Arrangement of the Subunits of the Nicotinic Acetylcholine Receptor of *Torpedo californica* As Determined by α-Neurotoxin Cross-Linking†

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ABSTRACT: [^{3}H]Methyl-α-neurotoxin prereacted with dithiobis(succinimidyl propionate) (DTSP) can be covalently linked to each of the subunits of the nicotinic acetylcholine receptor in membranes from the electric tissue of *Torpedo californica*. Pronounced changes in the cross-linking pattern are observed upon prior incubation with receptor specific ligands and upon reduction and/or alkylation of the receptor. *d*-Tubocurarine has been shown to bind to two different sites in receptor-rich membranes. These sites are present in equal numbers but have different affinities [Neubig, R. R., & Cohen, J. B. (1979) *Biochemistry 18*, 5464–5475; Sine, S., & Taylor, P. (1981) *J. Biol. Chem. 256*, 6692–6699]. Using *d*-tubocurarine inhibition of [^{3}H]-methyl-α-neurotoxin binding, we demonstrate two inhibitory constants for *d*-tubocurarine of 67 ± 21 nM and 4.9 ± 1.7 μM in unreduced membranes. We utilize the large difference in K_i's to preferentially block toxin cross-linking at the high affinity site for *d*-tubocurarine. Low concentrations of this competitive antagonist selectively block the cross-linking of toxin to the β and γ subunits of the receptor, suggesting that these subunits are located close to the toxin binding site which is also the high-affinity binding site for *d*-tubocurarine. Reduction of disulfide bonds alters the affinity of the receptor for α-neurotoxin. Alterations are also seen in the cross-linking pattern of DTSP-activated [^{3}H]methyl-α-neurotoxin to reduced and alkylated membranes in the presence of tubocurarine. The constants for *d*-tubocurarine inhibition of [^{3}H]methyl-α-neurotoxin binding to reduced and alkylated membranes are 172 ± 52 nM and 2.4 ± 0.4 μM. The effects of bromoacetylcholine, carbamoylcholine, gallamine, and procaine on the cross-linking pattern are also examined. Our observations are consistent with an arrangement of the subunits in the membrane of αβαγδ.

The nicotinic acetylcholine receptor of *Torpedo californica* is an integral membrane protein composed of four different subunits (α, β, γ, and δ). One subunit (α) is apparently present in two copies per receptor monomer and contains the binding sites for acetylcholine and α-neurotoxins. This subunit has an apparent molecular weight of 39K (Weill et al., 1974). The other subunits are present in one copy per monomer (Reynolds & Karlin, 1978; Lindstrom et al., 1978; Raftery et al., 1980) and have apparent molecular weights of 48K (β), 58K (γ), and 64K (δ) (Weill et al., 1974). Each subunit is glycosylated (Karlin et al., 1975) and is at least partially exposed at the external surface of the membrane (Hartig & Raftery, 1977; Nathanson & Hall, 1979; Klymkowsky et al., 1980; Strader & Raftery, 1980; Anderson & Blobel, 1981). The primary sequence of each of the receptor subunits has recently been determined from cDNA sequences (Noda et al., 1982, 1983a,b; Sumikawa et al., 1982; Claudio et al., 1983), and a comparison of the sequences shows a considerable amount of homology among subunits. This homology had also been previously demonstrated by partial sequencing of the polypeptides (Raftery et al., 1980). This observation suggests that all of the subunits may each be arranged in the membrane in a similar fashion.

The arrangement of the subunits with respect to one another is for the most part unknown. Using an electron microscopic approach with *Naja naja siamensis* toxin complexed with biotin–avidin and utilizing the ability of the receptor to form dimers via either δ or β chains (Hamilton et al., 1979), Karlin and co-workers have concluded that the arrangement is most likely αγαβδ (Holtzman et al., 1982; Karlin et al., 1983a,b). Other laboratories using chemical cross-linking and/or image reconstruction techniques favor an arrangement in which β rather than γ lies between the two α subunits (Kistler et al., 1982; Zingsheim et al., 1982).

The α subunits of reduced receptor can be radiolabeled with affinity ligands such as [^{3}H][4-(*N*-maleimido)benzyl]trimethylammonium (Damle & Karlin, 1978) and [^{3}H](bromoacetyl)choline (Damle et al., 1978; Wolosin et al., 1980), but one of the two sites reacts more readily than the second. Heterogeneity is also seen in the binding of the competitive antagonists such as *d*-tubocurarine to the receptor (Neubig & Cohen, 1979; Sine & Taylor, 1981).

α-Neurotoxins bind with high affinity and specificity to nicotinic acetylcholine receptors from both electric tissue (Changeux et al., 1970) and skeletal muscle (Lee, 1972; Berg et al., 1972; Patrick et al., 1973; Hartzell & Fambrough, 1973). Cross-linking of α-neurotoxin to its binding sites on the receptor has been used by a number of laboratories to confirm that the toxin binding sites are apparently on the α chains of the receptor and to demonstrate that the β, γ, and δ chains are near neighbors of the α subunit (Hamilton et al., 1978; Witzemann et al., 1979; Nathanson & Hall, 1980). Hamilton et al. (1978) demonstrated the cross-linking of chemically modified *Naja naja siamensis* [^{3}H]methyl-α-neurotoxin 3 to the α and δ subunits of the receptor. Using [^{125}I]-α-bungarotoxin derivatized with ethyl *N*-[(5-azido-2-nitrobenzoyl)amino]acetamidate, Nathanson & Hall (1980) demonstrated labeling of the α, β, γ, and δ subunits of the *Torpedo* receptor. Witzemann et al. (1977), after specifically reducing one disulfide bond per [^{125}I]-α-bungarotoxin molecule, prepared photoaffinity derivatives of the toxin with aryl azide side chains of different lengths. A modified toxin,

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containing a 14-Å side chain, cross-linked to the 40K (α) and the 65K (δ) receptor chains, while a modified toxin with a longer side chain (33Å) cross-linked exclusively to the 65K band. Using ultraviolet irradiation, Oswald & Changeux (1982) cross-linked [^{125}I]-α-bungarotoxin to receptor α, γ, and δ chains. In all cases, preincubation of receptor with unmodified α-neurotoxin blocked the cross-linking action.

In this paper we use the chemical cross-linking of [^{3}H]-methyl-α-neurotoxin to the subunits of the receptor and the modification of that cross-linking pattern by receptor specific ligands to propose a model for the arrangement of the subunits at the surface of the membrane.

Materials and Methods

Materials

Torpedo californica was obtained live from Pacific Biomarine Laboratories (Venice, CA). The electric organs were immediately dissected and stored in liquid nitrogen.

Carbamoylcholine, *d*-tubocurarine chloride, and *N*-ethylmaleimide were obtained from Sigma, and dithiothreitol (DTT)[1] was from Bethesda Research Laboratories. NaB^3H_4 was from New England Nuclear, and molecular weight standards for $NaDodSO_4$–PAGE were from Bio-Rad. Lyophilized *Naja naja siamensis* venom was obtained from Miami Serpentarium.

Methods

Preparation of Membranes. Receptor-rich membranes were prepared by the technique of de Souza Otero & Hamilton (1984).

Reduction and Alkylation of Membranes. Membranes were reduced by incubation with 1 mM DTT in NT50 (pH 8.3) for 30 min at room temperature and were alkylated with 10 mM *N*-ethylmaleimide for 30 min at 4 °C.

Purification and Tritiation of α-Neurotoxin. α-Neurotoxin 3 was purified from lyophilized *Naja naja siamensis* venom as described by Karlsson et al. (1971) and labeled by reaction with formaldehyde and NaB^3H_4 as described by Damle & Karlin (1978). The [^{3}H]methyl-α-neurotoxin used in these experiments had a specific activity of 11 mCi/mmol. Amino acid analysis of the radiolabeled toxin shows that greater than 90% of the incorporated label can be accounted for by methylated lysines and about 5% represents the methylated products of the isoleucine which is the N-terminal amino acid. Peptide mapping of the toxin indicates that the radiolabel is fairly uniformly distributed among the five lysines of the toxin, and each toxin is derivatized, on average, at two lysines per molecule.

Toxin Binding. The binding of [^{3}H]methyl-α-neurotoxin to receptor-rich membranes was performed as described by Damle & Karlin (1978) using Whatman DE81 filters.

Cross-Linking. [^{3}H]Methyl-α-neurotoxin (19.2 μM) was activated by incubation with 0.25 mg/mL DTSP (5 μL of 5 mg/mL DTSP in *N,N*-dimethylformamide per 100 μL of toxin solution) for 15 min at room temperature in 50 mM sodium phosphate (pH 7.0). Dimethylformamide alone does not affect the ability of toxin to bind to the receptor (data not shown). Activated toxin was separated from unreacted DTSP by passage of the reaction mix through a 13 × 0.7 cm Bio-Rad P-6 column. Peak fractions of [^{3}H]methyl-α-neurotoxin (eluting in the void volume) were pooled and aliquoted into glass tubes. In all of the experiments to be described in this paper, the DTSP-modified [^{3}H]methyl-α-neurotoxin was used immediately after elution from the column. Delays resulted in hydrolysis of the *N*-hydroxysuccinimide ester and loss of the ability of the toxin to bind to the receptor. The high molar excess of DTSP over amino groups results in some modification of all of the lysines of the toxin. No toxin oligomers were detected by gel filtration on a Bio-Rad P-30 column. Membranes (25–200 μg) were added directly to the DTSP-activated [^{3}H]methyl-α-neurotoxin and incubated for 15 or 30 min at room temperature. To stop the reaction, lysine was added to 0.5 mM and/or *N*-ethylmaleimide to 5 mM. Aliquots were removed to assay for toxin binding by the technique of Damle & Karlin (1978). For electrophoresis (see below), the membranes were pelleted in a Beckman airfuge for 2 min at 30 psi and the pellets resuspended in 25 or 50 μL of $NaDodSO_4$ sample buffer. The extent of cross-linking was determined either directly from the counts recovered from $NaDodSO_4$ gels or by one of two alternate methods: (1) A 10-μL aliquot of each sample was added to 100 μL of 0.1% $NaDodSO_4$ in NP50 and passed through a 13 × 0.7 cm Bio-Rad P-150 column to separate cross-linked toxin from free toxin and the fractions counted. (2) A 10-μL aliquot of the reaction mixed was added to 100 μL of 0.5 M NaCl, 0.1% $NaDodSO_4$, and 10 mM Tris (pH 7.4), and heated to 50 °C for 5 min; 3 mL of 0.2% Triton X-100/10 mM MOPS (pH 7.4)/10 mM NaCl/1 mM EDTA was added, and the samples were heated again for 5 min and filtered as before with DE-81 filters using 4 times 3 mL of 0.2% Triton X-100/10 mM MOPS (pH 7.4)/10 mM NaCl/buffer as a wash solution.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples in 2% $NaDodSO_4$, 62.4 mM Tris-HCl (pH 6.8), 10% w/v glycerol, and 0.001% (w/v) bromophenol blue ($NaDodSO_4$ sample buffer) were alkylated with 20 mM *N*-ethylmaleimide for 15 min at room temperature and heated at 50 °C for 1 h. Twenty-five microliter aliquots of each sample were applied to 10% $NaDodSO_4$–polyacrylamide gels prepared and run as described by Laemmli (1970). Individual lanes were removed from these gels, sliced into 1-mm slices, digested with NCS (Amersham), and counted in a liquid scintillation counter. Molecular weight markers were myosin (200K), β-galactosidase (116.5K), phosphorylase *b* (94K), bovine serum albumin (68K), and ovalbumin (43K).

Computer Analysis of Data. The apparent dissociation constants for [^{3}H]methyl-α-neurotoxin binding to the nicotinic acetylcholine receptor were calculated by computer fitting of equilibrium binding data. The program uses the Marquardt method and gives parameter uncertainities and a χ^2 value. The statistical tests involve a linearized version of the "*F*" test as used in the LIGAND program (Munson & Robard, 1980).

To obtain the inhibitory constants for *d*-tubocurarine, the data obtained from *d*-tubocurarine inhibition of [^{3}H]-methyl-α-neurotoxin binding to receptor-rich membranes were fit to an appropriate binding model with a nonlinear, least-squares fitting procedure similar to that described by Colquhoun (1971). The same least-squares fitting algorithm was used to obtain unbiased estimates of the peak areas from

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; BAC, (bromoacetyl)choline; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; α-neurotoxin, α-neurotoxin 3 of *Naja naja siamensis* venom; $NaDodSO_4$, sodium dodecyl sulfate; DTSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; DMF, *N,N*-dimethylformamide; PAGE, polyacrylamide gel electrophoresis; DTSP–[^{3}H]toxin (DTSP–toxin), [^{3}H]-methyl-α-neurotoxin reacted with DTSP; PMSF, phenylmethanesulfonyl fluoride; NP50, 50 mM NaCl, 10 mM sodium phosphate (pH 7.0), and 1 mM Na_2EDTA; TNT50, same as NP50 except with 0.2% Triton X-100; NT50, 50 mM NaCl, 10 mM Tris (pH 8.3), and 1 mM Na_2-EDTA; TM50, 0.2% Triton, 10 mM MOPS (pH 7.4), and 50 mM NaCl; Tx–α, Tx–β, Tx–γ, Tx–δ, and Tx–δ_2, toxin cross-linked to α, β, γ, δ, and δ_2 subunits.

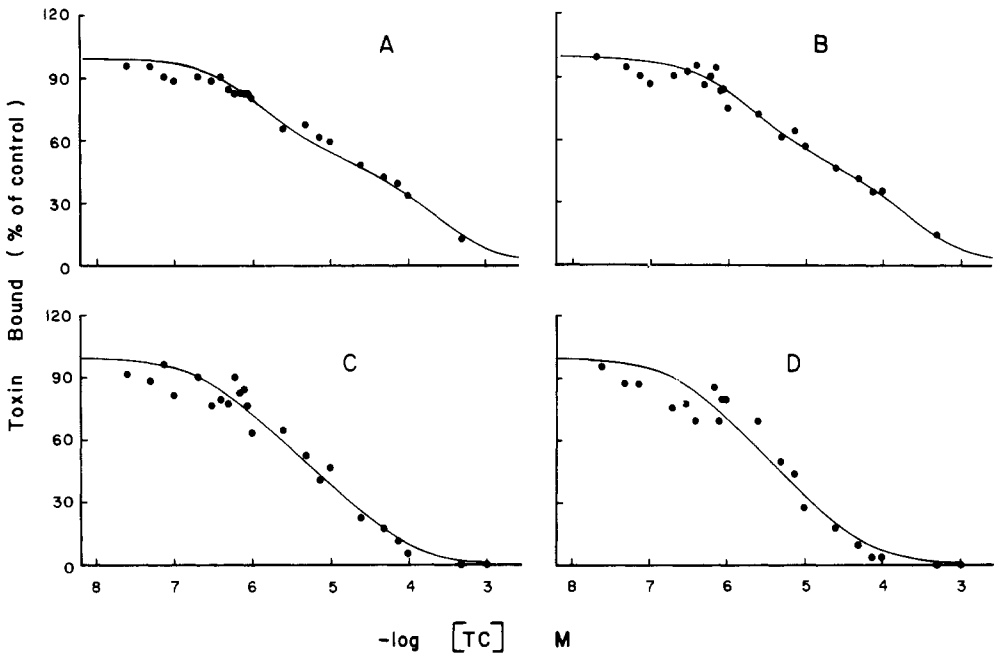

FIGURE 1: Effect of *d*-tubocurarine on the binding of [^{3}H]methyl-α-neurotoxin to receptor-rich membranes. Receptor-rich membranes (1.1 pmol of toxin binding sites) were left untreated or reduced and alkylated as described under Methods and were then incubated for 30 min in 900 μL of NP50 with increasing concentrations of *d*-tubocurarine. [^{3}H]Methyl-α-neurotoxin (9.3 pmol) in 100 μL of NP50 was added, and the samples were filtered after 3 h at room temperature through DE-81 filters as described under Methods. The solid line represents the computer fit to a two-site model. (A) Unreduced membranes, (B) unreduced, NEM reacted, (C) reduced, and (D) reduced, NEM reacted.

isotopically labeled sliced gels. The initial estimates of peak location and magnitude are determined iteratively in a manner similar to that previously described by Kitazoe et al. (1984).

RESULTS

Effect of Reduction and/or Alkylation on the Binding of [^{3}H]Methyl-α-neurotoxin to Receptor-Rich Membranes. Reduction of disulfide bonds in receptor-rich membranes alters the apparent affinity of the acetylcholine receptor for [^{3}H]-methyl-α-neurotoxin. The K_d for toxin binding to untreated membranes is 0.13 ± 0.04 nM (n = 4), to NEM-treated membranes is 0.15 nM (n = 2), to reduced membranes is 0.35 nM (n = 2), and to reduced and alkylated membranes is 0.42 ± 0.08 nM (n = 8). Reaction of 50% of the α-neurotoxin sites with BAC does not greatly alter the K_d for [^{3}H]methyl-α-neurotoxin binding to the second site (K_d = 0.38 ± 0.3 nM, n = 3). All of the data obtained in these experiments is best fit by a model in which there is only one class of binding site for toxin on the receptor in each state of alkylation or reduction. Neither reduction nor alkylation alters the maximum number of binding sites for [^{3}H]methyl-α-neurotoxin. Low concentrations of [^{3}H]methyl-α-neurotoxin give a downward curvature when the data are analyzed with a Scatchard plot. This is a result of the high site concentration. Only that data falling on the linear portion of the Scatchard were used in this analysis as suggested by Cuatrecasas (1975). Reduction lowers the apparent affinity of the receptor for [^{3}H]methyl-α-neurotoxin and alkylation with NEM does not appear to alter further the affinity of either unreduced or reduced membranes.

d-Tubocurarine has been reported to have two different dissociation constants for binding to the receptor (Neubig & Cohen, 1979; Sine & Taylor, 1981). Figure 1 and Table I show the effects of preincubation of membranes with *d*-tubocurarine on the equilibrium binding of [^{3}H]methyl-α-neurotoxin. The solid line in Figure 1 shows the best fit for a model in which there are two binding sites for *d*-tubocurarine present in equal numbers. For unreduced membranes using a value of 0.13 nM for the K_D of [^{3}H]methyl-α-neurotoxin binding, we calculate apparent K_i's for *d*-tubocurarine of 67 ± 21 nM and 4.9 ± 1.7 μM. Using a value of 0.42 nM for the equilibrium dissociation constant for [^{3}H]methyl-α-neurotoxin binding to reduced and alkylated membranes, we observed a decrease in the apparent K_i for the lower affinity site (K_{i_2} = 2.4 ± 0.4 μM) and an apparent increase in the K_i of the high-affinity site (K_{i_1} = 172 ± 52 nM).

Table I: Inhibition of [^{3}H]Methyl-α-neurotoxin Binding by *d*-Tubocurarine[a]

treatment of membranes	K_{i_1} (nM)	K_{i_2} (μM)
control (n = 5)	67 ± 21	4.9 ± 1.7
NEM (n = 3)	74 ± 43	6.1 ± 0.7
DTT (n = 2)	69	1.7
	19	2.9
DTT/NEM (n = 3)	172 ± 52	2.4 ± 0.4

[a] Control, alkylated, reduced, and reduced and alkylated receptor-rich membranes (0.2–1 nM sites in NP50) were incubated with *d*-tubocurarine at concentrations ranging from 1 nM to 1 mM for 15 min at room temperature. [^{3}H]Methyl-α-neurotoxin (4–10 nM) was added, and the membranes were incubated for 3 h at room temperature and filtered as previously described. Apparent K_i's were calculated as described under Methods.

These observed changes led us to examine not only the effects of reduction and alkylation but also the effect of *d*-tubocurarine in the incubation media on the chemical cross-linking of [^{3}H]methyl-α-neurotoxin to the subunits of the acetylcholine receptor.

Cross-Linking of DTSP-[^{3}H]Methyl-α-neurotoxin to Receptor-Rich Membranes. DTSP-activated [^{3}H]methyl-α-neurotoxin can be covalently linked to the subunits of the nicotinic acetylcholine receptor. Typical cross-linking patterns obtained by slicing, digesting, and counting 1-mm slices of the $NaDodSO_4$–polyacrylamide gels of cross-linked membranes which were untreated or reacted with dithiothreitol and/or *N*-ethylmaleimide are shown in Figure 2. The gels shown in this figure were sliced only to below the α subunit of the receptor. There are essentially no cross-linked products on the gel below this point. In the experiment depicted in Figure

Table II: Percent of Cross-Linked Toxin Associated with Each Subunit

treatment of membranes	% of bound toxin which is cross-linked	% of cross-linked toxin associated with receptor subunits				
		145K δ_2	70K δ	61K γ	53K β	47K α
(A) DTSP–[^{3}H]Methyl-α-neurotoxin Cross-Linking[a]						
control (n = 3)	39 ± 5	9 ± 1	5 ± 2	12 ± 1	18 ± 2	17 ± 2
NEM	37	10	3	14	23	24
DTT (n = 5)	48 ± 13		11 ± 5	4 ± 1	6 ± 2	47 ± 2
DTT/NEM (n = 7)	34 ± 3		19 ± 4	12 ± 3	20 ± 3	12 ± 2
(B) DSS–[^{3}H]Methyl-α-neurotoxin Cross-Linking[b]						
control	35	15	5	13	13	5
NEM	36	15	6	14	15	6
DTT	52		11	9	7	33
DTT/NEM	33		16	18	13	7
(C) Same as in (A) with DTSP in Excess[c]						
control (n = 3)	36 ± 3	15 ± 2		11 ± 3	13 ± 3	25 ± 3
NEM (n = 3)	35 ± 3	16 ± 2		13 ± 1	9 ± 2	31 ± 1
DTT (n = 5)	43 ± 3		8 ± 1	5 ± 1	4 ± 1	40 ± 2
DTT/NEM (n = 10)	25 ± 2		18 ± 8	11 ± 3	13 ± 3	16 ± 6
(D) DTSP Added to [^{3}H]Methyl-α-neurotoxin Bound to Receptor[d]						
control	2	11		13	8	27
NEM	3	11		16	9	29
DTT	9		5	4		50
DTT/NEM	3		18	4	12	16

[a] Percent of cross-linked toxin associated with each subunit. Toxin cross-linking was performed as described in Figure 2. The radioactivity associated with each subunit was calculated as described under Methods. [b] Cross-linking same as in Figure 3 except with DSS as the cross-linker. [c] Cross-linking of DTSP–[^{3}H]methyl-α-neurotoxin in the presence of excess DTSP. [^{3}H]Methyl-α-neurotoxin (3.5 μM) was activated by incubation with 0.25 mg/mL DTSP (5 μL of 5 mg/mL DTSP in N,N-dimethylformamide per 100 μL of toxin solution) for 30 min at room temperature in NP50 (pH 7.0). Membranes (100–200 μg) were added directly to this mixture, and the incubation was continued for another 30 min. [d] The percent of cross-linked toxin associated with each subunit when DTSP is added to membranes containing bound [^{3}H]methyl-α-neurotoxin. Control, alkylated, reduced, and reduced and alkylated membranes were prepared as previously described. Membranes (120 pmol of sites) in 200 μL of NP50 were incubated with 90 pmol of [^{3}H]methyl-α-neurotoxin for 10 min at room temperature; 5 μL of 5 mg/mL DTSP in DMF was added, and the mixture was incubated 30 min at room temperature. The reaction was stopped with both 10 μL of 10 mM lysine and 10 mL of 100 mM NEM.

2, the DTSP–toxin was added at a concentration (135 nM) that was smaller than the concentration of toxin binding sites (625 nM) on the membranes. Reaction of [^{3}H]methyl-α-neurotoxin with DTSP does not prevent toxin from binding to the receptor. Under conditions of receptor excess essentially all of the DTSP–toxin binds to the receptor.

Reaction of unreduced membranes with DTSP-activated [^{3}H]methyl-α-neurotoxin (Figure 2A) produces broad radioactive bands with apparent molecular weights of 145K, 70K, 61K, 53K, and 47K. Varying the amount of toxin to produce a toxin to binding sites ratio between 0.1 and 3 does not significantly alter the cross-linking pattern. The peak area of each band was determined by computer fitting as discussed under Methods and is summarized in Table II. The width of the bands most likely reflects the reaction of different lysines of the toxin with each subunit, giving rise to products with slightly different mobilities. The brackets shown in Figure 2 indicate the regions in which computer-fit peak areas were summed and assigned to each subunit. In this gel system the apparent molecular weights of the subunits of reduced and NEM-alkylated affinity purified receptor are 63K (δ), 54K (γ), 47K (β), and 39K (α). In addition, these membranes frequently contain relatively large amounts of a 95K protein (ϵ) and a 43K protein (ν). The expected molecular weights of [^{3}H]methylneurotoxin cross-linked to subunits of the receptor are 71K (δ–toxin), 62K (γ–toxin), 55K (β–toxin), and 47K (α–toxin). In unreduced membranes the δ subunit exists partially as a dimer having an apparent molecular weight of 140K (Hamilton et al., 1979). The expected molecular weight of the δ_2–toxin cross-link product is 148K. On the basis of the close agreement between the apparent molecular weights of observed radioactive cross-linked bands and the predicted toxin–subunit molecular weights, we propose that the cross-linked products observed represent δ_2–toxin (145K), δ–toxin (70K), γ–toxin (61K), β–toxin (53K), and α–toxin (47K). These assignments are supported by several other observations. The 145K band is decreased, the 71K band is increased in reduced membranes, and under these conditions, δ_2 is decreased and δ increased in the stained gel (Chang & Bock, 1979; Hamilton et al., 1979). Also, diamide, an oxidizing agent, decreases the radioactivity associated with 53K and increases higher molecular weight bands (data not shown). Diamide is known to oxidize β to a dimer or trimer (Hamilton et al., 1979). Pretreatment of membranes with high concentrations of p-(chloromercuri)benzoate alters the mobility of the β and γ bands on $NaDodSO_4$ gels and shifts the position of the 53K and 61K bands in a corresponding fashion (S. L. Hamilton, unpublished observation). Reaction of receptor-rich membranes with DTSP-activated [^{3}H]methyl-α-neurotoxin does not detectably alter the Coomassie Brilliant Blue staining pattern of the $NaDodSO_4$–polyacrylamide gels.

Alkylation of unreduced membranes with N-ethylmaleimide prior to cross-linking alters the pattern as shown in Figure 2B. The only major change is a decrease in the 70K band, suggesting that toxin is cross-linking either to a sulfhydryl on δ or to a lysine which readily reacts with NEM.

As shown previously, reduction of membranes alters the affinity of the receptor for [^{3}H]methyl-α-neurotoxin. Reduction also causes a very pronounced change in the cross-linking pattern (Figure 2D). There is a large increase in the cross-linking to both δ and α and a decrease in the cross-linking to both β and γ (Table IIA). Alkylation of reduced membranes with N-ethylmaleimide also changes the cross-linking pattern (Figure 2C). The amount of radioactivity associated with Tx–β and Tx–γ increases, while the amount with Tx–α and Tx–δ decreases relative to reduced membranes. Similar cross-linking patterns to those obtained with DTSP-activated [^{3}H]methyl-α-neurotoxin are obtained with DSS-activated

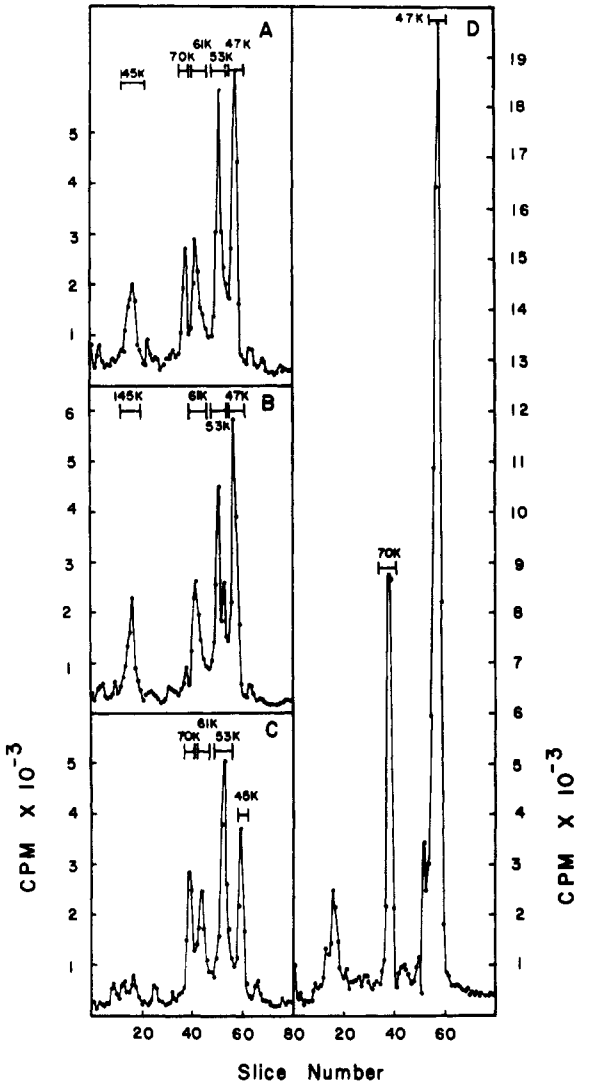

FIGURE 2: DTSP-activated toxin cross-linking to control, alkylated, reduced, and reduced and alkylated receptor-rich membranes. Reduction and alkylation of receptor-rich membranes was performed as described under Methods. Following these treatments the membranes were washed twice and resuspended in NP50. Membranes (125 pmol of binding sites) were incubated with DTSP-[^{3}H]-methyl-α-neurotoxin (27 pmol) for 30 min at room temperature. The reaction was stopped by the addition of 0.5 mM lysine and 10 mM *N*-ethylmaleimide. Samples were prepared for electrophoresis and following electrophoresis were sliced, digested, and counted as described under Methods. (A) Unreduced, (B) unreduced, NEM reacted, (C) reduced, NEM reacted, and (D) reduced.

[^{3}H]methyl-α-neurotoxin (Table IIB). DSS, disuccinimidyl suberate, is an analogue of DTSP that lacks an internal disulfide. The one major change in the observed cross-linking pattern with DSS is a decrease in Tx-α in all cases, suggesting the possibility that DTSP-activated [^{3}H]methyl-α-neurotoxin cross-linking to α can occur, at least partially, via interchange between the disulfide of the cross-linker and sulfhydryls on the receptor which are not accessible to alkylation by NEM in unreduced membranes.

Table IIC demonstrates that the patterns obtained with DTSP-activated [^{3}H]methyl-α-neurotoxin cross-linking are reproducible even when excess cross-linker is not removed. Also, the addition of DTSP to [^{3}H]methyl-α-neurotoxin already bound to receptor-rich membranes produces very similar patterns (Table IID); therefore, this treatment has not greatly altered the specificity of the toxin. Radiolabeling of one of the two α subunits with [^{3}H]BAC and cross-linking unlabeled

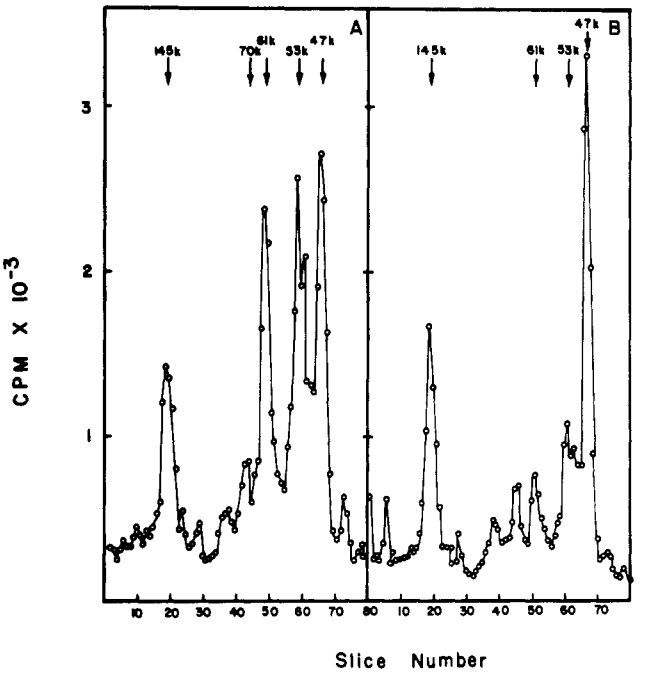

FIGURE 3: Effect of *d*-tubocurarine on α-neurotoxin cross-linking to unreduced membranes. Membranes (56 pmol of toxin binding sites in 100 μL of NP50) were incubated with *d*-tubocurarine (100 pmol) for 15 min at room temperature. A total of 100 μL of DTSP-[^{3}H]methyl-α-neurotoxin (18 pmol) was added, and after 15 min the reaction was stopped as described in Figure 2. (A) Control and (B) *d*-tubocurarine treated.

methyl-α-neurotoxin to receptor-rich membranes do not result in the incorporation of radiolabel into proteins with molecular weights higher than that of the α subunit. In addition, cross-linking of [^{3}H]methyl-α-neurotoxin to receptor-rich membranes does not alter receptor monomer to dimer ratios.

Specificity of Cross-Linking Reaction. The reaction of DTSP-modified [^{3}H]methyl-α-neurotoxin with receptor is specific in that (1) it can be blocked by excess underivatized toxin or by carbamoylcholine (Figure 5D,E), (2) all bands can be immunoprecipitated with antireceptor antibody prepared from rabbits immunized with affinity purified acetylcholine receptor (data not shown), and (3) alkaline extraction of peripheral proteins (including the 43K protein) by the technique of Neubig et al. (1979) does not alter the cross-linking pattern (data not shown).

Effect of Antagonists on Cross-Linking Pattern to Unreduced Membranes. Since *d*-tubocurarine binds to two sites on receptor-rich membranes with different affinities, it should be possible to selectively block DTSP–toxin binding and cross-linking at the high-affinity *d*-tubocurarine site by using low concentrations of this ligand. The cross-linking pattern obtained in the presence of 0.5 μM *d*-tubocurarine is shown in Figure 3. In these experiments the ratio of DTSP-[^{3}H]-methyl-α-neurotoxin to sites in the controls is about 0.3. *d*-Tubocurarine decreased DTSP-modified α-neurotoxin binding by less than 50% and inhibited the cross-linking of [^{3}H]methyl-α-neurotoxin to β and γ to a much greater extent than to α and δ. Tx-δ_2 and Tx-α have decreased by less than 10% while Tx-γ and Tx-β have decreased by about 70%. The effect of several different concentrations of *d*-tubocurarine on the amount of cross-linking to each subunit is shown in Figure 4A. The amount of radiolabel in Tx-β and Tx-γ declines at low concentrations of *d*-tubocurarine more than that in Tx-α and Tx-δ_2. High concentrations of *d*-tubocurarine block essentially all cross-linking and all binding of DTSP–toxin to these membranes.

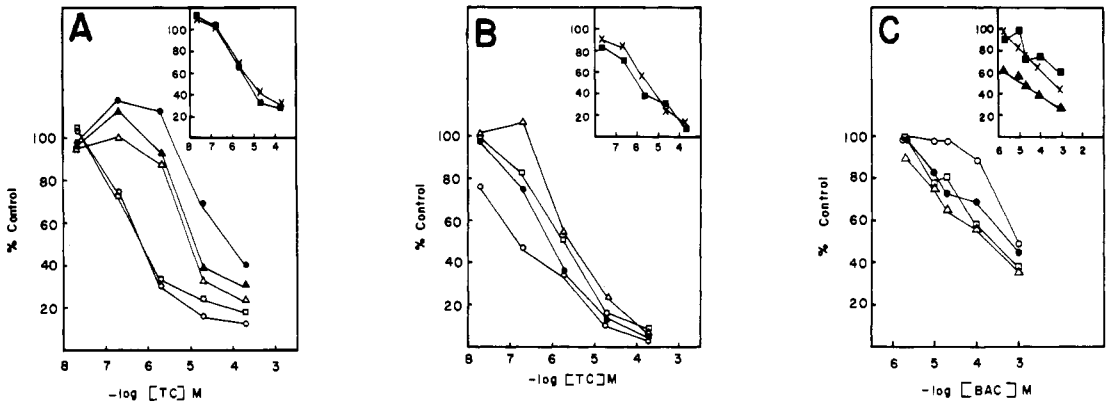

FIGURE 4: (A) Effect of *d*-tubocurarine on the amount of toxin cross-linked to each subunit in unreduced membranes. Membranes (56 pmol of toxin binding sites) in 100 μL of NP50 were incubated with increasing concentrations of *d*-tubocurarine followed by the addition of 100 μL of DTSP–[^{3}H]methyl-α-neurotoxin (18 pmol). Reaction and preparation of samples was performed as previously described. The amount of cross-linked toxin in each band at each concentration was determined as described under Methods and then normalized to the values obtained in the absence of *d*-tubocurarine. (●) Tx–α, (□) Tx–β, (○) Tx–γ, (△) Tx–δ, and (▲) Tx-δ_2. Insert: (■) [^{3}H]methyl-α-neurotoxin bound, percent control; (×) [^{3}H]methyl-α-neurotoxin cross-linked, percent control. Concentrations of *d*-tubocurarine shown here represent final concentrations. (B) The effect of increasing concentration of *d*-tubocurarine on [^{3}H]methyl-α-neurotoxin cross-linking to reduced and alkylated membranes. Reduced and alkylated membranes (56 pmol of toxin binding sites) in 100 μL of NP50 were incubated with increasing concentrations of *d*-tubocurarine followed 15 min later by the addition of 100 μL of DTSP–[^{3}H]methyl-α-neurotoxin (18 pmol). The amount of cross-linked toxin in each band at each concentration was determined as described under Methods and then normalized to the values obtained in the absence of *d*-turocurarine. The insert shows the percent of both toxin bound (■) and cross-linked (×) relative to controls. (●) Tx–α, (□) Tx–β, (○) Tx–γ, and (△) Tx–δ. (C) The effect of BAC on the cross-linking of [^{3}H]methyl-α-neurotoxin to receptor-rich membranes. Reduced membranes (400 pmol of toxin binding sites) in 100 μL of 10 μM neostigmine bromide NP50 were reacted with (bromoacetyl)choline at the concentrations shown in the figure for 20 min at room temperature. The reaction was stopped with 10 μL of 100 mM NEM, and the membranes were washed twice by pelleting. DTSP–[^{3}H]methyl-α-neurotoxin (62 pmol) was added to and incubated with the membranes (133 pmol of sites in 100 μL of 1 μM neostigmine NP50) for 30 min at room temperature. The reaction was stopped by the addition of lysine and NEM as previously described. The insert shows the percent of the control for total toxin binding (■) and cross-linking (×) and the percent of the total sites blocked by BAC (▲) determined in an independent assay (Methods). (●) Tx–α, (□) Tx–β, (○) Tx–γ, and (△) Tx–δ.

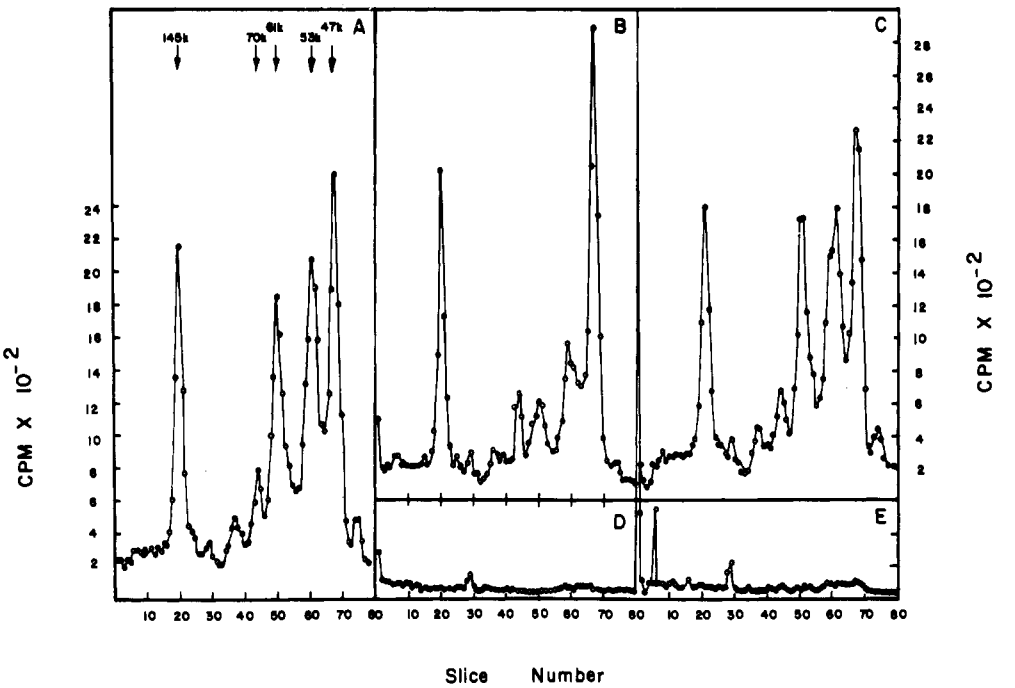

FIGURE 5: Effect of unlabeled toxin, carbamoylcholine, gallamine, and procaine on toxin cross-linking to unreduced membranes. Receptor-rich membranes (56 pmol of toxin binding sites in 100 μL of NP50) were incubated with (A) buffer, (D) unlabeled α-neurotoxin (77 nmol), (E) carbamoylcholine (100 μmol), (B) gallamine (100 μmol), or (C) procaine (100 μmol) for 15 min at room temperature. A total of 100 μL of DTSP–[^{3}H]methyl-α-neurotoxin (16 pmol) was added, and after 15 min at room temperature, the reaction was stopped by the addition of lysine (100 nmol) and *N*-ethylmaleimide (1 μmol). Electrophoresis and preparation of slices for liquid scintillation were as described under Methods.

We have also examined the effect of a second competitive antagonist on the cross-linking patterns. Figure 5B shows the pattern obtained from the reaction of DTSP-modified [^{3}H]-methyl-α-neurotoxin with receptor-rich membranes in the presence of 0.5 mM gallamine. Similar to the results with *d*-tubocurarine, gallamine inhibited DTSP-activated [^{3}H]-methyl-α-neurotoxin by about 50% and reduced the radioactivity in Tx–β (~70% inhibition) and Tx–γ (~70% inhibition) with little effect on Tx–α and Tx–δ. To determine if this effect is characteristic of only competitive antagonists, we also tested the effect of a noncompetitive antagonist, procaine, on the cross-linking reaction (Figure 5C). Procaine decreases the amount of DTSP-activated [^{3}H]methyl-α-neurotoxin bound by about 30% but, in contrast to competitive antagonists,

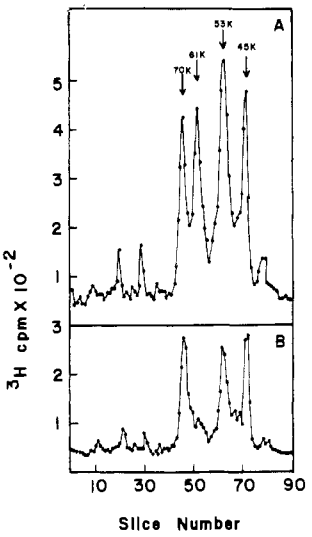

FIGURE 6: Effect of *d*-tubocurarine on [^{3}H]methyl-α-neurotoxin to reduced toxin cross-linking and alkylated membranes. Reduced and alkylated membranes (56 pmol of toxin binding sites in 100 μL of NP50) were incubated with *d*-tubocurarine (100 pmol) for 15 min at room temperature. A total of 75 μL of DTSP–[^{3}H]methyl-α-neurotoxin (16 pmol) was added, and after 15 min at room temperature the reaction was stopped by the addition of lysine (100 nmol) and *N*-ethylmaleimide (1 μmol). Electrophoresis and preparation of 1-mm slices for counting were as previously described.

decreases the amount of radioactivity associated with each subunit to approximately the same extent (about a 30% decrease for each band). This implies no specificity of effects of procaine on the cross-linking reaction.

Effects of Receptor-Specific Ligands on [^{3}H]Methyl-α-neurotoxin Cross-Linking to Reduced and Alkylated Receptor-Rich Membranes. One of our goals in these cross-linking studies was to examine the effect of blockage of DTSP-activated [^{3}H]methyl-α-neurotoxin binding to membranes by BAC covalently bound to one of the two toxin sites. Because BAC only reacts with reduced receptor, these studies required a more careful examination of the cross-linking of [^{3}H]methyl-α-neurotoxin to reduced and subsequently alkylated membranes.

As with unreduced membranes excess underivatized α-neurotoxin blocks essentially all of the cross-linking of DTSP–toxin to reduced and alkylated membranes. Carbamoylcholine, however, at 0.5 mM is less effective in blocking the cross-linking to reduced and alkylated membranes. This result is expected because of the lowered affinity of reduced and alkylated membranes for agonists (Walker et al., 1981; Blanchard et al., 1982). Carbamoylcholine blocks 67% of the binding of DTSP–toxin to these membranes and reduces the cross-linking in all bands by about 60%.

In unreduced membranes, *d*-tubocurarine inhibits cross-linking to the γ and β subunits about equally. In contrast, in reduced and alkylated membranes *d*-tubocurarine shows a greater effect on the cross-linking to the γ subunit of the receptor than to the β subunit (Figure 6). The effect of a range of *d*-tubocurarine concentrations on the cross-linking of [^{3}H]methyl-α-neurotoxin to each subunit of the receptor is shown in Figure 4B. Covalent reaction of the toxin with the γ subunit is affected to the greatest extent by *d*-tubocurarine.

Thus, in reduced and alkylated membranes it is possible to block selectively toxin binding at one of the two sites. It is, therefore, feasible to examine the effects on the [^{3}H]-methyl-α-neurotoxin cross-linking patterns of covalent reaction of reduced receptor with BAC prior to NEM alkylation (Figure 4C). BAC reduced the amount of radioactivity in

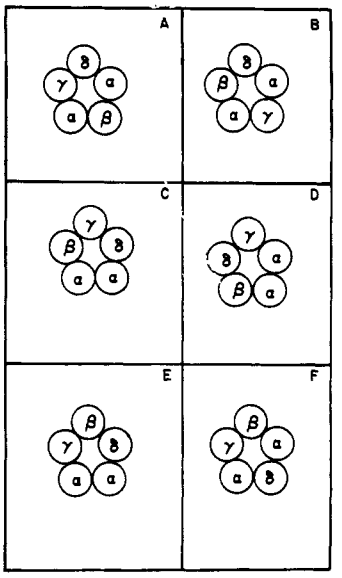

FIGURE 7: Models for the arrangements of the subunits in the membranes.

Tx–δ, Tx–β, and Tx–α at a lower concentration than Tx-γ, suggesting that toxin occupying the site to which BAC can covalently bind at low concentrations is not particularly close to the γ subunit of the receptor. A second possibility is that covalently bound BAC alters the conformation of the receptor such that reactive lysines of the γ subunit are no longer within cross-linking distance of the bound toxin.

Discussion

In this paper we describe a new approach to analyzing the arrangement of the nicotinic acetylcholine receptor subunits in the membrane. Some of the possible arrangements are shown in Figure 7. [^{3}H]Methyl-α-neurotoxin reacted with the bifunctional cross-linking reagent dithiobis(succinimidyl propionate) can be covalently attached to each of the subunits of the receptor. DTSP-activated [^{3}H]methyl-α-neurotoxin is shown to bind to the same site on the receptor as unmodified toxin since its binding can be blocked by excess unmodified toxin and by receptor agonists and competitive antagonists. Removal of as much as 50% of the total protein from these membranes by alkaline extraction does not alter the cross-linking pattern. In addition, all the cross-linked bands can be immunoprecipitated with antireceptor antibody and the addition of DTSP to [^{3}H]methyltoxin already bound to the receptor produces very similar patterns.

As the toxin molecule is large, it is possible that α-neurotoxin bound at one site is cross-linking to subunits in the vicinity of the second site. However, radiolabeling of one of the two α subunits with [^{3}H]BAC and cross-linking unlabeled methyl-α-neurotoxin to receptor-rich membranes do not result in the incorporation of the radiolabel into proteins with molecular weights higher than that of the α subunit. A second possibility is that α-neurotoxin bound to one receptor monomer is cross-linking to the subunits of another receptor located close to the first in the membrane. We do not, however, see any changes in the receptor monomer to dimer ratios on sucrose gradients of Triton X-100 solubilized membranes to which the DTSP–toxin has been cross-linked.

Reduction of disulfides of the receptor alters a number of its properties (Karlin & Bartels, 1966; Karlin, 1967, 1969; Karlin & Winnik, 1968; Podleski et al., 1969; Landau & Ben Haim et al., 1975; Terrar, 1978; Cox et al., 1979a,b; Walker et al., 1981). In this paper, we report that reduction also lowers

the affinity of the receptor for [^{3}H]methyl-α-neurotoxin and changes the apparent K_i's for *d*-tubocurarine inhibition of [^{3}H]methyl-α-neurotoxin binding.

The cross-linking patterns obtained when receptor-rich membranes are reduced prior to reaction with DTSP–toxin are also changed. There is a large increase in the cross-linking to δ and α and a decrease in the cross-linking to β and γ. There are a number of possible explanations of this observation: (1) increased numbers of sulfhydryls on δ and α could compete with groups on β and γ for the reaction with cross-linker, (2) a structural change in the protein upon reduction results in β and γ being oriented away from the toxin binding sites, or (3) reduction selectively alters the affinity of one binding site for DTSP-activated toxin relative to the other and at the low toxin to site ratios used in these experiments all of the toxin is binding to one site. The third possibility is unlikely for the following reasons. Although reduction alters the affinity of the receptor for toxin, [^{3}H]methyl-α-neurotoxin binding is best fit by a one-site model (or multiple sites with smaller K_d's) regardless of the state of reduction or alkylation of the membranes. The maximum number of binding sites for [^{3}H]-methyl-α-neurotoxin or DTSP–[^{3}H]methyl-α-neurotoxin is not altered by reduction, and finally, increasing the toxin to site ratio does not increase the relative amount of radioactivity in Tx–β and Tx–γ when DTSP–[^{3}H]methyl–α–neurotoxin is cross-linked to reduced membranes.

A noncleavable analogue of DTSP, disuccinimidyl suberate (DSS), when used in place of DTSP produces similar cross-linking patterns, and these patterns show similar changes upon reduction and/or alkylation of the membranes. The only major difference using the two different cross-linkers is a decrease in the relative amount of Tx–α with DSS as compared to DTSP, suggesting that disulfide interchange occurs between the disulfide of DTSP attached to toxin and sulfhydryls on the α subunit.

The effects of the competitive antagonists *d*-tubocurarine and gallamine on the cross-linking of toxin to the receptor have been examined. *d*-Tubocurarine has been shown to bind to two sites on the receptor which are present in equal numbers but with very different affinities (Neubig & Cohen, 1979; Sine & Taylor, 1981). Neubig & Cohen (1979) report values of 33 ± 6 nM and 7.7 ± 4.6 μM for the dissociation constants for [^{3}H]methyl-*d*-tubocurarine binding to *Torpedo* receptor-rich membranes. These values are comparable to the K_i's which we calculate for *d*-tubocurarine inhibition of [^{3}H]-methyl-α-neurotoxin binding (K_{i_1} = 67 ± 21 nM; K_{i_2} = 4.9 ± 1.7 μm). As discussed by Neubig & Cohen (1979) and Sine & Taylor (1981), several models can account for the two affinities of the nicotinic acetylcholine receptor for *d*-tubocurarine. There could be two types of receptor. It is known that the *Torpedo* receptor exists as a mixture of monomers and dimers (Chang & Bock, 1977; Hamilton et al., 1979). Neubig & Cohen (1979), however, have shown that receptor-rich membranes prepared in the presence of *N*-ethylmalemide in order to increase the proportion of dimers still possess equal numbers of *d*-tubocurarine binding sites. Sine & Taylor (1981), by comparing the ratio of the two dissociation constants with the concentration dependence for functional antagonism for a series of antagonists, also concluded that the two sites are confined to a single oligomer. A second possible explanation of the two affinities is that *d*-tubocurarine bound at one site inhibits binding at the second site either by steric hindrance or by causing a conformation change in the protein, thereby reducing the affinity of the second site. Sine & Taylor (1980), however, have shown that prior occupation of part of the sites by α-toxin does not alter the concentration dependence of antagonist binding to the remaining sites. The most probable explanation of the two affinities observed for *d*-tubocurarine is that there are two distinct binding sites on a single receptor molecule. As the receptor has five subunits, only two of which are apparently identical (the α's), the different local environments of the two binding sites could give rise to the differences in binding affinities. Alternatively the binding characteristics may be due to differences in structure of the two α chains. Conti-Tronconi et al. (1984) reported differences in the extent of glycosylation of the two α chains, and Lindstrom et al. (1983) have demonstrated that the α subunit with which MBTA covalently reacts is the one which is not glycosylated. The high-affinity site for *d*-tubocurarine, however, appears to be on the glycosylated α chain (Hall et al., 1983).

Low concentrations of both *d*-tubocurarine and gallamine selectively block the cross-linking of DTSP–[^{3}H]methyl-α-neurotoxin to the β and γ subunits of the unreduced receptor, suggesting that either the high-affinity site for these competitive antagonists is closer to the β and γ subunits than to the δ subunit of the receptor or that the [^{3}H]methyl-α-neurotoxin binding site has been reoriented. Most of the cross-linking to the α subunit in unreduced membranes appears to occur via α-neurotoxin bound at the low-affinity tubocurarine site. *d*-Tubocurarine at high concentrations blocks the cross-linking to all subunits.

Because both *d*-tubocurarine (Adams, 1975; Manalis, 1977; Katz & Miledi 1978) and gallamine (Colquhoun & Sheridan, 1981) have been reported to have channel blocking type activity in addition to their competitive blockage of agonist binding, we have also examined the effect of a noncompetitive channel blocker, procaine, on the [^{3}H]methyl-α-neurotoxin cross-linking patterns. Unlike *d*-tubocurarine and gallamine, procaine appears to affect the cross-linking of the toxin to all of the subunits of the receptor to a similar extent, and therefore, binding of *d*-tubocurarine and gallamine to a local anesthetic site does not explain their selective inhibition of [^{3}H]methyl-α-neurotoxin cross-linking to the β and γ subunits.

Alkylation of reduced membranes has been shown to result in further changes in receptor function (Walker et al., 1981; Blanchard et al., 1982). Alkylation of reduced membranes does not appear to result in further changes in the appearent K_d for [^{3}H]methyl-α-neurotoxin binding but changes the pattern produced by DTSP–[^{3}H]methyl-α-neurotoxin cross-linking to receptor-rich membranes. There is a decrease in Tx–α and increases in Tx–β and Tx–γ.

d-Tubocurarine also alters the cross-linking of [^{3}H]-methyl-α-neurotoxin to reduced and alkylated membranes but over a narrower concentration range, as would be expected from the changes in the apparent K_i's of *d*-tubocurarine inhibition of [^{3}H]methyl-α-neurotoxin binding. The ratio of K_{i_2} to K_{i_1} is higher for unreduced membranes than for reduced and alkylated membranes. *d*-Tubocurarine inhibits the cross-linking of [^{3}H]methyl-α-neurotoxin to γ to a greater extent than to the other subunits. In contrast to the situation observed with unreduced membranes, Tx–β is not decreased as much as Tx–γ, suggesting that toxin occupying the site with lower affinity for *d*-tubocurarine can cross-link to β in reduced and alkylated membranes, while formation of Tx–β in unreduced membranes occurs primarily via toxin bound at the high-affinity *d*-tubocurarine site.

Reaction of reduced membranes with (bromoacetyl)choline has been shown to block the binding of α-neurotoxin preferentially to one of its two binding sites. BAC reaction of

reduced membranes prior to NEM alkylation affects the cross-linking of toxin to α, β, and δ to a greater extent than to γ. The magnitude of the changes suggest that toxin occupying either site can cross-link to α, β, or δ, but only toxin occupying the site which reacts at high concentrations of BAC can cross-link to γ. One interpretation of this finding is that the low-affinity binding site for BAC is the high-affinity site for *d*-tubocurarine. This interpretation is supported by the findings of Lindstrom et al. (1983) and Hall et al. (1983). Alternatively, covalently bound BAC may be desensitizing the receptor and, thereby, causing reorientation of the γ subunits.

In Figure 7, several possible arrangements of the receptor subunits are shown. Our data suggest that toxin bound to one site can cross-link fairly selectively to the β and γ subunits of the unreduced receptor. As the toxin binding sites are probably located on the α subunits of the receptor and it is unlikely that the two α subunits are adjacent (Karlin, 1983), we propose that the models shown in either part A or part B of Figure 7 (or their mirror images) are the most probable arrangements of the subunits of the receptor. Our results with reduced and alkylated membranes suggest that toxin bound at either site can cross-link to α, β, and δ, but only toxin bound at the high-affinity site for *d*-tubocurarine can cross-link to γ. And if the high-affinity site for *d*-tubocurarine is the low-affinity site for BAC, we suggest that the $\alpha\beta\alpha\gamma\delta$ arrangement shown in Figure 7 is the most probably arrangement of the receptor subunits at the surface of the membrane.

In summary, we show that (1) certain competitive antagonists which have two binding sites on the receptor can be used to selectively inhibit toxin cross-linking at the higher affinity site, (2) this site for both gallamine and *d*-tubocurarine appears to be closer to the β and γ subunits of the receptor than to the δ subunit, (3) differential inhibition of cross-linking to β and γ is not seen with a noncompetitive antagonist, and finally (4) (bromoacetyl)choline at low concentrations, in contrast to the the competitive antagonists, does not affect toxin cross-linking to the γ subunit but reduces the cross-linking to α, β, and δ. One model for the arrangement of the subunits of the receptor which is consistent with these findings is one in which the β subunit lies between the two α subunits.

Registry No. *d*-Tubocurarine, 57-94-3.

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Bacteriophage T7 E Promoter: Identification and Measurement of Kinetics of Association with *Escherichia coli* RNA Polymerase†

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ABSTRACT: The initiation point for transcription from the *Escherichia coli* RNA polymerase E promoter on bacteriophage T7 has been determined to be at 36 835 base pairs (92.22 T7 units) from the left end of T7. The location was determined by RNA fingerprinting of a runoff transcription product. Kinetics of association for the E and the T7 A3 promoters were measured by using the abortive initiation assay for approach to steady-state turnover. The kinetic association constant, k_a ($=K_B k_2$), for E was found to be over 10-fold slower than k_a for A3. For the E promoter, $k_a = 1.2 \times 10^6$ M^{-1} s^{-1}. For A3, we report $k_a \geq 4 \times 10^7$ M^{-1} s^{-1}. This difference is due mostly to a 10-fold difference in the initial equilibrium constant, K_B, for formation of the initial polymerase–promoter complex. The rate of isomerization, k_2, of the initial complex to the open polymerase–promoter complex for the E promoter was only 2-fold slower than k_2 for the A3 promoter. Various numerical methods for calculation of the kinetic parameters are discussed and compared. We argue that a nonlinear analysis provides the most reliable means of data analysis.

Regulation of transcription is essential for the control of gene expression. Transcriptional regulation can occur by the presence or absence of effector molecules or simply by the differential affinities of promoters for RNA polymerase (RNAP).[1] Bacteriophage T7 is a standard example of the latter. During infection of *Escherichia coli*, the early region of T7 is transcribed by RNAP from the three tandem A promoters (A1, A2, and A3) at the left end of T7 (see Figure

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[1] Abbreviations: RNAP, RNA polymerase; poly[d(A-T)], poly[d(A-T)·d(A-T)]; poly[d(I-C)], poly[d(I-C)·d(I-C)]; DTT, dithiothreitol; bp, base pair(s); RNase, ribonuclease; TE, 10 mM Tris, pH 8 at 25 °C, and 1 mM EDTA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylenimine); TLC, thin-layer chromatography.

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