-NOS, succinimidyl 5-azido-2-nitrobenzoate; $^{125}I_1$-Lqtx, mono-^{125}I-labeled scorpion toxin; ANB-$^{125}I_1$-Lqtx, 5-azido-2-nitrobenzoyl derivative of mono-^{125}I-labeled scorpion toxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

sodium channel (Hartshorne & Catterall, 1984) as described by Costa & Catterall (1984). Antibodies were isolated from the serum by precipitation with an equal volume of saturated ammonium sulfate and 1 mM EDTA, pH 7.3. Covalently labeled synaptosomes were sedimented and solubilized by using 3% $NaDodSO_4$ (final concentration of 4 mg of $NaDodSO_4$ per mg protein). Labeled synaptosomes dissolved in detergent were diluted with the immunoprecipitation buffer (75 mM NaCl, 25 mM EDTA, 25 mM Tris, 1% Triton X-100, 1% sodium deoxycholate, 50 mM NaH_2PO_4, and 0.01% sodium azide, adjusted to pH 7.4 with NaOH) plus 5 mg/mL BSA, the antibodies were added, and the mixtures were incubated 24 h at 4 °C. *Staphylococcus aureus* cells (Pansorbin), which had been boiled in 3% $NaDodSO_4$ + 10% 2-mercaptoethanol and washed with immunoprecipitation buffer, were added, and the incubation was continued for 1 h. The suspensions were centrifuged in a microfuge for 5 min and the pellets resuspended in $NaDodSO_4$ gel sample buffer containing 5% (v/v) 2-mercaptoethanol and heated to 100 °C for 6 min. The cells were then removed by centrifugation in a microfuge and the supernates subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis.

Extraction of Peripheral Proteins from Covalently Labeled Synaptosomes. Aliquots of synaptosomes labeled by ANB-$^{125}I_1$-Lqtx were incubated in standard binding medium under the following conditions: 45 min at 0 °C for no additions, 30 min at 0 °C with 5 mM lithium diiodosalicylate, 30 min at 23 °C with 90 mM citraconic anhydride, 45 min at 0 °C with 8 M urea, 45 min at 0 °C with 2 M sodium thiocyanate, 60 min at 0 °C with 2.5 M NaCl, or 60 min at 0 °C with 2 mM EDTA. Following the incubation period, the samples were diluted 1:1 (v/v) with water and centrifuged for 20 min at maximum speed in a Beckman airfuge. The pellets were dissolved in $NaDodSO_4$ gel sample buffer, made 2.5% (v/v) with 2-mercaptoethanol, and heated to 100 °C for 3 min. Samples were subjected to electrophoresis, the gels were dried and autoradiographed, and the autoradiograms were scanned by densitometry.

Results

Separation of Photoreactive α-Scorpion Toxin Derivatives. In our first report on the covalent labeling of voltage-sensitive sodium channels with an azidonitrobenzoyl derivative of scorpion toxin, membranes were photolyzed in the presence of the unfractionated mixture of products from the reaction between $^{125}I_1$-Lqtx and ANB-NOS (Beneski & Catterall, 1980). Figure 1 shows, however, that three major peaks of radioactivity are resolved when the reaction mixture is subjected to ion-exchange chromatography. Peak III was identified as nonderivatized $^{125}I_1$-Lqtx based upon the evidence that peak III elutes in the same position as $^{125}I_1$-Lqtx, its binding to synaptosomes is blocked by the presence of nonradioactive Lqtx, and the irradiation of synaptosomes to which it is bound yields no covalently labeled polypeptides. Fractions composing peak I and peak II contain photoreactive derivatives of Lqtx which covalently label the subunits of the sodium channel as described below. In order to achieve an optimal yield of the derivatives in peaks I and II, reaction conditions were systematically varied. Because $^{122}I_1$-Lqtx rapidly adsorbs to labware, all solutions contained an excess of BSA, and reagent concentrations were calculated relative to the total concentration of free amino groups contributed by BSA and Lqtx. Table I summarizes the distribution of radioactivity after $^{125}I_1$-Lqtx has been reacted with ANB-NOS at different molar stoichiometries and passed over a cation-exchange column. When the ratio of reagent to amino groups is increased,

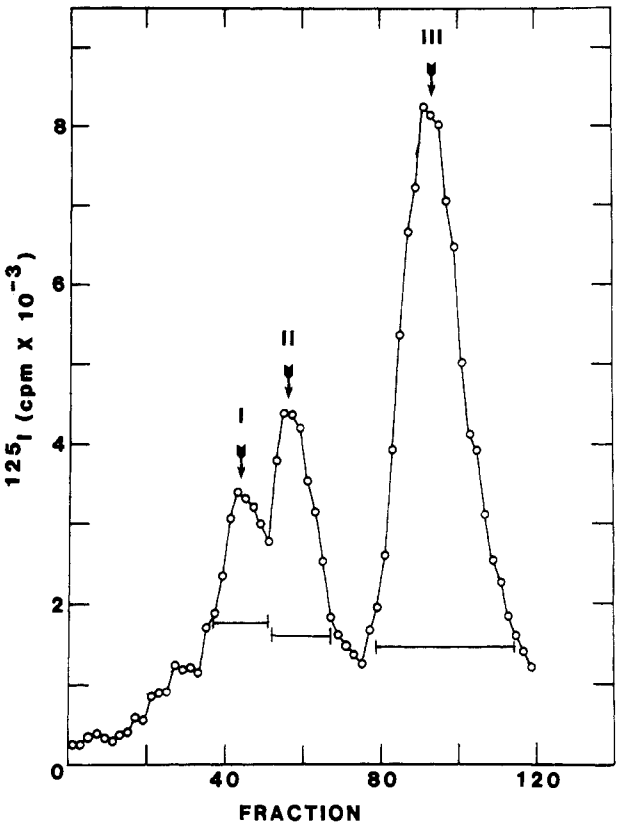

FIGURE 1: Resolution of photoreactive α-scorpion toxin derivatives by ion-exchange chromatography. ANB-NOS and $^{125}I_1$-Lqtx were incubated, and the reaction mixture was subjected to chromatography on Amberlite CG-50 as described under Experimental Procedures. Aliquots (3 μL) of the indicated fraction were analyzed for ^{125}I by γ counting. The bars under the three designated peaks mark the fractions pooled.

Table I: Distribution of cpm following Reaction of $^{125}I_1$-Lqtx with ANB-NOS and Chromatography on Amberlite CG-50[a]

reagent ratio	% of total cpm					
	flow through	pre-peak	peak I	peak II	peak III	resin
none	17	4	0	0	62	17
0.4:1	23	4	6	7	39	21
0.7:1	26	5	7	9	35	18
1:1	24	7	9	11	30	19
2:1	34	9	10	11	21	15
5:1	69	10	5	3	5	7

[a] ANB-NOS and $^{125}I_1$-Lqtx were incubated, and the reaction mixture was subjected to chromatography on Amberlite CG-50 as described under Experimental Procedures. ^{125}I cpm were determined in each fraction. The reagent ratio is expressed as moles of ANB-NOS to moles of lysine.

$^{125}I_1$-Lqtx is converted to less positively charged derivatives substituted with ANB as represented by the decrease in radioactivity in peak III and the increase in radioactivity in peaks I and II and the flow through. The fraction of radioactivity in peaks I and II increases in the range of reagent to amino group ratios from 0.4:1 to 2:1. Both peaks I and II increase as the reagent concentration increases, and their relative proportion is nearly identical at each reagent to amino group ratio examined. At the highest reagent concentration tested, 5:1, the majority of the radioactivity appears in the flow through with a corresponding decrease in peaks I, II, and III. Although not shown, further experimentation demonstrated that a slightly better yield of peaks I and II is obtained at a ratio of 1.5:1 than 1:1 or 2:1. Consequently, a molar ratio of 1.5:1 was used in subsequent experiments.

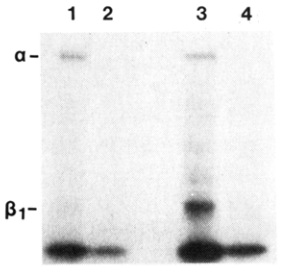

FIGURE 2: Analysis of $NaDodSO_4$ gel electrophoresis of $^{125}I_1$-Lqtx-labeled polypeptides from synaptosomes. Synaptosomes were prepared and covalently labeled according to Experimental Procedures by using a 1 nM sample of peak I (Figure 1) in the absence (lane 1) or presence (lane 2) of 200 nM unlabeled Lqtx or by using a 1 nM sample of peak II (Figure 1) in the absence (lane 3) or presence (lane 4) of 200 nM unlabeled Lqtx. An autoradiogram of the gel is shown. The film was exposed for 4 days.

The $^{125}I_1$-Lqtx derivatives in peaks I and II were pooled separately and used to covalently label synaptosomes. The autoradiogram from a $NaDodSO_4$–polyacrylamide gel of the labeled synaptosomal preparations (Figure 2) revealed that the protein bands labeled by using either derivative were predominantly α and $\beta 1$. However, the ratio of labeled α to labeled $\beta 1$ differed for the two derivatives. With peak I, the α polypeptide is labeled much more extensively than the $\beta 1$ polypeptide, whereas with peak II the $\beta 1$ polypeptide is labeled more intensely than α. Therefore, the two distinct arylazido scorpion toxin derivatives produced during the coupling reaction between $^{125}I_1$-Lqtx and ANB-NOS react selectively with the subunits of the sodium channel. Because the two peaks could not be separated completely, it is possible that the weak labeling of $\beta 1$ by peak I may have resulted from a minor contamination by peak II. Peak I may label α only. The results suggest that Lqtx binds near the interface of the α and $\beta 1$ subunits and can be covalently attached to either polypeptide depending upon the site of derivatization by ANB-NOS.

Enhancement of $^{125}I_1$-Lqtx Covalent Labeling. The alkaloid toxins batrchotoxin, veratridine, and aconitine have been found to enhance the specific binding of $^{125}I_1$-Lqtx to synaptosomes (Tamkun & Catterall, 1981). Batrachotoxin is the most potent of the three toxins. Since the binding of scorpion toxin is membrane potential dependent and batrachotoxin causes depolarization due to persistent activation of sodium channels, synaptosomes were incubated in sodium-free medium (standard binding medium) containing tetrodotoxin. The autoradiogram from a $NaDodSO_4$–polyacrylamide gel of synaptosomal preparations which were covalently labeled by ANB-$^{125}I_1$-Lqtx in the presence or absence of a saturating concentration of batrachotoxin is shown in Figure 3. The intensity of labeling of α and $\beta 1$ in the presence of batrachotoxin (lanes 3 and 4) is clearly greater than in its absence (lanes 1 and 2). Labeling with peak I in the presence of batrachotoxin gave similar results (data not shown). Thus, the enhancement of the yield of labeled polypeptides by the alkaloid toxin parallels that observed in binding experiments with $^{125}I_1$-Lqtx where a larger proportion of scorpion toxin binds in the presence of batrachotoxin.

Resolution of the $\beta 1$ and $\beta 2$ Polypeptides. Previous work from this laboratory established the presence of two nonidentical β polypeptides in sodium channels purified with respect to saxitoxin binding activity (Hartshorne et al., 1982). Furthermore, the amount of covalently bound $^{125}I_1$-Lqtx associated with the β-protein band on a $NaDodSO_4$–polyacrylamide gel is the same in unreduced samples in which only the $\beta 1$ subunit migrates in the β-protein band and in samples

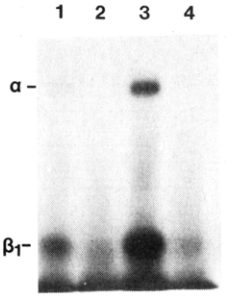

FIGURE 3: Enhancement by batrachotoxin of the covalent labeling of synaptosomes. Peak II (Figure 1) at 1 nM was used to label synaptosomes in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1 μM batrachotoxin. Nonspecific labeling was determined by the addition of 200 nM unlabeled Lqtx (lanes 2 and 4). An autoradiogram of the gel is shown. The film was exposed for 5 days.

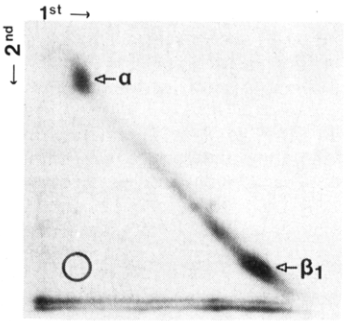

FIGURE 4: Two-dimensional gel of synaptosomes labeled by ANB-$^{125}I_1$-Lqtx. In this experiment and all that follow (Figures 5–7 and Table II), photoaffinity labeling was performed with peaks I and II combined (Figure 1). The polypeptides of covalently labeled synaptosomes were separated in the first dimension on a $NaDodSO_4$ slab gel as described under Experimental Procedures except that 2-mercaptoethanol was omitted. The entire lane, excluding the dye front, was sliced out and was washed with 15 mL of 30 mM Tris-HCl, 0.1% $NaDodSO_4$, and 1 mM EDTA, pH 6.7, for 15 min (Nathanson & Hall, 1979). In the second dimension, the excised lane was overlayed with a modified $NaDodSO_4$ sample buffer containing 0.1% $NaDodSO_4$ and 10% (v/v) 2-mercaptoethanol and resubjected to $NaDodSO_4$ gel electrophoresis on top of a slab gel with a stacking and separation gel composition identical with the first dimension. An autoradiogram of the second gel is shown. The circled area of the autoradiogram indicates where $\beta 2$ has migrated. The film was exposed for 3 weeks.

reduced with 2-mercaptoethanol in which both $\beta 1$ and $\beta 2$ migrate in the β-protein band. On the basis of this observation, it was proposed that the low molecular weight polypeptide labeled by ANB-$^{125}I_1$-Lqtx is $\beta 1$ and that there is no ^{125}I associated with the $\beta 2$ polypeptide. Since it would have been difficult to detect weak labeling of $\beta 2$ by comparison of ^{125}I associated with the β-protein band before and after disociation of $\beta 2$ from α, a two-dimensional gel electrophoresis experiment was performed to examine $\beta 2$ directly. The $\beta 1$ polypeptide was first separated from α and $\beta 2$ by subjecting ANB-$^{125}I_1$-Lqtx-labeled synaptosomes to $NaDodSO_4$–polyacrylamide gel electrophoresis after denaturation in the absence of a reducing agent. The entire lane was sliced out of the slab gel, placed horizontally on top of a second identical $NaDodSO_4$–polyacrylamide slab gel, overlaid with $NaDodSO_4$ sample buffer containing 2-mercaptoethanol, and subjected to electrophoresis. Upon reduction, $\beta 2$ is separated from α, thus permitting good resolution of the $\beta 1$, $\beta 2$, and α polypeptides.[2] The second gel when stained for protein with Coomassie brilliant blue revealed that the synaptosomal polypeptides fell on a straight diagonal as expected (Wang & Richards, 1974). The autoradiogram of the same gel (Figure 4) shows that α

[2] R. P. Hartshorne and W. A. Catterall, unpublished experiments.

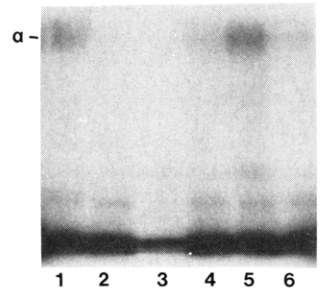

FIGURE 5: Immunoprecipitation of $^{125}I_1$-Lqtx-labeled polypeptides by antibodies against native sodium channel. Synaptosomes labeled by ANB-$^{125}I_1$-Lqtx were dissolved in $NaDodSO_4$ sample buffer (final concentration 4 mg of $NaDodSO_4$ per mg of protein) and heated to 100 °C for 3 min. The solution was diluted with the immunoprecipitation buffer to lower the $NaDodSO_4$ concentration from 3% to 0.19%, which is below its critical micellar concentration (Helenius & Simon, 1975), to prevent inactivation of the antibodies by denaturation. Incubation of the dissolved, labeled synaptosomes with antiserum and isolation and analysis of the immunoprecipitates were performed as described under Experimental Procedures. An autoradiogram of the gel is shown: lane 1, immunoprecipitation of labeled synaptosomes with antiserum; lane 2, immunoprecipitation of labeled synaptosomes with preimmune serum; lane 3, immunoprecipitation with antiserum of synaptosomes labeled in presence of 200 nM unlabeled Lqtx; lane 4, immunoprecipitation of labeled synaptosomes with antiserum in the presence of 1 pmol of purified saxitoxin receptor; lane 5, identical with lane 1; lane 6, same as lane 4 except with 10 pmol of purified saxitoxin receptor. The letter α marks the position to which the α polypeptide from ANB-$^{125}I_1$-Lqtx-labeled synaptosomes migrated. The film was exposed for 2 months.

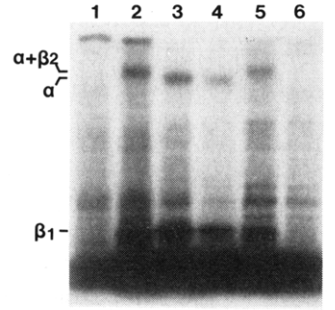

FIGURE 6: Comparison of radioactive polypeptides from synaptosomes and brain homogenate labeled by ANB-$^{125}I_1$-Lqtx. Synaptosomes and brain homogenate were prepared according to Experimental Procedures. On the basis of experimental results not shown, the following modifications to the covalent labeling protocol described under Experimental Procedures were made. The incubation period at 36 °C during which ANB-$^{125}I_1$-Lqtx equilibrates with the binding site in the dark was shortened to 5 min. The photolysis period was reduced to 5 min at 36 °C, and the centrifugation step following irradiation was decreased to 20 min. Brain homogenate and synaptosomes were covalently labeled simultaneously in neighboring wells of a multiwell plate. Labeled samples were dissolved in $NaDodSO_4$ and were reduced and alkylated or only alkylated as detailed under Experimental Procedures. An autoradiogram of the gel is shown: lane 1, synaptosomes labeled in presence of 200 nM unlabeled Lqtx; lane 2, labeled synaptosomes unreduced; lane 3, labeled synaptosomes reduced with 2-mercaptoethanol; lane 4, labeled brain homogenate reduced with 2-mercaptoethanol; lane 5, labeled brain homogenate unreduced; lane 6, brain homogenate labeled in the presence of 200 nM unlabeled Lqtx. Lanes 1–3 (synaptosomes) received 140 μg per lane, and lanes 4–6 (brain homogenate) received 200 μg per lane in order to deliver equal quantities of radioactivity from the two preparations. The film was exposed for 3 weeks.

and β2 were well resolved from β1 in the first dimension and that there is no autoradiographic spot in the region where β2 has migrated in the second dimension. Evidently, there is negligible incorporation of $^{125}I_1$-Lqtx into the β2 polypeptide.

Immunoprecipitation of Detergent-Dispersed, Labeled Synaptosomes. Results from our laboratory have suggested that the α and β polypeptides contain both the saxitoxin/tetrodotoxin binding site and the α-scorpion toxin binding site of the mammalian voltage-sensitive sodium channel (Hartshorne & Catterall, 1981; Tamkun et al., 1984). The proposal that the polypeptides identified as protein components of the sodium channel by purification and by photoaffinity labeling are identical is based upon their comigration on $NaDodSO_4$–polyacrylamide gels. The possibility, however, that different polypeptides coincidentally showed identical electrophoretic mobility could not be excluded. To demonstrate that the polypeptides characterized by either technique are in fact the same, synaptosomes labeled with ANB-$^{125}I_1$-Lqtx were solubilized in $NaDodSO_4$ and reacted with antiserum raised against purified, native sodium channels, and the immune complexes were isolated by immunoprecipitation with a protein A adsorbent. Figure 5 shows an autoradiogram of a $NaDodSO_4$–polyacrylamide gel in which the labeled and immunoprecipitated polypeptides were separated. The labeled α polypeptide was observed when labeled proteins were immunoprecipitated with specific antiserum (lanes 1 and 5). Three control experiments demonstrated the specificity of the immunoprecipitation reaction. At the position where α migrates, no band was observed with preimmune serum (lane 2) or with antiserum reacted with synaptosomes labeled in the presence of an excess of nonradioactive Lqtx (lane 3). Finally, the immunoprecipitation of α was nearly completely blocked by the addition of an excess of highly purified, native saxitoxin receptor (lanes 4 and 6). Therefore, antiserum against the purified, native saxitoxin receptor reacts specifically with the ANB-$^{125}I_1$-Lqtx-labeled α polypeptide. The labeled β1 subunit is also precipitated in lanes 1, 2, and 4–6. However, this precipitation is not blocked by unlabeled sodium channel and is observed with preimmune serum. It represents nonspecific adsorption of the β1 subunit to the protein A immunoadsorbent. The interaction of our antisera with the β1 subunit is too weak to clearly detect in these experiments.

Comparison of Covalent Labeling Patterns from Synaptosomes and Brain Homogenate. Purification of the tetrodotoxin binding component of the voltage-sensitive sodium channel from the electroplax of the electric eel has resulted in the isolation and partial characterization of a single large molecular weight polypeptide (Agnew et al., 1980; Nakayama et al., 1982; Norman et al., 1983; Miller et al., 1983) which appears to be analogous to the α polypeptide from rat brain. The lack of significant quantities of any lower molecular weight polypeptides led to the concern that the β1 and/or β2 polypeptides may be produced by partial proteolysis of the α polypeptide or a larger native form during the experiments with rat brain despite the substantial precautions taken in previous work. Because synaptosomes are isolated by using a series of centrifugation steps spanning several hours, the α, β1, and β2 polypeptides might be produced by specific cleavage of a single larger species before our photoaffinity labeling experiments are initiated. In order to explore the likelihood of this possibility, we have developed a method to covalently label the sodium channel subunits in a freshly prepared rat brain homogenate. As described in the legend to Figure 6, several experimental variables were manipulated to reduce the time which elapsed from sacrifice of the rat to denaturation of the labeled membranes in $NaDodSO_4$. Furthermore, the rat brain homogenate was freshly prepared and immediately labeled in a solution containing seven protease inhibitors, EDTA, and bacitracin as described under Experimental Procedures. The autoradiogram of a slab gel containing synaptosomal and brain homogenate preparations which were labeled in parallel is shown in Figure 6. A comparison of the two labeled prepa-

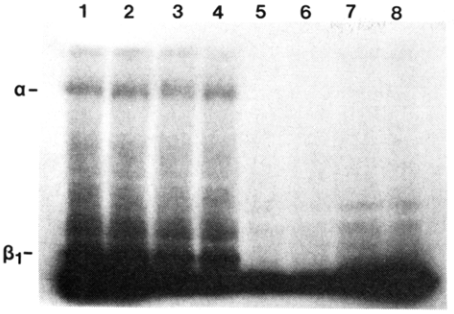

FIGURE 7: Extraction of covalently labeled synaptosomes by treatment with high ionic strength and metal ion chelating agent. Aliquots of synaptosomes labeled by ANB-$^{125}I_1$-Lqtx were incubated in standard binding medium alone (lanes 1 and 5) or containing 2 mM EDTA (lanes 2 and 6) or 2.5 M NaCl (lanes 3 and 7) or both 2 mM EDTA + 2.5 M NaCl (lanes 4 and 8). After 60 min on ice, the solns. were centrifuged for 10 min at maximum speed in a Beckman airfuge. The pellets (lanes 1–4) were prepared for and subjected to $NaDodSO_4$ gel electrophoresis according to Experimental Procedures. To the supernates were added 25 μg of BSA and trichloroacetic acid to a final concentration of 6.4% (w/v). Following centrifugation, the trichloroacetic acid pellets (lanes 5–8) were dissolved in $NaDodSO_4$ sample buffer, neutralized with NaOH, and prepared for and subjected to $NaDodSO_4$ gel electrophoresis as above. An autoradiogram of the gel is shown. The film was exposed for 15 days.

rations (lanes 1, 2, 5, and 6) reveals that the same polypeptides specifically labeled in synaptosomes (α and β1) are specifically labeled in brain homogenate. Furthermore, the same increase in the electrophoretic mobility of the α-protein band from labeled synaptosomes in the presence of reducing agent (lane 3) is observed in the high molecular weight band from labeled brain homogenate (lane 4). Since this increased mobility of α from labeled synaptosomes after reduction has been correlated with the dissociation of the unlabeled β2 polypeptide (Hartshorne et al., 1982), we assume that the decrease in apparent molecular weight of the α-protein band in the labeled brain homogenate is also due to the dissociation of β2 upon reduction. These results taken together demonstrate that the same three polypeptides detected by photoaffinity labeling of sodium channels in synaptosomes and isolated in the most purified sodium channel preparations from rat brain are present in the initial rat brain homogenate. It is likely that they are present in the intact brain.

Interactions of α and β1 with the Membrane. Synaptosomes labeled by ANB-$^{125}I_1$-Lqtx were treated with a diverse group of agents in order to investigate the nature of the attachment of the polypeptide chains of the sodium channel to the synaptosomal plasma membrane. Treatment of membranes with high ionic strength or metal ion chelating agents which both disrupt electrostatic noncovalent interactions has led to the release of intact proteins such as cytochrome *c* of mitochondria and spectrin of erythrocytes from their membranes [reviewed in Singer (1974)]. Extraction of the covalently labeled synaptosomes by a high concentration of NaCl, EDTA, or both resulted in no loss of labeled α or β1 from the membrane (Figure 7). A comparison of the protein content of Coomassie brilliant blue staining, however, demonstrated that some synaptosomal polypeptides are removed from the membrane fraction by these conditions. Further experiments were performed with chemicals that either covalently modify proteins or perturb their quaternary structure including 5 mM lithium diiodosalicylate, 90 mM citraconic anhydride, 8 M urea, or 2 M NaSCN as described under Experimental Procedures (Hatefi & Hanstein, 1969; Steck & Yu, 1973). The majority of these treatments caused the disappearance of the α band and the appearance of a new autoradiographic band at the top of the separation gel. Therefore, it appears that these treatments resulted in aggregation of the α polypeptide but did not remove it from the membrane fraction. In all cases, however, incubation of the labeled synaptosomes with these agents resulted in no loss of the β1 polypeptide from the membrane fraction. Recoveries of the labeled β1 subunit in the membrane fraction following these treatments as compared to controls were the following: 5 mM lithium diiodosalicylate, 105%; 90 mM citraconic anhydride, 126%; 8 M urea, 115%; 2 M NaSCN, 138%. Thus, the β1 subunit appears to be an integral membrane polypeptide interacting with the phospholipid bilayer as well as with the complex of α and β2 subunits.

Discussion

Differential Photoaffinity Labeling of the α and β1 Polypeptides. Aryl azide derivatives of scorpion toxins have previously been prepared and the unfractioned reaction mixtures utilized to covalently label components of the sodium channel in synaptosomal membranes (Beneski & Catterall, 1980; Darbon et al., 1983). This report describes improved procedures for the labeling reaction. Reaction conditions were developed to give an optimum and reproducible yield of photoreactive toxin derivatives, and two different toxin derivatives were purified and resolved by ion-exchange chromatography in the dark (Figure 1 and Table I). These two derivatives give distinct patterns of labeling of the α and β1 subunits of the sodium channel (Figure 2). The derivative in peak I labels the α subunit almost exclusively. The low level of incorporation into the β1 subunit may be due to residual peak II derivative since there is incomplete resolution of the two forms. The derivative in peak II preferentially labels the β1 subunit but also labels the α subunit significantly. Evidently, the location of the aryl azide substituent on ANB-$^{125}I_1$-Lqtx determines the site of reaction with the sodium channel.

The small amounts of ANB-$^{125}I_1$-Lqtx available for analysis, together with the necessity of maintaining a high concentration of BSA in all solutions containing $^{125}I_1$-Lqtx, have prevented a direct chemical determination of the nature of the derivatives in peaks I and II. Some indirect inferences can be made, however. Primary amino groups are the most likely sites of reaction of ANB-NOS. *Leiurus* scorpion toxin has eight lysine residues and a free amino terminus (Kopeyan et al., 1978). As ANB-NOS reacts with an amino group on $^{125}I_1$-Lqtx to convert it from a postively charged form to a neutral one, the derivatized toxin becomes less basic than native toxin, binds less well to the weakly acidic cation-exchange resin, and elutes earlier from the column. Peak I and II derivatives are the first products to be observed at low ratios of ANB-NOS to toxin (Table I) which suggests that one or both represent monosubstituted derivatives. They migrate closely together as though their net charge is similar (Figure 1). As the ratio of ANB-NOS to amino groups increases, the amount of these two products rises and falls together (Table I). A precursor–product relationship between peaks I and II is not evident. Rather, both peak I and peak II derivatives seem to be precursors of less positively charged derivatives which are eluted from the column at lower ionic strength. On the basis of these considerations, our working hypothesis is that the peak I and peak II azidonitrobenzoyl derivatives of Lqtx represent two different mono-substituted adducts resulting from reaction at different lysine residues.

Our photoaffinity labeling experiments place the Lqtx receptor site at or near the interface of the α and β1 subunits. The derivative of peak I has an ANB substituent located such that, when ANB-$^{125}I_1$-Lqtx is bound to its receptor site and

is photoactivated, the nitrene preferentially attacks the α subunit. The peak II derivative must have its ANB substituent located within several angstroms of both the α and $\beta 1$ subunits so that it can react with either polypeptide to give specific photoaffinity labeling. Identification of the sites of attachment of the peak II derivative to the α and $\beta 1$ subunits will give insight into the regions of contact of these two subunits. Further experiments will be required to determine whether α, $\beta 1$, or both make direct contact with noncovalently bound Lqtx.

Analysis of synaptosomes specifically labeled with ANB-$^{125}I_1$-Lqtx by using a sensitive two-dimensional gel electrophoresis procedure did not detect any incorporation of $^{125}I_1$-Lqtx into the $\beta 2$ subunit of the sodium channel (Figure 4). Labeling at a level of 5% of that of $\beta 1$ would have been detected. These results suggest that the contacts between the α and $\beta 2$ subunits that lead to the formation of one or more disulfide linkages are distant from the region of the α–$\beta 1$ interaction at or near the Lqtx receptor site.

Polypeptides of the same apparent molecular weights as α and $\beta 1$ are also labeled by neurotoxins that act at receptor sites 1 and 4. Darbon et al. (1983) found that both the α and $\beta 1$ polypeptides of the sodium channel in synaptosomes were specifically labeled by an azidonitrophenyl derivative of β-scorpion toxin from *Centruroides suffusus* that acts at neurotoxin receptor site 4 of the sodium channel. Barhanin et al. (1983) labeled only a peptide of M_r 260 000 in rat brain synaptosomes by covalent cross-linking of specifically bound β-scorpion toxin from *Tityus serrulatus* which also acts at neurotoxin receptor site 4. These results indicate that neurotoxin receptor site 4, like neurotoxin receptor site 3, lies near the regions of contact between the α and $\beta 1$ polypeptides and scorpion toxins bound at the receptor site can be specifically cross-linked to either polypeptide depending upon the amino acid residues with which the cross-linking reagents react.

The tetrodotoxin binding component of the sodium channel purified from eel electroplax has only a single polypeptide with a molecular weight of 260 000 (Agnew et al., 1980; Nakayama et al., 1982; Miller et al., 1983; Norman et al., 1983). This polypeptide is covalently labeled by the cross-linking of specifically bound derivatives of tetrodotoxin (Lombet et al., 1983). If the α subunit of the mammalian sodium channel is analogous to this polypeptide from the eel electroplax, these data would suggest that this single polypeptide subunit forms part of or is located adjacent to neurotoxin receptor sites 1, 3, and 4.

Polypeptide Components of the Sodium Channel in Situ. In previous work, we have shown that polypeptides having the same apparent molecular weights as the α and $\beta 1$ subunits of the purified sodium channel can be identified by photoaffinity labeling of intact synaptosomes with ANB-$^{125}I_1$-Lqtx (Beneski & Catterall, 1980; Hartshorne & Catterall, 1981; Hartshorne et al., 1982). Moreover, reduction of protein disulfide bonds with 2-mercaptoethanol caused an increase in the electrophoretic mobility of the high molecular weight component labeled by ANB-$^{125}I_1$-Lqtx which was consistent with the cleavage of the disulfide-linked $\beta 2$ subunit (Hartshorne et al., 1982). These results led us to propose that the α, $\beta 1$, and $\beta 2$ subunits of the purified sodium channel were also the polypeptide components of the native sodium channel in situ. The data presented here provide additional support for this proposal.

Antisera raised against the purified sodium channel from rat brain specifically recognize a protein with a molecular weight of 260 000 that comigrates with the α subunit in Western blot analyses of NaDodSO$_4$–polyacrylamide gels of synaptosomal membrane preparations.[3] We show here that these antisera also specifically immunoprecipitate the polypeptide with a molecular weight of 260 000 that is covalently labeled by ANB-$^{125}I_1$-Lqtx (Figure 5). These results show that the α subunit of the purified sodium channel is the same as the 260 000 molecular weight polypeptide identified in intact membranes by photoaffinity labeling and is therefore a subunit of the sodium channel in situ in synaptosomal membranes.

Isolation of synaptosomes followed by photoaffinity labeling with our previous methods requires several hours of work. During this time, modification of the sodium channel polypeptides by proteolysis or other means is possible. To minimize these changes, we have developed methods to photoaffinity label sodium channels rapidly in unfractionated brain homogenates. All manipulations were carried out in the presence of a mixture of nine protease inhibitors. Under these carefully controlled conditions, labeling of the α and $\beta 1$ subunits is observed in brain homogenates as in synaptosomes (Figure 6). Furthermore, reduction with 2-mercaptoethanol increases the electrophoretic mobility of the α subunit band consistent with loss of the disulfide-linked $\beta 2$ subunit. Evidently, the α, $\beta 1$, and $\beta 2$ subunits are present in brain tissue freshly homogenized at 0 °C in the presence of nine protease inhibitors. This represents the most direct demonstration to date that these polypeptides comprise the sodium channel in the intact brain.

The protein components of sodium channels of rat skeletal muscle and electric eel electropolax have been studied by analysis of preparations purified using tetrodotoxin or saxitoxin binding as an assay. The tetrodotoxin binding component of the sodium channel from electroplax consists of a single polypeptide with a molecular weight of 260 000 (Agnew et al., 1980; Nakayama et al., 1982; Miller et al., 1983; Norman et al., 1983). Presumably, this polypeptide is analogous to the α subunit of the sodium channel from rat brain. Polypeptides of the size of the $\beta 1$ and $\beta 2$ subunits have not been observed. These subunits may be absent in the electroplax or lost during purification due to dissociation or proteolysis. Functional reconstitution of the purified electroplax sodium channel has recently been reported, raising the possibility that subunits analogous to $\beta 1$ and $\beta 2$ may not be required for neurotoxin-activated ion flux mediated by the electroplax sodium channel (Rosenberg et al., 1984).

The most recent preparations of sodium channels from rat skeletal muscle contain a large glycoprotein component whose apparent molecular weight varies from 230 000 to 130 000 depending upon the conditions of NaDodSO$_4$–polyacrylamide gel electrophoresis (Barchi, 1983). In addition, a polypeptide with a molecular weight of 45 000 and a doublet with molecular weight of 38 000 are observed (Barchi, 1983). It seems likely that the large glycoprotein is analogous to the α subunit of the rat brain sodium channel and that the doublet with a molecular weight of 38 000 is analogous to $\beta 1$ and $\beta 2$. The polypeptide with a molecular weight of 45 000 has no counterpart in the sodium channel from rat brain. Functional reconstitution of the sodium channel from rat skeletal muscle has been achieved (Weigele & Barchi, 1983; Tanaka et al., 1983), indicating that, as for the rat brain sodium channel (Talvenheimo et al., 1982; Tamkun et al., 1984), the purified preparation contains the components necessary to mediate ion flux.

Association of the Sodium Channel Polypeptides with the Lipid Bilayer. The sodium channel is known to be glycosylated

[3] W. Downey and W. A. Catterall, unpublished experiments.

by its binding to lectins (Barchi et al., 1980; Hartshorne & Catterall, 1981; 1981; Cohen & Barchi, 1981) and by the reduction in the number of sodium channels when glycosylation is inhibited (Waechter et al., 1983; Bar-Sagi & Prives, 1983). Furthermore, the α polypeptide has been shown to be heavily glycosylated by chemical analysis (Miller et al., 1983), and this polypeptide specifically binds labeled lectins[4] (Barchi, 1983). The general observation that carbohydrate moieties are only attached to the extracytoplasmic surface of membrane proteins [reviewed in Rothman & Lenard (1977)] combined with the covalent labeling of this peptide by tetrodotoxin and scorpion toxins (Beneski & Catterall, 1980; Darbon et al., 1983; Darhanin et al., 1983; Lombet et al., 1983) that act on ly from the external side of the axon (Narahashi et al., 1967, 1972) argues strongly that the α polypeptide has a portion of its mass exposed extracytoplasmically. Studies of the selective phosphorylation of the α polypeptide by endogenous cAMP-dependent protein kinase (Costa et al., 1982; Costa & Catterall, 1984) demonstrate that this polypeptide must have part of its mass on the cytoplasmic side of the membrane since ATP and protein kinase are only available inside the cell. Therefore, the α polypeptide is a transmembrane protein, and because of its large size, it possesses the potential to traverse the membrane several times and contact an aqueous environment after each pass through the bilayer.

Less information is available concerning the two β polypeptides. Both $\beta 1$ and $\beta 2$ appear to be glycoproteins based upon their ability to bind radioactive lectin[4] and thus would be expected to have at least part of their mass on the extracytoplasmic side. The presence of a portion of $\beta 1$ on the external surface is also demonstrated by its ability to be specifically labeled with ANB-$^{125}I_1$-Lqtx and photoreactive derivatives of a β-scorpion toxin(Darbon et al., 1983) which cannot cross the lipid bilayer. $\beta 1$ may be anchored to the membrane only via noncovalent, protein–protein interactions with membrane-bound α or may also make direct contact with the membrane through noncovalent interactions between the hydrophobic interior of the bilayer and hydrophobic amino acids on the surface of the subunit. The results obtained here show that $\beta 1$ is still found in the membrane fraction after treatment with a variety of agents known to disrupt protein–protein interactions and to release peripheral proteins from the membrane (Figure 7 and Results). These observations suggest that a portion of the mass of the $\beta 1$ polypeptide resides within the membrane. Additional experiments will be required to determine whether the $\beta 2$ subunit interacts with the hydrophobic region of the membrane, to examine whether the $\beta 1$ and $\beta 2$ subunits span the lipid bilayer, and to define the portion of polypeptide mass of the three sodium channel subunits that is located on the cytoplasmic and extracytoplasmic sides of the membrane.

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References

Agnew, W. S., Moore, A. C., Levinson, S. R., & Raftery, M. A. (1980) *Biochem. Biophys. Res. Commun. 92*, 860–866.

Bailey, J. L. (1967) *Techniques in Protein Chemistry*, 2nd ed., p 340, American Elsevier Publishing Co., New York.

Barchi, R. L. (1983) *J. Neurochem. 40*, 1377–1385.

Barhanin, J., Schmid, A., Lombet, A., Wheeler, K. P., Lazdunski, M., & Ellory, J. C. (1983) *J. Biol. Chem. 258*, 700–702.

Bar-Sagi, D., & Prives, J. (1983) *J. Cell. Physiol. 114*, 77–81.

Beneski, D. A., & Catterall, W. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 639–643.

Catterall, W. A. (1976) *J. Biol. Chem. 251*, 5528–5536.

Catterall, W. A. (1977) *J. Biol. Chem. 252*, 8660–8668.

Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol. 20*, 15–43.

Catterall, W. A. (1984) *Science (Washington, D.C.) 223*, 653–661.

Costa, M. R. C., & Catterall, W. A. (1984) *J. Biol. Chem. 259*, 8210–8218.

Costa, M. R. C., Casnellie, J. E., & Catterall, W. A. (1982) *J. Biol. Chem. 257*, 7918–7921.

Darbon, H., Jover, E., Couraud, F., & Rochat, H. (1983) *Biochem. Biophys. Res. Commun. 115*, 415–422.

Gray, E. G., & Whittaker, V. P. (1962) *J. Anat. 96*, 79–88.

Hartshorne, R. P., & Catterall, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4620–4624.

Hartshorne, R. P., & Catterall, W. A. (1984) *J. Biol. Chem. 259*, 1667–1675.

Hartshorne, R. P., Messner, D. J., Coppersmith, J. C., & Catterall, W. A. (1982) *J. Biol. Chem. 257*, 13888–13891.

Hatefi, Y., & Hanstein, W. G. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1129–1136.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.

Hirs, C. H.W. (1955) *Methods Enzymol. 1*, 113–121.

Jover, E., Couraud, F., & Rochat, H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1607–1614.

Kopeyan, C., Martinez, G., & Rochat, H. (1978) *FEBS Lett. 89*, 54–58.

Lewis, R. V., Roberts, M. R., Dennis, E. A., & Allison, W. S. (1977) *Biochemistry 16*, 5650–5654.

Lombet, A., Norman, R. I., & Lazdunski, M. (1983) *Biochem. Biophys. Res. Commun. 114*, 126–130.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maizel, J. V. (1971) *Methods Virol. 5*, 179–224.

Miller, J. A., Agnew, W. S., & Levinson, S. R. (1983) *Biochemistry 22*, 462–470.

Nakayama, H., Withy, R. M., & Raftery, M. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7575–7579.

Narahashi, T. (1974) *Physiol. Rev. 54*, 813–889.

Narahashi, T., Anderson, N. C., & Moore, J. W. (1967) *J. Gen. Physiol. 50*, 1413–1428.

Narahashi, T., Shapiro, B. I., Deguchi, T., Scuka, M., & Wang, C. M. (1972) *Am. J. Physiol. 222*, 850–857.

Nathanson, N. M., & Hall, Z. W. (1979) *Biochemistry 18*, 3392–3401.

Norman, R. I., Schmid, A., Lombet, A., Barhanin, J., & Lazdunski, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4164–4168.

Ritchie, J. M., & Rogart, R. (1977) *Rev. Physiol., Biochem. Pharmacol. 79*, 1–50.

Rosenberg, R. L., Tomiko, S. A., & Agnew, W. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1239–1243.

Rothman, J. E., & Lenard, J. (1977) *Science (Washington, D.C.) 195*, 743–753.

[4] D. J. Messner and W. A. Catterall, unpublished experiments.

Singer, S. J. (1974) *Annu. Rev. Biochem. 43*, 805–833.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Steck, T. L., & Yu, J. (1973) *J. Supramol. Struct. 1*, 220–232.
Talvenheimo, J. A., Tamkun, M. M., & Catterall, W. A. (1982) *J. Biol. Chem. 257*, 11868–11871.
Tamkun, M. M., & Catterall, W. A. (1981) *Mol. Pharmacol. 19*, 78–86.
Tamkun, M. M., Talvenheimo, J. A., & Catterall, W. A. (1984) *J. Biol. Chem. 259*, 1676–1688.
Tanaka, J. C., Eccleston, J. F., & Barchi, R. L. (1983) *J. Biol. Chem. 258*, 7519–7526.
Waechter, C. J., Schmidt, J. W., & Catterall, W. A. (1983) *J. Biol. Chem. 258*, 5117–5123.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005–8018.
Weigele, J. B., & Barchi, R. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3651–3655.

Partition of Amphiphilic Molecules into Phospholipid Vesicles and Human Erythrocyte Ghosts: Measurements by Ultraviolet Difference Spectroscopy†

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ABSTRACT: Molar partition coefficients for chlorpromazine and methochlorpromazine between phospholipid vesicles or human erythrocyte ghosts and buffer are determined by ultraviolet difference spectroscopy. The partition coefficients between small unilamellar egg phosphatidylcholine vesicles and buffer at pH 7.4 are 4.4×10^5 for chlorpromazine and 0.8×10^5 for methochlorpromazine, determined with 10 μM amphiphile. An increase in the partition of chlorpromazine into vesicles is seen as the pH is increased to the pK_a of chlorpromazine at 9.2. Chlorpromazine also partitions preferentially into fluid-phase phospholipid compared to solid-phase phospholipid. Molar partition coefficients between unsealed human erythrocyte ghosts and buffer at pH 8.0 with 10 μM amphiphile are determined to be 6.5×10^5 for chlorpromazine and 2.5×10^5 for methochlorpromazine. Difference spectroscopy is an equilibrium technique that does not require separation of bound from free amphiphile, as do many other methods of determining membrane–buffer partition coefficients. This method is useful for any amphiphile that has an appreciable absorbance below its critical micelle concentration and whose absorbance is sensitive to environment.

Chlorpromazine and methochlorpromazine are amphiphilic amines that are clinically useful as tranquilizers. For many years the anesthetic properties of these amines and other amphiphilic compounds have been correlated with their membrane solubility. This correlation has recently been reviewed (Janoff et al., 1981). The shape changes produced in erythrocytes by chlorpromazine and methochlorpromazine have also been attributed to their membrane solubility (Deuticke, 1968; Sheetz & Singer, 1974). The current assessment of the molecular basis of chlorpromazine–lipid bilayer interaction derives from nuclear magnetic resonance spectroscopy (Frenzel et al., 1978; Kuroda & Kitamura, 1984) and suggests that the phenothiazine ring is located near the α-methylenes of fatty acyl chains and that the positively charged alkylamine group is in the proximity of the phosphate of the phospholipid polar head group. It is less clear how other effects of these drugs on membranes and membrane-related processes are related to the intercalation of amphiphile into the membrane lipid bilayer. For example, chlorpromazine affects the activity of a variety of phospholipases (Kunze et al., 1976; Vanderhoek & Feinstein, 1979), as well as phospholipid biosynthetic enzymes (Sturton & Brindley, 1977; Zborowski & Brindley, 1983). Recent work from this laboratory describes the inhibition of phosphatidylinositol transfer protein from bovine brain by both chlorpromazine and methochlorpromazine (Mullikin & Helmkamp, 1984).

A high solubility of chlorpromazine in red cell ghosts was first demonstrated directly by Roth & Seeman (1972). These studies involved mixing erythrocyte ghosts with amphiphiles, then centrifuging the ghosts, and determining the amount of ghost-associated amphiphile. However, later measurements of the partition of these compounds into cell membranes and phospholipid vesicles led to the conclusion that there was practically no cell membrane associated chlorpromazine or methochlorpromazine (Conrad & Singer, 1979, 1981). The latter method involved the separation of vesicles or membranes from the free amphiphile in buffer by a series of filters.

In order to resolve this discrepancy and to assess what factors affect the binding of chlorpromazine and methochlorpromazine to membranes, we undertook the present study. The vesicle–buffer and ghost–buffer partition properties of these compounds were investigated by ultraviolet difference spectroscopy. This method takes advantage of a shift in the absorption spectra of these drugs upon going from an aqueous to a hydrophobic environment. Furthermore, it is an equilibrium method that does not require the separation of bound and free amphiphile. We demonstrate that chlorpromazine and methochlorpromazine partition strongly into phospholipid vesicles and erythrocyte ghosts. Our results on ghosts support and extend those obtained by Bondy & Remien (1981) using

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