Functional Unit Size of the Neurotoxin Receptors on the Voltage-dependent Sodium Channel*

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Radiation inactivation was used in situ to determine the functional unit sizes of the neurotoxin receptors of the voltage-dependent sodium channel from rat brain. Frozen or lyophilized synaptosomes were irradiated with high energy electrons generated by a linear accelerator and assayed for [3H]saxitoxin, [3H]-Leiurus quinquestriatus quinquestriatus (α-scorpion toxin), [3H]-Centruroides suffusus suffusus (β-scorpion toxin), and batrachotoxinin-A 20a-[3H]benzoate binding activity. The functional unit size of the neurotoxin receptors determined in situ by target analysis are 220,000 for saxitoxin, 263,000 for α-scorpion toxin, and 45,000 for β-scorpion toxin. Analysis of the inactivation curve for batrachotoxinin-A 20α-benzoate binding to the channel yields two target sizes of Mr = 287,000 (50%) and ~51,000 (50%). The results are independent of the purity of the membrane preparation. Comparison of the radiation inactivation data with the protein composition of the rat brain sodium channel indicates that there are at least two functional components.

The molecular events which underlie the action potential in nerve and muscle are correlated with sequential time- and voltage-dependent openings and closings of a transmembrane protein, the sodium channel, which mediates the flow of sodium ions across the membrane.

A number of naturally occurring neurotoxins are able to modulate the action potential specifically by exerting their effects on this channel. At least four distinct receptors and actions have been identified for these toxins on Na⁺ channel function (for a review see Ref. 1). These toxins include 1) TTX, 5TX, heterocyclic guanidinium compounds which block sodium movement through the channel when applied to the extracellular side, 2) veratridine and batrachotoxin which cause persistent activation of the channel and result in a permanently depolarized cell membrane, 3) the α-scorpion toxins such as toxin V from Leiurus quinquestriatus quinquestriatus which prolong the action potential and bind to the channel in a voltage-dependent manner, and 4) the β-scorpion toxins such as toxin II from Centruroides suffusus suffusus which bind to a voltage-independent site and induce repetitive firing by altering the kinetics of channel opening (2). Binding and action of the α-scorpion toxins is enhanced in the presence of veratridine or BTX, indicating allosteric coupling between these two receptor sites on the channel (3).

These neurotoxins have been indispensable in elucidating mechanisms of ion selection and gating as well as providing tools to probe the molecular structure and purification of the channel.

Sodium channels from rat brain, muscle sarcolemma, and eel electroplax have been characterized, through their ability in a solubilized form to bind TTX, as membrane glycoproteins with an assembled weight of ~300,000 (4–8). Subunit studies using a purified preparation from rat brain and photolabeling with a Lqq derivative reveal that the channel is composed of at least three subunits: α of Mr ~270,000, and β1 and β2 of Mr 39,000 and 37,000, respectively. The β1 subunit constitutes a portion of the Lqq receptor site, and the β2 subunit is apparently linked to α by disulfide bonds (9). The Na⁺ channel from eel electroplax, on the other hand, appears to be composed of a single chain of apparent Mr ~270,000 with carbohydrate representing 30% of this mass. Thus far the molecular composition of the sarcolemmal Na⁺ channel appears different from both the rat brain synaptosomal or eel electroplax Na⁺ channels, since the STX-binding component is reported to consist of four polypeptides of Mr 150,000–200,000, 45,000, 38,000, and 37,000 (6). Other neurotoxin sites and functions for the sarcolemmal channel have not yet been described.

The molecular arrangement of these important receptor sites and functions on the rat brain sodium channel has recently been provided by fluorescence resonance energy transfer measurements using a series of biologically active and high affinity fluorescent neurotoxin derivatives (10–14). These studies have revealed the spatial relationships between the important neurotoxin receptors on the channel in situ and, together with the available biochemical information, have led to a three-dimensional working model of the Na⁺ channel in synaptosomes (15).

Further insight into the structural features of the in situ Na⁺ channel can be gained by irradiating the native channel in its membrane environment with high energy electrons and determining the functional unit target size of the membrane channel STX, α- and β-scorpion toxin, and BTX receptors using target size analysis (16). This technique has been successfully employed for polyenzymes (17) and for a number of membrane protein systems which include the glucagon (18), insulin (19, 20), IgE (21), opiate (22), and eel electroplax TTX receptor (23). There are a number of distinct advantages of
Functional Unit Size of Neurotoxin Receptors

EXPERIMENTAL PROCEDURES

Materials

Chemicals were obtained from the following sources: tetrodotoxin (citrate-free) from Calbiochem-Behring; *L. quinquestriatus* quinquestriatus venom, acetylethicholine, 5,5'-dithio-bis(2-nitrobenzoic) acid, horse liver alcohol dehydrogenase, and lactoperoxidase from Sigma: veratridine from K&K Laboratories; and mercycyanine 540 from Eastman. [H]Saxitoxin was provided by the Toxicology Study Section, National Institutes of Health, and had a specific activity of 1.57 Ci/mmol. TTX V from the venom of *L. quinquestriatus* quinquestriatus was purified and characterized as described previously (11). Toxin II from C. sulfusis sulfusus and anti-rabbit anti-Lqq V and anti-Cas II were generous gifts from the Laboratory of Professor H. Rochat, Université d'Aix-Marseille. Batrachotoxinin-A 20α-[H]benzoate was very generously provided by Dr. Georgio Bove, New Jersey Medical School, University of Alabama in Birmingham School of Medicine, and had specific radioactivity of 12 Ci/mmol and radiochemical purity of 90%. Lqq V and Cas II were radiolabeled by [3H]labeled toxins coupled by iodoination with H2O2 as the initiator, and repurified by immunoprecipitation or ion-exchange chromatography as described (11, 14). The five preparations of radiolabeled toxins used in these studies had specific radioactivities between 613 and 883 Ci/mmol for Lqq and 896 and 1833 Ci/mmol for Cas II. All other reagents were the best grade commercially available, and all solutions were made with distilled-deionized water.

Methods

Preparation of Synaptosomes—Synaptosomes were prepared from rat brain by a combination of differential and density gradient centrifugation as described by Catterall (25). The membrane fraction at the 1.0 to 1.2 M sucrose interface (“1.0 M” membranes) was suspended at a concentration of 7 mg/ml in standard binding medium (130 mM choline chloride, 50 mM Hepes adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM MgSO4, and 5.4 mM KCl) and rapidly frozen and stored at -70 °C. The 1.0 M fraction was used for most of the experiments because it has the highest STX binding site capacity and is able to maintain a membrane potential. PAS synaptosomal fraction was prepared (53), frozen immediately, and stored at -70 °C. Protein content was determined by the method of Peterson (26) with bovine serum albumin as a standard. Freezing at -70 °C and thawing the synaptosomes alone had relatively small effects (<5%) on the Kd or Bmax, as shown below. For some experiments 0.5 ml of bovine serum albumin (5-4 mg of protein) was each placed in 2-ml Kimble ampoules and lyophilized overnight at <0.06 torr and -60 °C. The ampoules containing frozen or lyophilized membranes were sealed and kept at -70 °C before and after irradiation. For neurotoxin binding studies samples were rehydrated to 0.5 ml to a final concentration of 130 mM choline chloride, 50 mM Hepes, 5.5 mM glucose, 0.8 mM MgSO4, and 5.4 mM KCl, pH 7.4, for 30 min at 4 °C.

Equilibrium Binding Measurements—The unexposed controls consisted of two groups; one remained at the University of Florida and one was transported to Bethesda. Both were treated in an identical manner to those ampoules which were also irradiated. All assays of neurotoxin receptors were measured by a rapid filtration assay (3, 11, 14, 27) after irradiation of the membranes. The STX receptor was assayed by adding membranes (200-500 μg) suspended in 100 μl of standard binding medium to an incubation mixture of 18 mM [3H]STX in a final volume of 500 μl. The samples were mixed and incubated for 60-90 min at room temperature, 10 μl aliquots were removed for determination of total ligand, and the remaining samples were rapidly diluted with 3.0 ml of ice-cold wash buffer consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM CaCl2, and 0.8 mM MgSO4, and immediately filtered onto Whatman GF/B and washed three times with ice-cold standard binding medium containing 1 mg/ml of bovine serum albumin. The filters and 10-μl aliquots were placed in test tubes and the radioactivity was determined by γ-counting at an efficiency of 75%. Non-specific binding measured in the presence of 200-400 nM unlabeled Lqq V was subtracted from the total. All data are presented as a fraction of the free [3H]Lqq V toxin concentration (bound/free).

Binding of [3H]labeled Cas II to synaptosomal membranes was measured as for Lqq V. The samples containing 10-33 nM [3H]Cas II were incubated for 30 min at 25 °C, and the membranes were collected by filtration onto Whatman GF/B filters in ice-cold standard binding medium containing 1 mg/ml of bovine serum albumin. Nonspecific binding of iodinated toxin was measured in the presence of a saturating concentration of Lqq V (1 μM) and was subtracted from the total.

Batrachotoxinin-A 20α-[H]benzoate binding to synaptosomes was measured routinely by a rapid filtration procedure similar to that for STX and scorpion toxin binding. Membranes (1 mg of protein) were added to assay tubes containing 8.9 nM [3H]BTX-B. 50 μg of crude *L. quinquestriatus* venom or in the presence of 1 μM Lqq V and 1 μM TTX in a final volume of 335 μl of standard binding medium. The samples were incubated for 60-90 min at room temperature, 10 μl aliquots were removed for determination of total ligand, and the remaining samples were rapidly diluted with 5.0 ml of ice-cold wash buffer consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM CaCl2, and 0.8 mM MgSO4, and immediately filtered onto Whatman GF/B filters under vacuum and washed twice with ice-cold binding medium. Counting for tritium was performed as described above for [3H]STX. Non-specific binding was determined in parallel assay tubes containing 235 μM veratridine and subtracted from the total data, which are presented as bound/free.

Equilibrium binding of the neurotoxins to synaptosomes was measured at selected doses which were also measured as a function of the concentration ratio of specifically bound to free ligand. The dissociation constant of the neurotoxin ligand is described by

\[
K_d = \frac{B_{max}}{1} \frac{L}{1}
\]

*rate of inactivation,* which is related to the mass associated with the functional unit size of the receptor complex and to the evaluation binding capacity of the membrane receptors. The nonspecific binding component was measured as described above and subtracted from the total. Specific binding was plotted in the form of a Scatchard analysis: 

\[
(B/F)/(E/F) = -(1/K_d)B + B_{max}/K_d
\]

where B and F refer to the specifically and bound free ligand, respectively, Kd is the dissociation constant, and Bmax is the maximal capacity of the synaptosomal membrane receptors for this ligand.

Radiation Inactivation—Synaptosomal membranes were irradiated at the Armed Forces Radiobiology Research Institute (Bethesda, MD) employing a linear accelerator generating electrons with an energy of 13 MeV. The beam was dispersed with a water scatterer to give a dose rate of 0.5 Mrad/min uniformly over a 225 cm2 exposure area (less than 10% variation) at 90 cm from the beam port. Dosimetry was accomplished with thermoluminescent dosimeters and radiographic dye films. The temperature was controlled as previously described (26).

The reduction in capacity of the synaptosomes to bind a given neurotoxin ligand is described by 

\[
(B/F)/(B/F_0) = e^{-kD}
\]

where (B/F), the ratio of specifically bound to free ligand at a given dose, is normalized to the bound/free ratio of unexposed controls; D is the dose of absorbed radiation in rads, and k is a constant reflecting the rate of inactivation, which is related to the mass associated with the measured function. The decay curve—ln(B/F)/(B/F_0) versus D—is a linear function. The slope, k, was determined by linear regression analysis constrained to pass through 1.0 at zero dose which primarily weights the data at high doses. The radiation dose which reduces the ligand binding to 1/e of the control value is defined as D50, k = (D50)^-1. The molecular weight of the functional binding unit is calculated
binding capacity and protein concentration.

From the irradiation ampoules, resuspended, and placed in test tubes, after which appropriate aliquots were removed to determine the binding capacity and protein concentration.

In this study, the functional unit/molecular weight determinations for the Na+ channel receptor sites are the results of 12 separate irradiations utilizing several membrane preparations (frozen, 1.6 M and P2; lyophilized, 1.0 M and P2). All four neurotoxin receptors were assayed on the same membrane preparation. For each experiment a constrained least squares line was obtained, and the target size was calculated. The errors shown in the figures are due to the variability between different irradiations and do not refer to the variability of the assay within one experiment. The errors quoted in the table refer to the variability between different irradiations and do not refer to the scatter of experimental points about the calculated lines in each experiment.

Radiation Inactivation of Known Enzymes—A number of internal protein standards of known composition and structure were used (5'-nucleotidase, acetylcholinesterase, and horse liver alcohol dehydrogenase). Target sizes for these were determined both to validate the temperature factor equal to 2.8 for irradiations performed at -135 °C (28). Accordingly, the correction factor of 2.8 was used in all subsequent radiation analyses for the neurotoxin receptors of the Na+ channel. For example, the calculated target sizes from the inactivation curves of a few of the enzymes assayed yielded 89,600 for 5'-nucleotidase, 70,000 for acetylcholinesterase, and 81,500 for horse liver alcohol dehydrogenase. These are in excellent agreement with the radiation inactivation results of others (16, 33) and correspond to the known biochemical composition of these proteins.

RESULTS

Analysis of the equilibrium binding isotherms for STX, Lqq, Cas II, and BTX on rat brain synaptosomes have been previously shown to be linear and indicate the presence of a homogeneous class of high affinity receptors (3, 27, 34). This homogeneity in binding site class for each ligand permits the use of the maximum binding site capacity on the synaptosomal membranes for receptor site target analysis.

In order to characterize the functional unit of the respective neurotoxin receptors on the sodium channel, synaptosomal membranes were irradiated with high energy electrons. The method determines the sensitivity of a given receptor function (in this case equilibrium binding) to high energy radiation by measuring the number of functional units remaining after increasing radiation doses. The method is predicated on the

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**Table I**

<table>
<thead>
<tr>
<th>Receptor site</th>
<th>Membrane preparation</th>
<th>$R_{max}$ (Mrad)</th>
<th>$K_d$ (Mrad)</th>
<th>Target size</th>
<th>Target size</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td>nM</td>
<td>kDa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STX</td>
<td>1.0 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3.7 (0)</td>
<td>1.3 (0)</td>
<td>220 ± 9</td>
<td>270,000 (a)</td>
<td>39,000 (b)</td>
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<tr>
<td></td>
<td></td>
<td>1.8 (6)</td>
<td>1.5 (6)</td>
<td></td>
<td>37,000 (b2)</td>
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<td>2.5 (0)</td>
<td>1.5 (0)</td>
<td>224 ± 6</td>
<td>316,000(b)</td>
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<td>Lyophilized 1.0 M</td>
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<td>2.6 (0)</td>
<td>266 ± 14</td>
<td>229(f)</td>
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<td></td>
<td>Lyophilized P2</td>
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<td>2.5 (0)</td>
<td>244 ± 11</td>
<td>266(b)</td>
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<td></td>
<td>0.075 (9)</td>
<td>2.2 (9)</td>
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<td>Lqq V (o-scorpion toxin)</td>
<td>1.0 M</td>
<td>1.4 (0)</td>
<td>2.4 (0)</td>
<td>263 ± 6</td>
<td>ND(a)</td>
<td>256,000 (a)</td>
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<td></td>
<td>0.55 (6)</td>
<td>2.5 (6)</td>
<td></td>
<td>39,000 (b)</td>
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<td>2.1 (0)</td>
<td>255 ± 9</td>
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<td>0.06 (0)</td>
<td>ND (0)</td>
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<td>0.01 (18)</td>
<td>ND (18)</td>
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<tr>
<td>Cas II (β-scorpion toxin)</td>
<td>1.0 M</td>
<td>2.4 (0)</td>
<td>3.3 (0)</td>
<td>43 ± 2</td>
<td>253,600 (a)</td>
<td>35,000 (b)</td>
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<td></td>
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<td>0.48 (60)</td>
<td>3.5 (60)</td>
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<td>P2</td>
<td>1.3 (0)</td>
<td>3.8 (0)</td>
<td>47 ± 3</td>
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<td></td>
<td>Lyophilized P2</td>
<td>0.33 (60)</td>
<td>3.6 (60)</td>
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<td>0.17 (0)</td>
<td>3.6 (0)</td>
<td>198 ± 25</td>
<td>266(b)</td>
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<tr>
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<td>0.024 (9)</td>
<td>4.2 (9)</td>
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<tr>
<td>BTX</td>
<td>1.0 M</td>
<td>2.3 (9)</td>
<td>86 (0)</td>
<td>287 ± 12 (50%); 51 ± 5 (50%)</td>
<td>ND</td>
<td></td>
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<tr>
<td></td>
<td>Lyophilized P2</td>
<td>0.65 (18)</td>
<td>108 (18)</td>
<td>472</td>
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</tr>
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</table>

* Determined in this study; STX and BTX, n = 5; Lqq V and Cas II, n = 11.

**Summary of results**

Table I

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from

$$M_r = 6.4 \times 10^4 S_H$$

where $S$ is a temperature factor equal to 2.8 for irradiations performed at -135 °C (28).

After irradiation, the frozen vials were opened and purged with dry nitrogen gas. The samples were then gradually thawed to ensure that the temperature remained lower than 5 °C. The samples were removed from the irradiation ampoules, resuspended, and placed in test tubes, after which appropriate aliquots were removed to determine the binding capacity and protein concentration.

In this study, the functional unit/molecular weight determinations for the Na+ channel receptor sites are the results of 12 separate irradiations utilizing several membrane preparations (frozen, 1.0 M and P2; lyophilized, 1.0 M and P2). All four neurotoxin receptors were assayed on the same membrane preparation. For each experiment a constrained least squares line was obtained, and the target size was calculated. The errors shown in the figures are due to the variability between different irradiations and do not refer to the variability of the assay within one experiment. The errors quoted in the table refer to the variability between different irradiations and do not refer to the scatter of experimental points about the calculated lines in each experiment.

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fact that each primary ionization in the macromolecular site deposits sufficient energy to completely destroy the activity of the functional binding unit.

**Target Size of the Saxitoxin Receptor**—In unirradiated frozen samples of purified 1.0 M membranes the maximal binding capacity for [3H]STX was 3.3-3.7 pmol/mg of protein and in the P2 preparation was 2.5-3.3 pmol/mg of protein. When 1.0 M and P2 synaptosomes were lyophilized and assayed for [3H]STX binding after rehydration of the membranes, losses in [3H]STX binding ranging from 70-90% were observed. \( B_{\text{max}} \) values for [3H]STX binding to control lyophilized 1.0 M and P2 membranes were 0.58-0.77 pmol/mg of protein and 0.34-0.56 pmol/mg of protein, respectively, independent of the method of lyophilization (Table I).

Fig. 1 shows a representative experiment of the specific binding of [3H]STX to synaptosomes (1.0 M fraction) after irradiation of both frozen and lyophilized membranes. Since irradiation had no effect on the values of the dissociation constant (Fig. 1, inset, and Table I), the observed loss of [3H]STX binding results from a decrease in the number of functional binding units.

**Fig. 1.** Representative radiation inactivation experiment of the specific binding of [3H]STX to irradiated synaptosomes. 1.0 M synaptosomes were prepared frozen (○) or lyophilized (▲) and irradiated at the indicated doses at -135 °C. The channel receptor specific for STX was assayed by the specific binding of 18 nM [3H]STX. Nonspecific binding was measured in the presence of 2 \( \mu \)M TTX and was subtracted from the total. The ratio of specific to nonspecific binding was measured in the presence of 200 nM Lqq V and subtracted from the results. The ratio of specific to nonspecific binding for Lqq V was 3.3 at 0 Mrad and 0.54 at 12 Mrad. The loss in specific binding is represented as the ratio \( (B/F)/(B/F)_0 \) of unirradiated controls. The error bar is standard deviation in quadruplicate assays. The line is the best fit to the data from frozen samples and yields a \( D_{\text{RQ}} \) of 6.8 Mrad and a \( M_r \) = 264,000. Scatchard analysis of the specific binding of [3H]STX to synaptosomes irradiated at 0 and 6 Mrad is shown in the inset. Protein concentrations for the equilibrium binding experiments were 0.38 mg of protein/ml at 0 Mrad and 0.28 mg of protein/ml at 6 Mrad. The loss of binding activity in the frozen 1.0 M preparations. Slightly greater rates of inactivation were observed in lyophilized samples. Radiation-induced losses of binding activity in the P2 fraction mirrored those of the 1.0 M fraction. The decrease in activity was independent of the purity of the preparation as predicted by the principles of target analysis.

The target sizes calculated from the inactivation of the various preparations are given in Table I. The values are the averages from combined analysis of five independent experiments. Lyophilized samples consistently displayed target sizes just larger than those from frozen samples.

**Target Analysis of the \( \alpha \)- and \( \beta \)-Scorpion Toxin Receptors on the Na+ Channel**—The binding of Lqq V and Cas II was comparable in either frozen 1.0 M or P2 membrane fractions (Table I). \( B_{\text{max}} \) values of 1.2-1.4 pmol/mg of protein were found for Lqq V and 1.9-2.4 pmol/mg of protein for Cas II. However, lyophilized and rehydrated samples retained only...
small amounts of activity: 125I-Lqq V binding was only 7% of that measured in membranes which had been frozen at -70°C.

Irradiation of the synaptosomes had no significant effect on the value of the dissociation constants (Table I) or the specificities of the sites for either Lqq V or Css II binding (e.g. α-scorpion toxin now having an affinity for β-scorpion toxin receptors), but the values of $B_{max}$ at these irradiations demonstrate that, indeed, irradiation resulted in loss of functional binding sites for this receptor. The difference cannot be attributed to the membranes prepared at each dose  showed no significant changes as determined by the fluorescence dye method (36). The following membrane potentials at selected doses were calculated: 0 M rad, -58 mV; 3 Mrad, -55 mV; 9 Mrad, -57 mV; 12 Mrad, -51 mV; 18 Mrad, -56 mV; 36 Mrad, -48 mV; 60 Mrad, -50 mV; 84 Mrad, -63 mV. Under our assay conditions the membrane potential is primarily created by the distribution of the impermeable cation choline and not by the Na⁺/K⁺-ATPase. Thus, even though the Na⁺/K⁺-ATPase might be destroyed by high doses of radiation, the membrane potential would not be altered significantly by loss of this enzyme system. Therefore, the loss in Lqq V binding capacity of the irradiated synaptosomes cannot be due to alteration in membrane potential. Secondly, the Lqq V binding to the membranes was measured at concentrations of Lqq V which were 10-20-fold in excess of the $K_a$. Under these conditions less than 5% of the iodinated scorpion toxin was bound even in unirradiated controls.

Since the apparent loss of binding capacity measured at a single ligand concentration could reflect an alteration in the affinity of the receptor, experiments were carried out in which a set of irradiated samples and control were incubated at two concentrations of labeled Lqq V (2.8 nM: about the $K_a$ and 40 nM: 20 x the $K_a$) which differed from our standard concentration of 10 nM (Fig. 2). Both concentrations gave similar inactivation curves, indicating that under those equilibrium...
binding conditions the loss of binding was not dependent on the levels of Lqq V. All experiments show that synaptosomes irradiated at 0 or 6 Mrad bind Lqq V with approximately the same apparent affinity ($K_a = 2.6 \text{ nM}$), indicating that the observed loss in binding reflects only loss in the number of binding sites (inset, Fig. 2).

Thus, because the total labeled Lqq V concentration is well above the $K_a$ in the irradiation experiments, it can be concluded that radiation produces no change in the affinity of the toxin for the receptor, that no alteration in membrane potential occurs as a result of the membrane preparation or irradiation, and that the observed loss in binding capacity reflects only loss in number of sites due to radiation damage.

Because of the small amount of $^{125}$I-Lqq V binding which survived lyophilization, there was a great deal of scatter in the data in radiation inactivation studies (Fig. 2, △). This variability precluded the determination of a target size.

The loss of Css II receptor sites on the Na' channel irradiated in the frozen state yielded a target size of 45,000. Evaluation of the dissociation constant by Scatchard analysis at 0 and 60 Mrad shows a single class of binding sites with similar values of the dissociation constant (Fig. 3, inset). These plots indicate that irradiation of the synaptosomes reduces the number of binding sites without changing the dissociation constant of the toxin-receptor complex.

Titrations of synaptosomes irradiated with doses of 0.8 to 15 Mrad of synaptosomes were kept frozen. Analysis of the inactivation curve for the Css II receptor yields a target size of 190,000. A portion of the samples was then stored overnight at 4°C (9% of the binding activity compared to frozen membranes) or frozen for 1 week at −20°C (13% of the binding activity compared to frozen membranes). When these samples were reassayed, the target size yield apparent $M_s = 250,000$ and 260,000. However, with frozen membranes treated in an identical fashion, 95% of the activity is retained compared to freshly isolated membranes. Moreover, refreezing and rethawing does not change the target size when frozen membranes were used.

These results substantiate the results obtained by titration with the α-scorpion toxins: 1) that lyophilization of the synaptosomes itself induced irreversible damage to the Na' channel structure or membrane environment such that only about 10% residual binding activity could be detected even in the unirradiated rehydrated samples, although the $K_a$ values for the Css II receptor were not appreciably altered, and 2) the target size was not dependent upon the purity of the membrane preparation since the $D_{37}$ values for the Css II site with frozen $P_2$ fraction and purified synaptosomes irradiated at −135°C yielded nearly identical values and corresponding target sizes.

In all the experiments presented above the concentration of $^{125}$I-Css II was between 5 and 10 times the dissociation constant (10−30 nM) to ensure receptor site saturation at all radiation doses. In some experiments the concentration of labeled toxin was varied between 2 and 5 nM with no change in the irradiation curve. At high concentrations the free ligand was equal to the total ligand in the incubation mixture, and the bound ligand was less than 5% of the concentration of ligand added to the assay tube. Therefore, in all cases the available ligand in the assay was clearly saturating and the apparent decrease in receptor site number with increasing dose reflects the destruction of this neurotoxin receptor on the Na' channel by ionizing radiation. Separate plots of total binding and nonspecific binding as a function of dose at high ligand concentrations (data not shown) indicated that total binding decreases while the nonspecific component remains relatively constant throughout the radiation range employed (0−120 Mrad).

**Target Size Analysis of the Batrachotoxin Receptor**—Fig. 4 shows the inactivation profile for specific BTX-B binding to the receptor on 1.0 M synaptosomes in the presence of α-scorpion toxin added to the assay medium after irradiation. The $D_{37}$ of a single exponential fit by linear regression analysis constrained to pass through 1.0 at zero dose is 16.5 Mrad corresponding to a $M_s = 110,000$. However, it can be seen that at low doses there is a deviation in the inactivation profile which represents approximately 50% of the total receptor sites. Fitting the inactivation curve with a nonlinear least squares routine for a two-exponential decay yields two target sizes. The $D_{37}$ for the second component is 40 Mrad and the target size is 30,000.
enhanced BTX binding. Therefore, the complexity is not a result of interaction of multiple components in the venom.

In some experiments a set of irradiated samples was incubated at two concentrations of labeled BTX-B (4.5 and 6.8 nM) which differed from our standard concentration of 8.9 nM. This was done to examine the effect of subsaturating concentrations of BTX on the inactivation profile. Both concentrations gave similar inactivation curves, indicating that the radiation produced a loss of binding that was not dependent on the level of [H]BTX-B used. Nevertheless, the data are plotted in a form which accounts for the bound/free ratio, i.e. B/F of the irradiated samples compared to that B/F ratio for the control unirradiated samples.

The above experiments were carried out with synaptosome samples which were frozen and maintained at -70°C and irradiated at -135°C. When lyophilized and rehydrated membrane samples were used, little specific binding of [3H]BTX-B was detected (20% of that of frozen membranes). Thus, substantial losses occur in the Na+ channel BTX receptor either through direct alteration or through alteration of the Lqq V receptor necessary for the preservation of high affinity specific BTX-B binding. When lyophilized samples were irradiated at 30°C (data not shown) or -135°C (Fig. 4) analysis of the BTX-binding data yields a target size of approximately 472,000.

The radiation inactivation data for all four neurotoxin receptor sites on the Na+ channel are summarized in Table I. The target sizes presented for the STX and the BTX receptors are the means of five separate irradiations and the target sizes for the α-scorpion and β-scorpion toxin receptors are the means of 11 separate determinations. Each sample in each irradiation was measured in three to eight replicate assays.

DISCUSSION

Biochemical studies on the structure of the voltage-dependent Na+ channel from rat brain indicate that it is a large macromolecular structure composed of at least three major subunits (4, 5, 9, 37). Identification of these components by solubilization (4) and photolabeling (5, 38) with site-specific neurotoxins has revealed that the receptor sites on the channel are presented by a combination of these subunits often forming overlapping receptor binding sites.

Only recently has structural information appeared on the arrangement of these receptors and subunits in the synaptosomal membrane. A model has been proposed in which the TTX receptors reside on 250,000-dalton tubular α subunits of the channel that span the membrane bilayer (15). The β1 subunit contains both the Lqq V and Css II neurotoxin receptors and was viewed as having a regulatory role, undergoing conformational transitions as a result of changes in the transmembrane voltage. The α and β1 components appear juxtaposed since photolabeling with either α- or β-scorpion toxins labels both the α and β1 polypeptides (5, 38).

Fluorescence resonance energy measurements on the in situ synaptosomal Na+ channel have served to place these receptors and components in a three-dimensional framework. A portion of the β1 polypeptide that contains the Lqq V and Css II receptors is embedded 15 Å into the membrane bilayer, adjacent to the α subunit, and 35 Å from the externally placed TTX receptors (15). Addition of BTX alters the TTX-Lqq V distance by 7 Å and was interpreted as reflecting a change in the interaction between the α and β1 components. The α- and β-scorpion toxin receptors are 22 Å apart and are able to communicate conformationally (14). Furthermore, the α-scorpion toxin and BTX receptors, which are allosterically coupled, are 37 Å apart. This indicates that the binding of these ligands must involve conformational changes that extend over large distances from the binding loci via the matrix of each polypeptide and/or through alterations in the subunit/subunit contacts (12). From the analysis of the spectra of a fluorescent BTX derivative, the BTX receptor is located in a hydrophobic environment that becomes more hydrophilic with α-scorpion toxin binding (13).

Radiation target size analysis provides a complementary method for obtaining structural information and for defining structural dynamics of the Na+ channel in intact membranes. The method has been applied to the insulin receptor (19, 20), benzodiazepine receptor (39), the nicotinic acetylcholine receptor (33), hormone-activated adenylate cyclase (18), and the n-mydriplasia and diltiazem receptors of the Ca++ channel (40). This method potentially yields the molecular weight of the receptor in situ. Most importantly, however, the procedure provides information on the target size of the functional unit as revealed by the use of the specific assay for individual functions. Unless the various ligands assay for the final product or disclose the rat-limiting-step (as would be the case of an enzymatic analysis) the target size or radiation sensitivity will correspond only to the partial function which is being assayed. Together with other biochemical and structural information these data on the functional unit size of each channel component become very important.

Table I summarizes the results of radiation inactivation studies of the Na+ channel receptors and compares the available biochemical data with radiation inactivation data for the size of the functional binding unit. The standard deviations given in Table I refer to the variability between different irradiations, and not within each experiment. The number of separate determinations is also listed in the table.

The radiation sensitivity of the STX/TTX receptor appears to parallel the molecular weight of a single α subunit of the channel identified in the purified channel preparation and seemingly does not require the smaller β2 subunit for binding. Although energy transfer measurements indicate that in synaptosomes two or more TTX sites may lie within 70 Å of each other, radiation inactivation and equilibrium binding indicate that these components function quite independently of one another. The target size of this polypeptide appears somewhat smaller than that estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (270,000) for two reasons. 1) The target size refers only to the polypeptide portion of the chain. The STX/TTX receptor is a glycoprotein in which carbohydrate represents 30% of the mass (41). Radiation inactivation measurements of invertase, which contains 50% carbohydrate, showed that oligosaccharides did not contribute to the target size (42). The same observation has been reported for the Na+/K+-ATPase (43) and the receptors for IgE (21) and low density lipoprotein (44). 2) The molecular weight derived by gel electrophoresis under denaturing conditions is only an apparent molecular weight since it is known that the α subunit binds unusual amounts of sodium dodecyl sulfate, resulting in anomalous migratory behavior on sodium dodecyl sulfate-polyacrylamide gels (6, 37, 41).

The radiation sensitivity of the α- and β-scorpion toxin receptors on the channel indicate very different functional binding unit sizes. The loss in binding of Lqq V with increasing dose appears to follow the target size of the α subunit and the STX receptor, rather than the smaller 38,000 β1 compo-
The proper conformation in from the voltage-dependent LqV binding requires the integrity of the α subunit of the channel. There are three possible interpretations for these observations: 1) Transfer of ionizing energy from the α to the smaller β1 component could result in the loss of binding function for LqV as a secondary event and not reflect the true target size of the channel receptor. This, however, seems unlikely in view of the radiation inactivation results obtained with glutamate dehydrogenase in which the primary ionization results in damage to a single polypeptide unit, with no large transfer of radiation energy to other units in the hexamer (45). 2) Ionizing energy damages only a portion of the α subunit at the interface of α and β1 which is required for maintenance of the α-β1 subunit interactions. As a result, the proper conformation in β1 is lost and the binding capacity for LqV declines, yielding the large target size for the Lqq V site. If there were such a domain, a target size smaller than α would be observed. This was not found. 3) α-Scorpion toxins bind to a macromolecular complex of α and β1. The radiation kinetics then should be the sum of α and β1. The sum of one STX binding unit (220,000) and the molecular weight for a β1 subunit (39,000) is close to the target size of the α-scorpion toxin receptor site (e.g. 263,000). To distinguish between 220,000 and 263,000 is at the limit of resolution of the technique. Nevertheless, from 11 separate determinations the mean and standard deviation of the target size for the α-scorpion toxin receptor is 263,000 ± 6,000. In each experiment the target size for the α-scorpion toxin receptor was always larger than that obtained for the STX receptor. Photolabeling and fluorescence energy transfer experiments lend further support to this interpretation. The photolabeling pattern identifies α and β1 in these synaptosomal membrane preparations. Integration of the autoradiogram intensities of proteins labeled by α-scorpion toxin indicate that the β1 component is labeled at least 5 times more strongly than α (see Fig. 4 in both Refs. 9 and 38). Because the dissociation rate of the scorpion toxin is slower than the lifetime of the nitrene generated, it is unlikely that dissociation and reassociation of Lqq V accounts for those components labeled. Regardless of the precise mechanism of specificity, the data imply that the Lqq V binding domain is either shared or close to each subunit of the Na+ channel complex. The radiation inactivation experiment then points to the idea that the functional α-scorpion toxin receptor is an associated complex of α and β1 where the functional binding site is donated by both the α and β1 subunits.

Taken together then, we suggest that the α-scorpion toxin receptor site is formed by portions and interactions of both α and β1 subunits that are insulated from each other so that no radiation energy transfer occurs between them. However, the β1 subunit and α subunit are conformationally coupled in that subtle changes in protein conformation are transmitted by alterations in subunit/subunit interactions and associations (12, 13, 15).

On the other hand, the voltage-independent Css II receptor site depends upon a much smaller functional binding unit of Mr, 45,000. Photoaffinity labeling of rat brain synaptosomes with β-scorpion toxin derivatives show that two polypeptides of apparent molecular weights 263,000 and 35,000, corresponding to α and either β1 or β2, are labeled with the lower molecular weight band, contributing to more than 90% of the autoradiogram intensity (38). Although Css II photolabeling labels the large α subunit as well as a smaller β (1 or 2) unit, the radiation inactivation studies indicate that the Css II β-scorpion toxin receptor does not require the α subunit for binding and is functionally associated with the β subunit. If the Css II receptor were located on β2, the radiation sensitivity would correspond to the sum of α and β2 since these two are linked by disulfide bonds. It is known from radiation inactivation studies with ricin, in which the two polypeptide chains are linked by a single disulfide bond, that deposition of ionizing radiation in one chain is transmitted through such a covalent bond to destroy the enzymatic activity located in the linked subunit. Thus, it appears that both α- and β-scorpion toxin receptors are associated with the same polypeptide component of the channel. This is not unreasonable since fluorescence studies have reported on conformational interactions between these sites (14), energy transfer measurement show that these two sites are separated by 22 Å, consistent with the dimensions of a globular polypeptide of 39,000 daltons, and since some cooperativity in the action of these two toxins on Na+ channel in myelinated nerve has been reported (46).

Previous radiation inactivation studies on the TTX and Css II receptors indicate target sizes of 260,000 for both receptors (23, 24). Although our target sizes for the TTX/STX binding component are in reasonable agreement, there is an apparent discrepancy in the target size for the Css II receptor. The previous studies utilized lyophilized preparations whereas in the present report both frozen and lyophilized synaptosomal preparations were irradiated. Both earlier and the present studies found no dramatic changes in the Kn values of these ligands after lyophilization of the membranes. However, the data of Table I show a significant loss of binding site capacity for each ligand studied due exclusively to lyophilization. Comparable data were not reported in the earlier studies. Possible sources of error are introduced by examining activities of the control membranes which are 90% below that of fresh or frozen preparations (see Refs. 47 and 49 for discussion). Similar losses in [3H]nitrendipine binding to the Ca2+ channel have been reported using lyophilized preparations with apparent discrepancies in the target sizes obtained (47, 48).

One possibility is that lyophilization induces a conformational change in the receptor which in turn gives rise to a larger target size. We have tested the idea that lyophilization and subsequent assay yield the results previously obtained. It appears that methods of sample handling could give rise to the discrepancy in target sizes and that lyophilization may cause an irreversible destruction of the channel protein or environment to yield the larger target size. At least for those ligands whose binding is voltage-dependent the use of frozen membranes is superior for radiation inactivation studies. Thus, it appears that methods of sample handling could give rise to the discrepancy in target sizes and that lyophilization causes an irreversible destruction of the channel protein or environment to yield the larger target size.

When the inactivation curve for BTX-B binding to synaptosomes is fitted to a single exponential decay a target size of 110,000 is obtained. In preparations of the purified STX/TTX binding component no protein of this size has been identified. It is obvious that a single exponential is inadequate to describe the loss of BTX-B binding with increasing radiation, and that the target size of 110,000 is not an accurate representation of this receptor size. The deviation from linearity at low doses in the inactivation curve indicates a structure composed of Mr, ~287,000 and 51,000. The analysis for two target sizes assumes that each component is independ-
ently functional in binding BTX. The difficulties in resolving the inactivation curves into two simple exponentials leads to uncertainties in the ultimate target size determinations. Nevertheless, it appears that BTX may be bound by either of two different structures and the sizes determined are in reasonable agreement with those of the α and β subunits. Either or both may have been enhanced by the presence of LqV.

The application of the radiation inactivation technique has revealed the functional binding components of the voltage-dependent Na⁺ channel and has correlated this with the known biochemical and topological properties of the channel. Some discussion has arisen from the apparent compositional differences between the eel and rat brain sodium channels (6, 37, 50, 51). From our radiation inactivation studies it appears that functionally the mammalian channel requires at least two components. All measurements and studies indicate that a large protein of Mr between 220,000 and 250,000 is an integral and major functional part of the channel and that the β1 component does indeed constitute a part of the functional substructure of the mammalian sodium channel. Presently, the functional role of β2 is unclear. Although gel electrophoresis of the solubilized purified STX-binding component of mammalian sarcolemma shows polypeptides of 150,000-200,000, 45,000, 35,000, and 37,000, radiation inactivation shows that the functional unit size of the STX receptor in purified rat sarcolemma is 230,000, similar to that obtained in the present study with rat brain synaptosomes. At present it appears that a functionally active eel Na⁺ channel requires only a single 270-kDa polypeptide (52). Further, radiation inactivation of the in situ sarcolemmal and electropalax Na⁺ channels will be required to examine these compositional differences.

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