Stabilization of a Sodium Channel State with High Affinity for Saxitoxin by Intramolecular Cross-Linking. Evidence for Allosteric Effects of Saxitoxin Binding†

Francisco J. Tejedor,‡ Elizabeth McHugh,§ and William A. Catterall*
Department of Pharmacology, SJ-30, University of Washington, Seattle, Washington 98195
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ABSTRACT: Incubation of purified rat brain sodium channels at 37 °C or at high ionic strength causes a concomitant loss of saxitoxin-binding activity and dissociation of β1 subunits. Reaction with hydrophilic carbodiimides produced a resistance against the loss of saxitoxin binding and caused covalent cross-linking of α, β1, and β2 subunits. In the presence of saxitoxin, this cross-linking reaction led to formation of a state with increased affinity for saxitoxin. However, analysis of the concentration dependence of covalent cross-linking and its inhibition by hydrophilic nucleophiles showed that the stabilization of the saxitoxin-binding activity was due to the formation of a small number of isopeptide bonds in the α subunit rather than to cross-linking of α and β1 subunits. In the presence of amine nucleophiles, carbodiimides caused loss of saxitoxin binding, which was prevented in the presence of the toxin. Nucleophiles yielding positively charged amide products were more effective than those forming uncharged or negatively charged products. Under conditions where saxitoxin protected the binding activity of the sodium channel from inactivation, the overall availability of carboxyl groups for reaction was increased, providing evidence for a toxin-induced conformational change on binding. These results are considered in terms of an allosteric model of saxitoxin binding, in which the functional form of the sodium channel having high affinity for saxitoxin can be stabilized against inactivation by noncovalent interactions with β1 subunits, binding of saxitoxin and tetrodotoxin, or intramolecular cross-linking of amino acid residues within the α subunit.

The voltage-sensitive sodium channel is a transmembrane protein that mediates increases in sodium conductance during the action potential in a wide variety of electrically excitable tissues. In skeletal muscle, brain, and eel electroplax, the principal component of the sodium channel is a glycoprotein of approximately 260 kDa [reviewed in Agnew (1984), Barchi (1984), and Catterall (1984, 1986)]. Sodium channels purified in functional form from rat brain consist of a stoichiometric complex of three subunits designated α (M_r 260 000), β1 (M_r 36 000), and β2 (M_r 33 000) [reviewed in Catterall (1984, 1986)]. The β2 subunit is covalently attached to α by one or more disulfide bonds, while the β1 subunit is noncovalently bound. The β2 subunit, but not the β1 subunit, can be removed from purified sodium channels without loss of functional activity (Messner & Catterall, 1986; Messner et al., 1986). High molecular weight mRNA from rat brain (Sumikawa et al., 1984), α subunit mRNA purified by hybrid selection (Goldin et al., 1986), and α subunit mRNA synthesized from cloned cDNA (Noda et al., 1986) all direct the synthesis of functional, tetrodotoxin-sensitive sodium channels in *Xenopus* oocytes, suggesting that the α subunit alone can function as a voltage-gated ion channel which is inhibited by tetrodotoxin.

In our previous experiments (Messner & Catterall, 1986; Messner et al., 1986), incubation of purified sodium channels in solutions of high ionic strength or at 37 °C was found to cause concomitant dissociation of β1 subunits and loss of sodium channel function. Both of these effects were prevented in the presence of tetrodotoxin, leading to the conclusion that a complex of α and β1 subunits was both necessary and sufficient for maintenance of a functional state of the purified sodium channel having high affinity for tetrodotoxin and saxitoxin. The dissociation of the β1 subunit and the loss of sodium channel function caused by incubation at high ionic strength or 37 °C could arise from an initial dissociation of the β1 subunit, which then destabilizes the functional form of the α subunit, or from an initial change in the state of the α subunit, which leads to loss of activity and dissociation of β1. In the experiments described here, chemical cross-linking agents and carboxyl-specific reagents were used to determine the relationships between binding of saxitoxin and tetrodotoxin, association of α and β1 subunits, and maintenance of a functional state of the purified sodium channel having high affinity for tetrodotoxin and saxitoxin.

Experimental Procedures

Materials. Saxitoxin (STX)[1] was obtained from the National Institutes of Health, labeled by New England Nuclear

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‡ Present address: Instituto Cajal, CSIC, Velazquez 144, Madrid 28006, Spain.

§ Present address: Molecular Pharmacology Laboratory, Food and Drug Administration, Bldg. 29, Room 208, Rockville Pike, Bethesda, MD 20892.

[1] Abbreviations: ArgMe, arginine methyl ester; BSA, bovine serum albumin; CMT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; DCC, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; GlyMe, glycine methyl ester; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; STX, saxitoxin; TTX, tetrodotoxin; TMO, trimethyloxonium; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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using a ^3H_2O exchange procedure (Ritchie et al., 1976), and purified and characterized in our laboratory as described previously (Waechter et al., 1983). The rat brain sodium channel was solubilized with Triton X-100 and purified by chromatography on DEAE-Sephadex, hydroxylapatite, and wheat germ agglutinin–Sepharose as described previously (Hartshorne & Catterall, 1984).

CMT was obtained from Aldrich, and EDC and DCC were obtained from Sigma. Concentrated solutions of carbodiimides in the reaction buffer were prepared fresh before use. Gel electrophoresis reagents came from Bio-Rad. Other supplies and reagents were of the highest purity available from commercial sources. L-[2,3,4,5-^{3}H]Arginine monohydrochloride was purchased from Amersham.

Synthesis of [^{3}H]ArgMe. L-[2,3,4,5-^{3}H]Arginine monohydrochloride (1 mCi; 40 Ci/mmol) in 1 mL of water was lyophilized after an excess of concentrated hydrochloric acid was added to force the conversion to the dihydrochloride salt. The resultant solid was suspended in 100 μL of absolute methanol containing approximately 0.5 μL of 10.2 N HCl and incubated at 70 °C in a tightly closed vial. The course of the reaction was followed by thin-layer chromatography on cellulose sheets using EtOH/NH_4OH (30%)/H_2O (20:1:4) as eluant. After 36–48 h of incubation, [^{3}H]ArgMe represented 80–90% of the radioactivity in the reaction mixture. This product was purified by preparative thin-layer chromatography on cellulose sheets eluting with 2-propanol/H_2O/0.5 M Tris-HCl, pH 6.7 (17:2:1) and stored in absolute ethanol solution at −20 °C. Its purity was frequently tested by thin-layer chromatography.

Rapid Gel Filtration and Ion-Exchange Chromatography. These methods were used to assay STX binding activity (Catterall et al., 1979), desalt samples, exchange buffer solutions, and remove excess reagents. Columns were prepared by placing 2 mL of either Sephadex G-50–150 or carboxymethyl-Sephadex C-25–120 in a 3-mL disposable syringe. The syringes were equilibrated in the appropriate buffer and prespun in a clinical centrifuge. Samples containing sodium channels were sedimented through these columns in 150–200-μL aliquots.

Reconstitution of Sodium Channels. Purified sodium channels in buffer 2 (50 mM potassium phosphate, 100 mM choline chloride, 0.1% Triton X-100, 0.025% phosphatidylcholine, pH 7.4) were reconstituted in phosphatidylcholine vesicles as described previously (Feller et al., 1985).

Measurements of [^{3}H]STX Binding. The STX-binding activity of control and chemically modified samples was measured by rapid gel filtration assay through Sephadex G-50 columns as described previously (Catterall et al., 1979).

SDS–PAGE. Samples were prepared and analyzed by SDS–PAGE in gradient (3–10%) gels using the discontinuous gel system of Maizel (1971). When necessary, 7–20% acrylamide gradients were prepared under conditions that provide optimum separation of the β1 and β2 subunits as described previously (Messner & Catterall, 1985). Following electrophoresis, gels were silver stained according to the method of Oakley et al. (1980). When necessary, gels were dried, and autoradiography was performed at −70 °C.

Purification of β1 and β2 Subunits and Preparation of Antisera. β1 and β2 subunits were selectively removed from sodium channels prebound to hydroxylapatite resin as described previously (Wollner et al., 1987). The β1 and β2 subunit containing eluates were pooled, concentrated, and used to immunize rabbits. A partially purified IgG fraction was prepared by ammonium sulfate precipitation from the anti-β2 antiserum. Anti-β1 antibodies were affinity purified by adsorption of any contaminating anti-α or anti-β2 subunit antibodies to a nitrocellulose strip containing αβ2 that had been purified by SDS–PAGE and electroblotted, and then by specific adsorption and elution of anti-β1 antibodies from a nitrocellulose strip containing β1 subunits that had been purified by SDS–PAGE and electroblotted as described previously (Wollner et al., 1987).

Treatment of Sodium Channel with Carbodiimides and Nucleophiles. Purified sodium channel at a concentration of 50–80 nM in buffer 1 (50 mM potassium phosphate, pH 6.0, 0.1% Triton X-100, 0.025% PC) was incubated at 0 °C with carbodiimide alone or carbodiimide plus a nucleophile in the absence or presence of saxitoxin at the concentrations indicated in the figure legends. After 1–3 h, EDTA was added to a final concentration of 10 mM, and the pH of the medium was increased to 7.4 by careful addition of 1 M Hepes/Tris, pH 7.4, and 1 M KOH. Excess reagents and saxitoxin if present were removed immediately by rapid ion exchange on Sephadex CM-25 columns preequilibrated in buffer 3 (25 mM Hepes/Tris, pH 7.4, 100 mM choline chloride, 10 mM EDTA, 0.1% Triton X-100, and 0.025% PC).

Reconstituted sodium channels prepared as described above were diluted to 50 nM in isotonic 50 mM potassium phosphate, pH 5.8, and 100 mM choline chloride and incubated with carbodiimide at the concentrations indicated in the figure legends for 2 h at 0 °C in the presence of 5 μM saxitoxin. Saxitoxin and excess reagents were then removed by rapid gel filtration through columns of Sephadex G-50 preequilibrated in the same reaction buffer but at pH 7.4.

In order to determine the incorporation of radioactivity into the channel when tritiated nucleophiles were used, 10 μL of 2 mg/mL BSA was added as carrier to 100-μL aliquots of the final reaction media, and the protein was precipitated with 1.5 mL of cold 10% TCA at 0 °C for 5 min. Samples were then filtered on Whatman GF/C filters and washed 3 times with 2 mL of cold 5% TCA. Filters were counted after equilibration with scintillation cocktail for at least 2 h.

Treatment of the Sodium Channel with TMO. TMO tetrafluoroborate was weighed just before use, dissolved in ice-cold water at 20 mg/mL, and added immediately to purified sodium channel (50 nM) in buffer 2 in the presence or absence of saxitoxin. Samples were incubated at 0 °C for 10 min. The excess of reagents was removed by rapid ion exchange with Sephadex CM-25 columns equilibrated in buffer 2 as indicated above. [^{3}H]Saxitoxin binding was immediately determined in aliquots of the samples.

Determination of Covalent Cross-Linking of αβ1 and αβ2 Complexes. To dissociate disulfide-linked αβ2 complexes, control and cross-linked sodium channels were incubated in buffer 2 containing 25 mM DTT at 0 °C for 1 h. The excess of reducing agent was removed by rapid gel filtration through Sephadex G-50 columns. Samples were immediately used for immunoprecipitation and phosphorylation experiments as described below.

The dissociation of the β1 subunit from the solubilized sodium channel was carried out as described by Messner and Catterall (1986). Control and carbodiimide-modified samples obtained as described above were either incubated at 37 °C for 3–5 min or treated with 1 M $MgCl_2$ at 0 °C for 90 min. In order to remove the $MgCl_2$ after the treatment, samples were passed through Sephadex G-50 columns preequilibrated in buffer 3 as described above.

To determine whether α subunits were covalently cross-linked to β1 or β2 subunits after incubation with carbodiimides,

aliquots containing 0.5 pmol of control and cross-linked sodium channels, which had been treated as described above to dissociate reversibly associated $\beta 1$ and $\beta 2$ subunits, were incubated for 3 h on ice in buffer 3 containing 0.1% BSA with affinity-purified anti-$\beta 1$ or anti-$\beta 2$ antibodies. In order to stabilize the $\alpha\beta 1$ complex, 10 μM TTX was present during all the steps of the immunoprecipitation and phosphorylation with anti-$\beta 1$ antibodies. Protein A–Sepharose was added, and the samples were mixed by rotation for 30 min at 4 °C. The resin was then sedimented and washed 3 times with cold buffer 3 and once with phosphorylation buffer (25 mM Hepes/Tris, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, 0.1% Triton X-100, 0.025% PC). The α subunits covalently associated with $\beta 1$ or $\beta 2$ subunits in the immunoprecipitates were radioactively labeled by phosphorylation with cAMP-dependent protein kinase as described previously (Schmidt et al., 1985). Samples were prepared for SDS–PAGE by incubating the pellets with SDS–PAGE loading solution containing 5% 2-mercaptoethanol in boiling water for 5 min.

Iodination of Sodium Channels. Sodium channels were solubilized and denatured by incubating in boiling water for 2 min with 1% SDS. The samples were filtered through Sephadex G-50 columns preequilibrated in 50 mM sodium phosphate, pH 7.4, 80 mM choline chloride, and 0.5% SDS. Approximately 5 pmol of sodium channel in 100 μL of this buffer was treated with 25 μg of chloramine T at room temperature for 1 min in the presence of 200 μCi of $Na^{125}I$. The reaction was stopped by addition of 50 μg of sodium metabisulfite. The iodine not incorporated into the protein was removed by rapid gel filtration through Sephadex-G50 columns equilibrated in the same buffer containing 0.5 mg/mL of BSA. Samples obtained in this way were used for SDS–PAGE.

For quantitation of cross-linking of the $\beta 1$ subunit, 25 μL of 50 mM sodium phosphate, pH 8.2, and 1% SDS were mixed with 25 μL of buffer 2 containing 1–2 pmol of sodium channel and incubated in boiling water for 2 min. Bolton–Hunter reagent (50 μCi) in benzene solution was evaporated to dryness in the bottom of a reacti-vial, and the solution containing the sodium channel was added immediately with vigorous agitation. After 90 min at room temperature, 5 μL of 0.5 M Tris-HCl, pH 6.8, was added to stop the reaction and destroy the remaining reagent. Samples were analyzed by SDS–PAGE without reduction. After an autoradiograph of the fixed and dried gels was obtained, the bands corresponding to $\alpha\beta 2$ and $\beta 1$ were localized, cut out, and counted in order to determine the percentage of $\beta 1$ subunit cross-linked.

Peptide Maps of Iodinated α Subunits. The protein band corresponding to the α subunit was localized by autoradiography of fixed and dried polyacrylamide gels containing iodinated sodium channels. Gel slices were excised and swollen in 50 mM NH_4HCO_3 and 0.1 mM $CaCl_2$. The slices were minced into pieces and incubated at 37 °C for 20–24 h in 200 μL of fresh solution containing 150 μg of TPCK–trypsin in a shaking water bath. The solution was separated, and the gel pieces were washed with 100 μL of the same buffer. The supernatant and the wash were mixed and lyophilized. Two-dimensional peptide mapping was carried out on cellulose sheets by combination of electrophoresis in pyridine/acetic acid/water (1:10:189), pH 3.5, at 500 V for 90 min followed by ascending chromatography in 1-butanol/pyridine/acetic acid/water (15:3:12:10).

Results

Stabilization of the Saxitoxin-Binding Activity of Purified Sodium Channels by Reaction with Water-Soluble Carbodiimides. If dissociation of α and $\beta 1$ subunits is the primary cause of loss of sodium channel function due to incubation at high ionic strength or 37 °C, covalent cross-linking of these two subunits might stabilize sodium channels against loss of activity under these conditions. Chemical modification of purified sodium channels with the water-soluble carbodiimide EDC produces a concentration-dependent stabilization of the STX-binding activity during incubation at high ionic strength or 37 °C (Figure 1). In the absence of saxitoxin, EDC causes a progressive loss of total saxitoxin-binding activity measured at a saturating concentration of toxin and a progressive increase in the saxitoxin-binding activity retained after incubation at 37 °C or in 1 M $MgCl_2$ (Figure 1A). In the absence of EDC treatment, no saxitoxin binding is recovered after incubation at 37 °C or in 1 M $MgCl_2$. In contrast, at the highest concentration of EDC tested, 60% of total saxitoxin binding remained after EDC treatment, and 56% or 25% was recovered after further incubation at 37 °C or in 1 M $MgCl_2$, respectively. In the presence of saxitoxin, total binding activity did not decrease significantly during treatment with EDC, and 52% or 39% was recovered after further incubation at 37 °C or in 1 M $MgCl_2$ (Figure 1B). These results show that reaction with EDC can stabilize the saxitoxin-binding activity of purified sodium channels.

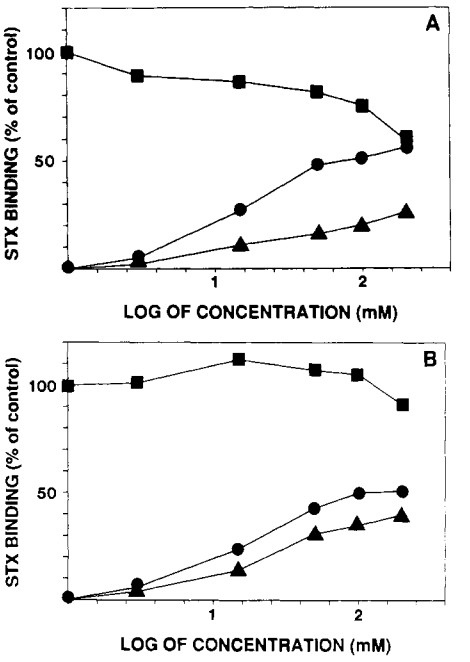

FIGURE 1: Effect of water-soluble carbodiimide on the STX-binding activity under conditions which cause dissociation of $\beta 1$ subunits. (A) Purified sodium channel (80 nM) was incubated at 0 °C with the indicated concentrations of EDC. After completion of the reaction as described under Experimental Procedures, the resulting samples in buffer 3 were assayed for [^{3}H]STX-binding activity directly (■) or after treatment with 1 M $MgCl_2$ (▲) or incubation at 37 °C (●). (B) A similar experiment was carried out with incubation with EDC in the presence of 5 μM STX.

The effects of these treatments on K_D and B_{max} for saxitoxin binding were examined by Scatchard analysis of equilibrium binding isotherms (Figure 2). In the absence of saxitoxin (Figure 2A), incubation with 60 mM EDC alone reduced B_{max} to 85% of control without effect on K_D. Further incubation with 1 M $MgCl_2$ reduced B_{max} to 51% of control without effect on K_D. Thus, under these conditions the number of sodium channels retaining high affinity for saxitoxin is affected by EDC and $MgCl_2$, but their affinity for saxitoxin (K_D = 4.4 nM) is unchanged.

In the presence of saxitoxin (Figure 2B), similar effects on B_{max} were observed. EDC alone had no effect on B_{max}, as

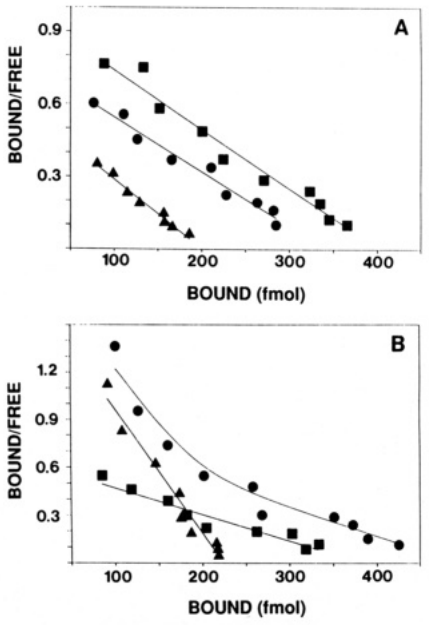

FIGURE 2: STX binding to carbodiimide-modified sodium channel. (A) Purified sodium channel (80 nM) was incubated at 0 °C for 2 h without any reagent (■) or with 60 mM EDC (●, ▲). After completion of the reaction as described under Experimental Procedures, half of the sample was incubated with 1 M $MgCl_2$ in buffer 3 at 0 °C for 65 min (▲) while the other half was incubated in buffer for the same period of time (●). Samples were desalted by rapid gel filtration. Aliquots were diluted to 10% of the original concentration, and the binding activity was determined with increasing concentrations of [^{3}H]STX from 0.5 to 20 nM. (B) A similar experiment was carried out with incubation with EDC in the presence of 5 μM STX. The data are presented as Scatchard plots. The curves presented were derived from a least-squares fit to a two binding site model by using the program LIGAND.

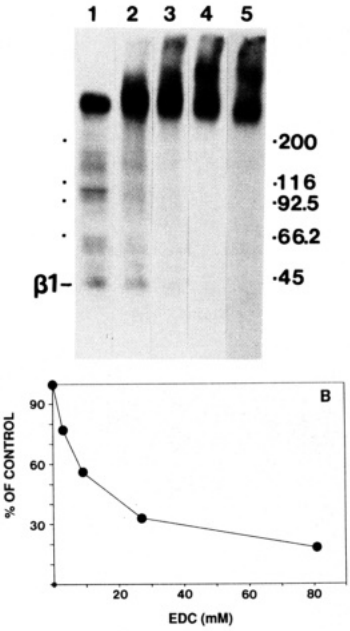

FIGURE 3: Cross-linking of sodium channel subunits with a water-soluble carbodiimide. (A) Purified sodium channel was incubated at 0 °C with 0 (lane 1), 3 (lane 2), 9 (lane 3), 27 (lane 4), and 81 mM (lane 5) EDC in the presence of 5 μM STX. After 2 h, STX and the excess of reagents were removed by rapid ion-exchange chromatography. The samples were radiolabeled with Bolton–Hunter reagent and the protein analyzed by SDS–PAGE and autoradiography. The protein bands migrating between α and β1 are not sodium channel components and are removed by sucrose gradient sedimentation when all four of the purification steps of Hartshorne and Catterall (1984) are used. (B) The radioactivity associated with the β1 band was determined as described under Experimental Procedures and plotted as the percentage of an untreated sample.

expected from the results of Figure 1, while EDC followed by 1 M $MgCl_2$ reduced B_{max} to 55% of control. However, in contrast to the results in the absence of saxitoxin, treatment with EDC in the presence of saxitoxin reduced the K_D for saxitoxin binding from 3.4 to 1.0 nM for appoximately 50% of the binding sites (Figure 2B, ●). After treatment with 1 M $MgCl_2$, primarily these new higher affinity binding sites were recovered (Figure 2B, ▲). These results suggest that binding of saxitoxin and reaction with EDC act synergistically to produce a stable, high-affinity form of the purified sodium channel. Evidence that this effect is due to a conformational change induced by saxitoxin binding is considered in the last section under Results.

Covalent Cross-Linking of α Subunits to β1 and β2 Subunits. In order to relate the stabilization of saxitoxin-binding activity to covalent cross-linking of α and β1 subunits, the subunit compositions of the sodium channel samples treated with increasing concentrations of EDC were analyzed by SDS–PAGE without reduction of disulfide bonds to separate the β1 subunits from the disulfide-linked αβ2 complexes. A concentration-dependent decrease in the protein migrating in the position of β1 subunits was observed in EDC-modified samples, as illustrated in Figure 3A. Concomitant with the loss of β1 subunits, the αβ2 complexes migrated in a broadened band consistent with covalent attachment to β1 subunits. The percent of β1 subunits recovered in the β1 protein band after treatment with EDC decreased sharply with increasing EDC concentration (Figure 3B). Since the loss of β1 subunits is prevented by addition of a nucleophile such as taurine to the reaction mixture (data not shown), it is likely that the loss of the β1 subunits is due to the formation of intersubunit isopeptide bonds between α and β1 subunits.

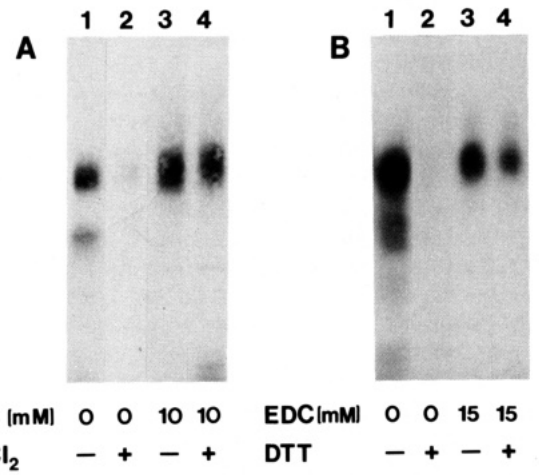

FIGURE 4: Immunoprecipitation of carbodiimide-modified sodium channel α subunits with antibodies against the β1 and β2 subunits. (A) Purified sodium channel was incubated at 0 °C for 2 h in the presence of 5 μM STX and the indicated concentrations of EDC. After completion of the reaction, samples 2 and 4 were incubated with 1 M $MgCl_2$ as described under Experimental Procedures. All four samples were immediately subjected to immunoprecipitation with specific anti-β1 antibodies followed by phosphorylation with cAMP-dependent protein kinase and [γ-^{32}P]ATP. Immunoprecipitated samples were eluted from the protein A–Sepharose pellets and analyzed by SDS–PAGE as described under Experimental Procedures. (B) A similar experiment was carried out to determine cross-linking of β2 subunits by incubation of control and EDC-treated sodium channel with DTT and immunoprecipitation with antibodies against the β2 subunit as described under Experimental Procedures.

In order to examine the formation of covalent complexes between α, β1, and β2 subunits directly, control and EDC-treated samples were immunoprecipitated with specific anti-β1

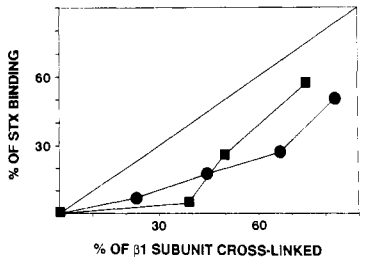

FIGURE 5: Covalent cross-linking of $\beta 1$ subunits is not sufficient for stabilization of STX-binding activity. Purified sodium channel was treated at 0 °C with 0, 3, 9, 27, or 81 mM EDC in the presence of 5 μM STX. After completion of the reaction, the percentage of [^{3}H]STX binding resistant to incubation at 37 °C was determined. Aliquots of the samples were radioactively labeled with Bolton–Hunter reagent and analyzed by SDS–PAGE in 7–20% gradient gels in order to determine the percentage of $\beta 1$ covalently cross-linked to $\alpha\beta 2$ as described under Experimental Procedures. The correlation between the percentage of cross-linked $\beta 1$ and [^{3}H]saxitoxin binding resistant to incubation at 37 °C (●) was plotted in comparison the theoretical line for perfect correlation. A similar experiment was carried out using 0, 5, 20, and 80 mM EDC and incubation in 1 M $MgCl_2$ as a method to dissociate noncovalently associated $\beta 1$ subunits, and the correlation between the percentage of cross-linked $\beta 1$ and [^{3}H]saxitoxin binding resistant to 1 M $MgCl_2$ was plotted (■).

and anti-$\beta 2$ antibodies, and the immunoprecipitated α subunits were radiolabeled by phosphorylation with cAMP-dependent protein kinase and analyzed by SDS–PAGE (Figure 4). In the absence of EDC, anti-$\beta 1$ antibodies immunoprecipitate α subunits under control conditions but not after treatment with 1 M $MgCl_2$ (Figure 4A, lanes 1 and 2). After treatment with 10 mM EDC, anti-$\beta 1$ antibodies immunoprecipitate equivalent amounts of α subunits under control conditions or after treatment with 1 M $MgCl_2$ (Figure 4A, lanes 3 and 4). These results show that EDC efficiently cross-links α and $\beta 1$ subunits at low concentration. Similar experiments with anti-$\beta 2$ antibodies are illustrated in Figure 4B. In the absence of EDC, anti-$\beta 2$ antibodies immunoprecipitate α subunits under control conditions but not after reduction of disulfide bonds with DTT (Figure 4B, lanes 1 and 2). After treatment with 15 mM EDC, equivalent amounts of α subunits are immunoprecipitated with or without reduction of disulfide bonds, indicating the α and $\beta 2$ subunits have been covalently cross-linked (Figure 4A, lanes 3 and 4).

Relationship between Stabilization of Saxitoxin-Binding Activity and Cross-Linking of $\beta 1$ Subunits. Although treatment of solubilized sodium channel with carbodiimide produces both stabilization of STX-binding activity (Figure 1) and cross-linking of the α and $\beta 1$ subunits (Figures 3 and 4), there is not a good quantitative correlation between these two effects. At each concentration of EDC studied, the percentage of cross-linking of $\beta 1$ was much greater than the percentage of stabilization of saxitoxin binding. This discrepancy was most striking at low concentrations of EDC, where up to 40% of the $\beta 1$ subunits are cross-linked to α subunits, but little of the binding activity is stabilized against treatment at 37 °C or in 1 M $MgCl_2$. The poor correlation between cross-linking and stabilization is illustrated clearly in a plot of percent stabilization of saxitoxin binding vs percent cross-linking of $\beta 1$ subunits (Figure 5). All the experimental points fall far from the expected line for correlated events, supporting the conclusion that covalent cross-linking of α and $\beta 1$ subunits is not sufficient to stabilize purified sodium channels against loss of saxitoxin-binding activity during treatment at 37 °C and in 1 M $MgCl_2$.

The correlation between cross-linking of α and $\beta 1$ subunits and stabilization of saxitoxin-binding activity was also examined in sodium channels reconstituted in phosphatidylcholine vesicles. For these experiments, the less hydrophilic carbodiimide CMT was used to allow the reagent better access to reactive groups that might form isopeptide bonds within the hydrophobic regions of the α and $\beta 1$ subunits, and, in some samples, taurine (a zwitterionic amine) was added to reduce the probability of formation of isopeptide bonds at the hydrophilic interface between α and $\beta 1$ subunits, where high ionic strength might act to dissociate the two subunits. In the absence of taurine, incubation with increasing concentrations of CMT produces progressive cross-linking of α and $\beta 1$ subunits and progressive stabilization of saxitoxin binding against treatment with 1 M $MgCl_2$ after solubilization (Table I, samples 1–3). In contrast, in the presence of taurine, CMT does not cross-link α and $\beta 1$ subunits but does provide an equivalent stabilization of saxitoxin-binding activity (Table I, samples 4 and 5). At 30 mM CMT in the presence of 200 mM taurine, 30% of saxitoxin binding remains after treatment with 1 M $MgCl_2$, while there is no detectable cross-linking of α and $\beta 1$ subunits. Thus, these experiments with reconstituted sodium channels also support the conclusion that covalent cross-linking of α and $\beta 1$ subunits is not required for stabilization of purified sodium channels by carbodiimides.

Table I: Stabilization of STX-Binding Activity after Competition of the Cross-Linking of $\beta 1$ Subunit[a]

sample	CMT (mM)	taurine (mM)	% of $\beta 1$ cross-linked	% of STX binding
1	0	0	0	3.0
2	20	0	30	30.0
3	30	0	45	24.7
4	20	200	0	26.1
5	30	200	0	29.9

[a] Purified reconstituted sodium channel was modified with water-soluble carbodiimide (CMT) at the indicated concentration in the presence or absence of taurine. After completion of the reaction, samples were solubilized at 0 °C with 0.2% Triton X-100 by adding the detergent in small aliquots over the course of 1 h. The percentage of $\beta 1$ cross-linked and the [^{3}H]STX binding remaining after treatment with 1 M $MgCl_2$ were determined in aliquots of the samples as described under Experimental Procedures.

Evidence for Intramolecular Cross-Linking of the α Subunit. An alternative mechanism by which carbodiimides might stabilize a sodium channel state having high affinity for binding of saxitoxin is by formation of intrasubunit isopeptide bonds between adjacent carboxyl and amino groups whose interaction is important in maintaining the functional state of the α subunit. Indirect evidence in favor of this mechanism comes from inspection of the α subunit band in SDS–PAGE analyses of sodium channel samples incubated with increasing concentrations of EDC (Figure 3A). As the concentration of EDC is increased, the α subunit exhibits a smaller apparent size consistent with formation of covalent cross-links that prevent complete unfolding of the polypeptide. Although the change in electrophoretic mobility is small, it is important to note that $\beta 1$ and $\beta 2$ subunits are being covalently cross-linked at the same time (Figure 4), which should make the apparent size of α larger.

More direct evidence for formation of intrasubunit isopeptide bonds was derived from analysis of α subunit peptides by two-dimensional peptide mapping (Figure 6). Samples 1–4 from Table I were chosen for comparison since sample 4 should have only intra-α-subunit cross-links in comparison to sample 1 as a control. Analysis of these two preparations by SDS–PAGE shows that the cross-linked sample has an apparent molecular weight of 240 000 compared to the expected value of 260 000 for α subunits (Figure 6A,B). When the tryptic peptide maps of those two samples were compared,

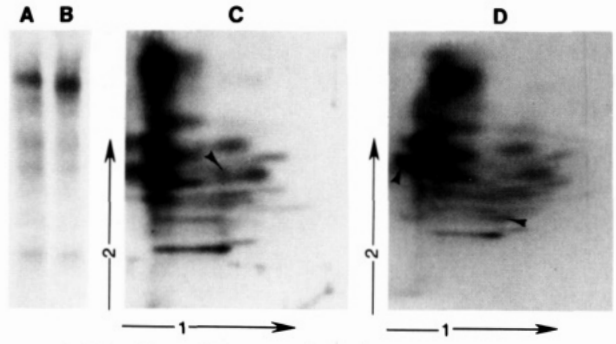

FIGURE 6: Tryptic peptide maps of covalently cross-linked α subunit. (A) Aliquots from the control sample from the experiment described in Table I were radiolabeled by iodination in the presence of chloramine T. The labeled proteins were analyzed by SDS–PAGE and autoradiography. (B) A similar experiment was carried out with sample 4 from the experiment of Table I. (C) The band corresponding to the α subunit in panel A was excised and digested with trypsin. A two-dimensional peptide map was prepared as described under Experimental Procedures, and the radiolabeled peptides were visualized by autoradiography. (D) A similar experiment was carried out with the sample from panel B. The arrows indicate the most significant differences observed in the patterns from three independent experiments. The recovery of radioactivity from the gel slices was approximately of 80% for both control and carbodiimide-treated samples.

a small, but reproducible, number of different peptide fragments corresponding to intramolecular cross-links were found. These are marked by arrows in Figure 6C,D. These results indicate that introduction of a small number of intra-α-subunit isopeptide bonds is sufficient to stabilize the α subunits against loss of functional properties upon incubation at 37 °C or in 1 M $MgCl_2$.

Loss of Saxitoxin-Binding Activity Due to Reaction with Carbodiimides and Nucleophiles. Previous studies have shown that loss of saxitoxin binding to sodium channels is caused by treatment with reagents that modify carboxyl groups including carbodiimides plus nucleophiles (Shrager & Profera, 1973; Baker & Rubinson, 1975) and TMO (Reed & Raftery, 1976; Spalding, 1980; Moore et al., 1982; Gulden & Vogel, 1985; Worley et al., 1986). Sodium channels modified in this way remain functional but are no longer inhibited by saxitoxin or tetrodotoxin and have reduced single-channel conductance (Baker & Rubinson, 1975; Spalding, 1980; Sigworth & Spalding, 1980). These modifications are considered to be specific for carboxyl groups in or near the saxitoxin-binding site since they are prevented in the presence of saxitoxin. The effects of these reagents on saxitoxin binding might result from modification of carboxyl groups within the toxin-binding site or from more widespread effects on sodium channel conformation.

Although reaction with EDC prevents loss of saxitoxin-binding activity during incubation at 37 °C or in 1 M $MgCl_2$, EDC itself destroys the saxitoxin-binding activity of a fraction of sodium channels, and saxitoxin can prevent the loss of saxitoxin-binding activity caused by EDC (Table II, samples 3 and 4, and Figure 1). Greater loss of saxitoxin binding is observed when purified sodium channels are incubated with the more hydrophobic carbodiimides CMT and DCC (Table II, samples 1 and 2), and this loss of binding activity is also prevented in the presence of saxitoxin. Loss of saxitoxin binding due to reaction with carbodiimides is also increased by the presence of amine nucleophiles that can react to form amides with the activated carboxyl species produced by carbodiimides (Table II, samples 4–7). The carboxyl reagent TMO causes loss of saxitoxin binding (Table II, sample 8). Saxitoxin protects against the loss of saxitoxin-binding activity caused by all of these reagents. These results verify for a

Table II: Effect of Carboxylate Reagents on the STX-Binding Activity of the Purified Channel[a]

sample	reagent	reaction time (min)	recovery of saxitoxin binding (%) unprotected[b]	protected[c]
1	25 mM CMT	120	39	108
2	25 mM DCC	120	51	100
3	25 mM EDC	120	87	111
4	75 mM EDC	70	83	100
5	75 mM EDC, 200 mM taurine	70	71	96
6	75 mM EDC, 200 mM GlyMe	70	54	98
7	75 mM EDC, 200 mM ArgMe	70	43	75
8	1 mg/mL TMO	10	50	91

[a] Purified sodium channel (50 nM) was treated at 0 °C with the indicated carboxylate reagents in the absence or presence or saturating concentrations of STX. The excess of reagents and STX (when present) were removed by rapid ion-exchange chromatography. After completion of the reaction, [^{3}H]STX binding was measured in aliquots of the samples and compared with the binding activity of parallel control samples. The percentage of the [^{3}H]STX binding destroyed by the reagents that was protected by STX is also shown. [b] In the absence of saturating concentrations of STX. [c] In the presence of saturating concentrations of STX.

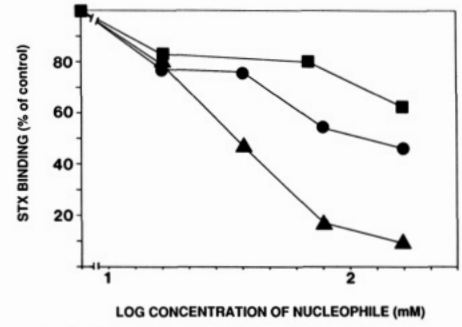

FIGURE 7: Effect of incubation with carbodiimide plus nucleophiles on STX binding. Purified sodium channel (50 nM) was treated at 0 °C for 2 h with 50 mM EDC and the indicated concentrations of taurine (■), GlyMe (●), or ArgMe (▲). Excess reagents were removed by rapid ion-exchange chromatography as described under Experimental Procedures, and the [^{3}H]STX-binding activity was determined in aliquots of the samples.

purified brain sodium channel preparation the previous results with intact and solubilized membrane fractions (Shrager & Profera, 1973; Reed & Raftery, 1976; Moore et al., 1982), showing that modification of carboxyl groups causes loss of saxitoxin-binding activity that is prevented by saxitoxin.

The nature of the nucleophile used in the modification reaction affects the extent of loss of saxitoxin binding (Figure 7). Taurine, which will produce an amide product having a negative charge, is least effective in causing loss of binding, while GlyMe, which yields a neutral amide, and ArgMe, which yields a positively charged amide, are more effective. The action of all three nucleophiles is prevented by saxitoxin (Table II), although the protection is only partial in the case of ArgMe. The different effects of these three reagents seem likely to be due to the nature of the amide derivative formed. Changing the charge of the carboxyl group from negative to positive would be more likely to interfere directly with binding of saxitoxin, a divalent cation, and also would have a greater effect on protein structure.

The differential efficiency of these three nucleophiles in inactivating saxitoxin binding suggested that prior reaction of sensitive carboxyl groups with EDC/taurine might retain their negative charge and protect them from subsequent re-

Table III: Effects of Prior Reaction with Saxitoxin and EDC/Taurine on the Loss of Saxitoxin Binding Caused by EDC/ArgMe and on Reactivity with EDC/[^{3}H]ArgMe[a]

sample	reaction I	reaction II	saxitoxin binding[b] (%)	incorporation[c] (mol/mol)
control	75 mM EDC, 200 mM taurine		69	
1	7 μM STX	75 mM EDC, 200 mM ArgMe[b] or 50 μM [^{3}H]ArgMe[c]	23	2.3
2	75 mM EDC, 200 mM taurine	75 mM EDC, 200 mM ArgMe[b] or 50 μM [^{3}H]ArgMe[c]	0	1.1
3	75 μM EDC, 200 mM taurine, 7 μM STX	75 mM EDC, 200 mM ArgMe[b] or 50 μM [^{3}H]ArgMe[c]	83	7.0

[a] Purified sodium channel was incubated at 0 °C with the indicated reagents (reaction I) in the absence or presence of 7 μM STX. After 2.5 h of incubation, the pH of the samples was increased to 7.4 by careful addition of 1 M KOH, and excess reagents were immediately removed by rapid ion exchange with Sephadex CM-25 columns equilibrated in buffer 1, pH 7.4. Diluted HCl was then added to adjust the pH to 6.0, and samples were again incubated in the absence of STX at 0 °C for 45 min (reaction II). The reaction was terminated as described under Experimental Procedures. [b] Reaction II was carried out in the presence of 200 mM ArgMe, and [^{3}H]saxitoxin binding was determined in aliquots of the samples. [c] Reaction II was carried out in the presence of 50 μM [^{3}H]ArgMe (35 μCi/mL) and incorporation of radioactivity was determined by TCA precipitation as described under Experimental Procedures.

action with EDC/ArgMe. Pretreatment with EDC/taurine in the presence of saxitoxin might therefore prevent loss of saxitoxin binding due to modification of carboxyl groups required for maintenance of a functional conformation of the channel but should leave carboxyl groups at the saxitoxin-binding site available for a subsequent reaction when saxitoxin is removed. To examine this point, sodium channel samples were first incubated with 75 mM EDC plus 200 mM taurine (control), 7 μM saxitoxin (sample 1), 75 mM EDC plus 200 mM taurine (sample 2), or 75 mM EDC, 200 mM taurine, and 7 μM saxitoxin (sample 3, Table III). Following removal of reagents and adjustment of pH, samples 1–3 were then incubated with 75 mM EDC and 200 mM ArgMe in the absence of saxitoxin, and saxitoxin binding was measured (Table III, third column). Reaction with EDC/taurine alone (control) reduced saxitoxin binding to 69% of the untreated control, whereas reaction with EDC/ArgMe (sample 1) reduced it to 23%. Sequential reaction with EDC/taurine followed by EDC/ArgMe (sample 2) caused additive loss of saxitoxin binding rather than preventing the loss of binding due to reaction with EDC/ArgMe. Surprisingly, addition of saxitoxin to the first reaction with EDC/taurine (sample 3) prevented not only the loss of binding in reaction I but also much of the loss of binding due to reaction with EDC/ArgMe in reaction II. On the basis of the results described above, it is likely that the loss of binding is prevented by internal cross-linking of the α subunit by EDC, even in the presence of taurine, to stabilize a conformation with high affinity for saxitoxin.

Reaction of Carboxyl Groups with Radiolabeled Nucleophiles in the Presence of Carbodiimides. The experiment of Figure 2B shows that binding of saxitoxin induces a state of sodium channels with increased affinity for saxitoxin and that the channel can be locked in this state by reaction with EDC. Our analysis of the action of EDC and other carbodiimides on sodium channels provides evidence that their effects on saxitoxin binding and stabilization of an active state of sodium channels derives from their formation of a small number of isopeptide bonds within the α subunit. It is expected, therefore, that the presence of saxitoxin will affect the reactivity of carboxyl groups on the sodium channel in two ways. First, binding of saxitoxin should prevent access of reagents to any carboxyl groups located within the toxin-binding site. Second, in causing a change of channel state to a form with higher affinity for saxitoxin, binding of saxitoxin may alter the reactivity of carboxyl groups located at a distance from the toxin-binding site through allosteric effects. The relative contribution of these two actions to the prevention by saxitoxin of the loss of STX binding caused by carboxyl reagents was examined by measurements of the reactivity of carboxyl groups after prior modification in the presence and absence of saxitoxin (Table III, fourth column). The overall design of the experiment was as for the saxitoxin-binding studies except that reaction II contained 75 mM EDC and a tracer level (50 μM) of [^{3}H]ArgMe to compare the availability of carboxyl groups for reaction with positively charged nucleophiles. Under control conditions (sample 1), 2.3 mol of ArgMe are incorporated. Prior reaction with EDC/taurine (sample 2) does reduce subsequent incorporation of ArgMe to 1.1 mol, even though the results of the third column of Table III show that there is no protection against the loss of saxitoxin binding caused by EDC/ArgMe. Surprisingly, addition of saxitoxin to reaction I (sample 3) under conditions where it prevents the loss of saxitoxin binding (Table III) increases the availability of carboxyl groups for reaction with ArgMe to 7 mol rather than reducing it. These results indicate that the conformational effects of saxitoxin are quantitatively more important than protection of carboxyl groups in the toxin-binding site in determining the availability of carbodiimide-activated carboxyl groups for reaction with nucleophiles. Evidently, binding of saxitoxin to purified sodium channels induces a conformational change in the α subunit that modifies its carboxyl group reactivity, results in higher affinity for saxitoxin binding, and, as shown previously (Messner & Catterall, 1986), stabilizes its association with β1 subunits.

Discussion

Carbodiimides Stabilize a State of the Purified Sodium Channel Having High Affinity for Saxitoxin. In previous work, we have shown that mild treatments of purified sodium channels, including incubation at 37 °C or in 1 M $MgCl_2$, result in concomitant dissociation of β1 subunits, loss of high-affinity saxitoxin binding, and loss of ion-transport activity (Messner & Catterall, 1986; Messner et al., 1986). All these effects were prevented in the presence of saturating concentrations of saxitoxin or tetrodotoxin, demonstrating strong free energy coupling (Weber, 1975) between high-affinity binding of these toxins, maintenance of a functional state of the purified sodium channel, and association of β1 subunits. These results led to the conclusion that the functional state of the purified sodium channel in which the α subunit has high affinity for saxitoxin and tetrodotoxin also contains an associated β1 subunit (Messner & Catterall, 1986; Messner et al., 1986). In this paper, we show that reaction with hydrophilic carbodiimides in the absence of nucleophiles can partially prevent the loss of saxitoxin binding during incubation of sodium channels at 37 °C or in 1 M $MgCl_2$. Reaction with these reagents can therefore either prevent the dissociation of β1 subunits during incubation at 37 °C or in 1 M $MgCl_2$ or can replace the apparent requirement for association of α and β1

subunits for maintenance of a state of the purified sodium channel retaining high affinity for saxitoxin and tetrodotoxin.

Stabilization of Saxitoxin-Binding Activity by Covalent Cross-Linking Correlates with Formation of Intramolecular Isopeptide Bonds in the α Subunit. Chemical modification with carbodiimides has been used frequently in studies of structure–function relationships of different proteins [for example, Lotscher et al. (1984) and Pedemonte and Kaplan (1986)]. These compounds react with carboxylic acid moieties to produce chemically reactive adducts that are subject to attack by nucleophiles to form stable derivatives. In biochemical preparations, the usual nucleophiles are the α-amino groups of the N-terminal amino acid residue and the ε-amino groups of the lysine residues of the protein itself and the amino groups of any low molecular weight compounds that may be present. Thus, in the absence of added nucleophile, carbodiimides are expected to form intramolecular isopeptide bonds between adjacent amino and carboxyl groups in proteins. Since amino and carboxyl groups often interact in salt bridges to stabilize the tertiary and quaternary structures of proteins, covalent cross-linking of these groups can lock native conformations of proteins in place without major perturbation of the overall protein structure and prevent denaturation (Labrude et al., 1979).

We have examined the nature of the intramolecular cross-links in the purified sodium channel that may be responsible for the stabilization of a functional state. Reaction with hydrophilic carbodiimides causes the cross-linking of β1 and β2 subunits to α subunits, as indicated by the disappearance of the β subunit bands from the electrophoresis gels. This cross-linking is likely to be a consequence of the formation of isopeptide bonds between amino and carboxyl groups from the side chains of amino acids in the α and β subunits since cross-linking was competitively inhibited in the presence of soluble amines such as taurine. Definitive evidence for intersubunit cross-linking is provided by immunoprecipitation of phosphorylated α subunits with specific anti-β1 and anti-β2 antibodies. Treatments that dissociate α and β subunits prevent immunoprecipitation of α subunits in control samples but not in carbodiimide-treated samples. These results provide direct evidence for the formation of covalent bonds between α and β subunits. The fact that only hydrophilic carbodiimides produce cross-linking between α and β1 subunits suggests that hydrophilic regions of the interface between these subunits contain salt bridges between the carboxyl and amino groups of the two subunits. The importance of such ionic interactions in the association of the α and β1 subunits after solubilization is also indicated by the sensitivity of this association to treatments such as high ionic strength, high temperature, and low pH that destabilize ionic interactions (Messner & Catterall, 1986).

Although carbodiimides cause covalent cross-linking of α and β1 subunits, our results show that these intersubunit isopeptide bonds are not responsible for the stabilization of the sodium channel caused by carbodiimides. Comparison of the concentration dependence of intersubunit cross-linking and stabilization of saxitoxin binding indicates that covalent cross-linking of α and β1 subunits in solubilized sodium channels is not sufficient to provide stabilization because cross-linking occurs at lower carbodiimide concentrations than stabilization of binding. In addition, in reconstituted sodium channel preparations, we were able to establish conditions in which low molecular weight, hydrophilic nucleophiles like taurine can prevent intersubunit cross-linking by carbodiimides without altering their effect on stabilization of saxitoxin-binding activity, indicating that intersubunit cross-links are not required for stabilization. Considered together, these results provide clear evidence against a primary role for formation of isopeptide bonds between the α and β1 subunits as the mechanism of stabilization of saxitoxin-binding activity.

The second chemical reaction expected of carbodiimides in the absence of nucleophiles is the formation of isopeptide bonds within the individual subunits of the sodium channel. Since formation of isopeptide bonds within the α subunit could be the mechanism of stabilization of the saxitoxin-binding activity, we sought evidence for the presence of such intramolecular cross-links. Both anomalous migration of the α subunit itself and the appearance of new peptides in tryptic peptide maps of the cross-linked α subunits indicated that intramolecular cross-links were formed under the conditions that give stabilization of saxitoxin binding but do not cause intersubunit cross-links. In contrast to the formation of intersubunit cross-links, the concentration dependence of formation of intramolecular cross-links did appear to correlate with the extent of stabilization of saxitoxin binding as judged from the migration of the α subunit band on SDS–PAGE. Considered together, our results support the conclusion that formation of a small number of isopeptide bonds between adjacent carboxyl and amino groups within the α subunit can stabilize a functional state of the purified sodium channel that retains normal affinity for saxitoxin binding under conditions which normally cause loss of function and dissociation of β1 subunits.

Saxitoxin Binding Induces a High-Affinity State That Can Be Stabilized by Covalent Cross-Linking. Saxitoxin and tetrodotoxin have generally been considered to inhibit sodium channels by binding to a site in or near the extracellular end of the transmembrane pore and blocking the access of sodium ions to the pore (Hille, 1975; Kao & Nishiyama, 1965; Spalding, 1980). Recent studies have expanded this mechanism of toxin action to include modulation of toxin binding dependent upon the functional state of the sodium channel. When membrane-bound (Krueger et al., 1983; Green et al., 1987) or purified (Hartshorne et al., 1985) sodium channels are persistently activated by batrachotoxin and incorporated into planar lipid bilayers, inhibition of the channels by tetrodotoxin, saxitoxin, and congeners is voltage-dependent. This voltage dependence arises from changes of state in the channel itself since it does not depend on the charge of the toxin (Moczydlowski et al., 1985; Green et al., 1987). Similarly, saxitoxin and tetrodotoxin reduce the affinity of the sodium channel for batrachotoxin under some conditions, suggesting that the toxins alter the conformation and binding properties of the batrachotoxin receptor site, which is located in a different region of the sodium channel structure (Brown, 1986).

Our results provide further direct evidence for a toxin-induced conformational change associated with binding of saxitoxin. When saxitoxin is bound, formation of isopeptide bonds within the α subunit by EDC stabilizes a fraction of the sodium channels in a state with an affinity for saxitoxin that is 3–4-fold higher than that of the untreated channels. Evidently, saxitoxin induces a high-affinity conformation by its binding, and EDC can lock the channel in this high-affinity state. This covalently cross-linked state is characterized by resistance to treatment at 37 °C and in 1 M $MgCl_2$ as described above. Moreover, the availability of carboxyl groups for reaction with nucleophiles having different charge properties is altered, providing direct chemical evidence for an altered channel conformation. These results argue for an allosteric model of saxitoxin and tetrodotoxin binding in which the interaction of these two toxins with their receptor site not

only blocks the transmembrane pore but also induces structural changes in the α subunit that are voltage and state dependent and affects the chemical reactivity of carboxyl groups at a distance from the toxin-binding site. It will be important to determine whether this toxin-induced conformational change may also play a role in impeding the passage of ions through the activated sodium channel.

Registry No. [^{3}H]ArgMe, 112987-89-0; [^{3}H]Arg·HCl, 112987-87-8; [^{3}H]Arg·2HCl, 112987-88-9; saxitoxin, 35523-89-8.

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