Formation of Disulfide-Linked Oligomers of Acetylcholine Receptor in Membrane from Torpedo Electric Tissue†

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ABSTRACT: Acetylcholine receptor in electric tissue of *Torpedo californica* occurs predominantly as a disulfide cross-linked dimer which is converted to monomer by dithiothreitol. Reduced monomers in membrane (but not in solution) are cross-linked to dimers and higher oligomers in the presence of oxidizing agents. The cross-links are disulfide bonds, probably symmetrical, between identical chains on separate monomers. In native dimer, the disulfide cross-links are between δ chains. The oxidizing agent, diamide, however, preferentially cross-links reduced monomers via β chains. Copper phenanthroline cross-links reduced monomers via δ chains and β chains. The sulfhydryl groups involved in cross-linking β chains preexist dithiothreitol reduction; the sulfhydryls involved in cross-linking δ chains are generated by dithiothreitol reduction. The specificity of the oxidative cross-linking reactions leading exclusively to dimers of β chains and/or of δ chains and the kinetics of the loss of receptor monomer and the generation of receptor oligomers with each of the oxidizing agents are consistent with the receptor monomer in membrane containing one β chain and one δ chain as in the proposed chain composition [Reynolds, J. A., & Karlin, A. (1978) *Biochemistry 17*, 2035] of $\alpha_2\beta\gamma\delta$. The kinetics of the reactions are also consistent with the random diffusion in two dimensions of the reacting receptor species in the membrane; i.e., it is not necessary to invoke specific association between reduced monomers in the membrane. The functional significance of disulfide cross-linked receptor dimers in torpedo electroplax is not obvious; such dimers are not present in electrophorus electroplax and are apparently not necessary for the translocation of sodium ions by activated receptor in torpedo or electrophorus.

The nicotinic acetylcholine receptors convert the binding of acetylcholine into a change in membrane permeability to cations. These receptors are assayed in isolation by their binding of acetylcholine and congeners and of α-neurotoxins and by their reaction with affinity labels and have been purified based on such assays by affinity chromatography in detergent solution (for reviews see Karlin, 1977, and Heidmann & Changeux, 1978). The receptor purified from the electric tissue of *Torpedo californica* occurs as a mixture of monomer and dimer of molecular weights 250 000 and 500 000, respectively (Reynolds & Karlin, 1978). Two sites bind acetylcholine or α-neurotoxin per 250 000 daltons of receptor protein, but only one of the two sites is susceptible to affinity labeling by 4-(*N*-maleimido)benzyltrimethylammonium iodide (Damle & Karlin, 1978). Torpedo receptor, when reduced, saturated with sodium dodecyl sulfate, and subjected to polyacrylamide gel electrophoresis, yields four bands corresponding to chains of apparent molecular weights of approximately 39 000 (α), 48 000 (β), 58 000 (γ), and 64 000 (δ) (Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1976; Chang & Bock, 1977; Lindstrom et al., 1978). These chains are also prominent components of receptor-rich plasma membrane fractions of torpedo electric tissue (Reed et al., 1975; Hamilton et al., 1977; Sobel et al., 1977, Hucho et al., 1978).

The function of only one of the four types of chains associated with purified receptor is known. The α chain is uniquely and specifically affinity labeled by 4-(*N*-maleimido)benzyltrimethylammonium iodide in mildly reduced receptor from *T. californica* (Weill et al., 1974; Karlin et al., 1975), *T. marmorata* (Sobel et al., 1978), and *Electrophorus electricus* (Reiter et al., 1972; Karlin & Cowburn, 1973; Karlin et al., 1975). This localization of the site of reaction and the known physiological effects of mild reduction and affinity alkylation (Karlin, 1969) lead to the conclusion that the α chain bears all or part of a functionally significant acetylcholine binding site. In receptor purified from denervated rat muscle two chains are specifically affinity labeled, one of which approximately corresponds in electrophoretic mobility to the α chains of receptors from electric organs (Froehner et al., 1977).

The chains other than α are associated with purified Torpedo receptor in a well-defined complex (Karlin et al., 1975; Reynolds & Karlin, 1978; Hucho et al., 1978; but cf. Sobel et al., 1978). There is additional evidence that these chains are integral components of the receptor. Immunization of rats with each of the isolated chains induces the production of antibodies which cross-react with rat muscle receptor (Lindstrom et al., 1978). The individual chains do not, however, cross-react among themselves (Lindstrom et al, 1978; Claudio & Raftery, 1977). Rat receptor, therefore, has antigenic determinants comparable to those present on α, β, γ, and δ individually. Furthermore, [^{3}H]methyl-α-neurotoxin can be covalently cross-linked to the δ chain as well as to the α chain in receptor-rich membrane from *T. californica* electric tissue; thus, the δ chain is at least in close proximity to an α-neurotoxin binding site in the membrane (Hamilton et al., 1978).

Although a physiological role cannot yet be assigned to the δ chain, there is evidence for a structural role. *T. californica* receptor, in crude detergent extracts of membrane and in purified form, contains a disulfide cross-linked dimer of δ (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977). The dimer of δ (δ_2) is a component of the dimer (but not the monomer) form of receptor (Hamilton et al., 1977). Receptor dimer is converted to monomer by reducing agents (e.g., dithiothreitol) (Sobel et al., 1977; Chang & Bock, 1977; Hamilton et al., 1977), and concomitantly δ_2 is converted to δ (Chang & Bock, 1977; Hamilton et al., 1977). About two-thirds of receptor in Triton X-100 extracts of receptor-rich membrane from *T. californica* electric tissue is in the form of dimer, and dimer is covalently cross-linked by disulfide

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bridges mainly between δ chains, one δ in each monomer (Hamilton et al., 1977). It is not known whether or not in the membrane receptor dimers further associate noncovalently.

In order to probe the state of association and the chain composition of the receptor in the membrane, we have applied agents, such as diamide (Kosower et al., 1969; Hosey et al., 1978) and copper *o*-phenanthroline (Kobashi, 1968; Steck, 1972; Wang & Richards, 1974; Kyte, 1975; Giotta, 1976; Murphy, 1976), which form cross-links by oxidizing sulfhydryl groups to disulfides. We find that reduced receptor monomer is reoxidized in the membrane, but not in solution, to dimer and to higher oligomeric forms. Cross-linking is between identical chains, either β chains or δ chains. Diamide and copper phenanthroline, however, preferentially cross-link different pairs of chains.

Experimental Procedures

Solutions frequently used are as follows: NP50—50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, and 3 mM NaN_3 (pH 7.0); NP150—as NP50 except 150 mM NaCl; NT150—as NP150 except 20 mM Tris buffer (pH 8.3) instead of $NaPO_4$; TNP50—0.2% Triton X-100 in NP50; TNP150—0.2% Triton X-100 in NP150; TNT150—0.2% Triton X-100 in NT150; NCM—100 mM NaCl, 1 mM $CaCl_2$, and 10 mM Mops (pH 7.4); MEP—10 mM Mops,[1] 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (pH 7.4).

Diamide [diazenedicarboxylic acid bis(dimethylamide)] was obtained from Calbiochem and copper *o*-phenanthroline from Sigma Chemical Co. [^{3}H]Methyl-α-neurotoxin from *Naja naja siamensis* was prepared and its binding to receptor assayed as before (Damle & Karlin, 1978).

Receptor-rich membranes were isolated from the electric tissue of *T. californica* by a modification of a procedure of Sobel et al. (1977). Typically 120 g of torpedo electric tissue (stored in liquid nitrogen) was thawed, minced with scissors in 100 mL of MEP, homogenized in a 1-qt container of a 1-gal capacity commercial Waring Blendor for 2 min at a setting of high, and centrifuged at 6000 rpm for 10 min in a Beckman 60 Ti rotor. The pellet was suspended in 50 mL of MEP, homogenized, and centrifuged as above. The second supernatant was removed and pooled with the first, and the combined supernatants were centrifuged at 45000 rpm for 30 min. The resultant pellet was resuspended in 14 mL of MEP, and 7-mL portions were layered over 15 mL of 35% (w/w) sucrose which was over 5 mL of 44% sucrose, in MEP, and centrifuged at 60000 rpm for 2 h. A dense band at the top of the 35% sucrose layer was removed (along with the clear solution above it) and discarded. All material below this band and above the pellet was collected, diluted with one volume of MEP, and centrifuged at 45000 rpm for 30 min. The pellet was resuspended in 10 mL of MEP. Membrane prepared in this manner contains 2–3 mg of protein/mL and 0.7–1.5 nmol of α-neurotoxin binding sites/mg of protein and was used for most experiments. For further purification, the membranes were layered on 32–44% (w/w) sucrose density gradients, 35-mL volume, and centrifuged at 27000 rpm for 12–14 h in a SW 27 rotor. One-milliliter fractions were collected and assayed for protein and toxin binding activity. The fractions with the highest specific activity occur between 36 and 39% sucrose and contain 5–10 mg of protein and 2–4 nmol of α-neurotoxin binding sites per mg of protein.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; Cu(*o*-phen)$_2$, copper bis(*o*-phenanthroline).

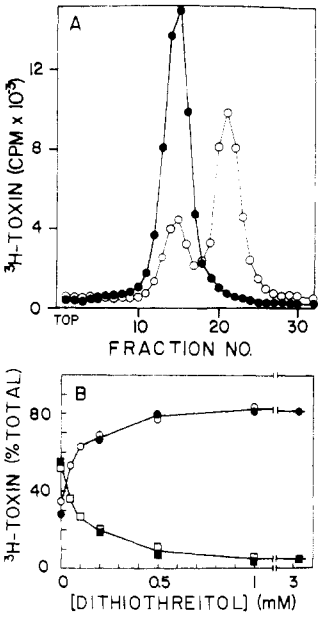

FIGURE 1: The conversion of receptor dimer to monomer by dithiothreitol. To 25 μL of a suspension of membrane containing 1.2 nmol of α-neutrotoxin binding sites and 1.8 mg of protein per mL of MEP was added 25 μL of dithiothreitol in NT150 (pH 8.2), to give pH 8.0 and a final concentration of dithiothreitol (A) of 10 mM or (B) as indicated on the abscissa. After 30 min at 25 °C, additions were made as follows: 5 μL of 0.56 M NaH_2PO_4 (pH 6.7), to bring the pH to 7.0, 50 μL of 1% Triton X-100 in NP50, to solubilize the receptor, and 10 μL of 2.6 μM [^{3}H]methyl-α-neurotoxin, sufficient to saturate 87% of the binding sites. After 30 min at 4 °C, 100 μL of the mixture was layered over a 17-mL, 5–20% sucrose density gradient, containing also TNP50, and the mixture was centrifuged in a Beckman SW 27.1 rotor at 27000 rpm for 19 h at 5 °C. Fractions of 0.3 mL were collected in vials, 5 mL of Scintisol Complete (Isolab) was added, and the ^{3}H activity was determined by scintillation counting. (A) The distribution of ^{3}H radioactivity in the gradient in the absence of dithiothreitol (○) and after reduction with 10 mM dithiothreitol (●). (B) The fractions of the total recovered [^{3}H]-methyl-α-neurotoxin in the monomer (○, ●) and dimer (□, ■) peaks plotted vs. the dithiothreitol concentration. The results of two independent experiments are shown.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out using either the system of Laemmli (1970) (gel system A) or the system of Fairbanks et al. (1971) as modified by Karlin et al. (1976b) (gel system B). The two-dimensional electrophoresis was performed as previously described (Hamilton et al., 1977).

Results

Oxidation of Reduced Receptor Monomers to Oligomers. Both in membrane (Figure 1A) and in solution, receptor dimer is converted to monomer by reduction of disulfide bonds (Hamilton et al., 1977; Chang & Bock, 1977; Sobel et al., 1977). Reaction of the receptor in membrane with 1 mM dithiothreitol results in nearly complete conversion of the dimer form to monomer (Figure 1B). It should be noted that 0.2 mM dithiothreitol, which under the same conditions completely reduces a disulfide bond in the vicinity of the acetylcholine binding site (Karlin et al., 1976a), does not completely convert receptor dimer to monomer.

Diamide, which oxidizes sulfhydryl groups to disulfides (Kossower et al., 1969), converts reduced receptor monomer in the membrane to oligomers (Figure 2). At 100 μM diamide and 4 °C, 75% of reduced monomer is converted to oligomers, 85% of which is dimer (Figure 2 inset). At 25 °C (not shown) 100 μM diamide converts 85% of monomer to oligomers, only 25% of which is dimer.

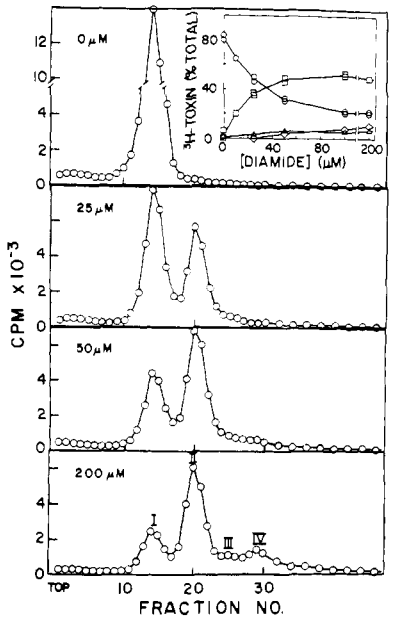

FIGURE 2: The conversion of reduced receptor monomer to oligomers by diamide. To 150 μL of membrane was added 150 μL of 2 mM dithiothreitol. The compositions of the mixtures were otherwise the same as those in the legend of Figure 1. After 30 min at 25 °C, 50 μL of water was added, and the mixture was centrifuged in two tubes in a Beckman Airfuge at 100 000 rpm (~160000*g*) for 3 min. The pellets were each resuspended in 175 μL of cold NP50 solution by repeated uptake and expulsion through an Eppendorf pipet tip. The centrifugation and resuspension were repeated. To 50-μL aliquots of the suspension was added 50 μL of cold diamide solution in NP50. After 30 min at 4 °C, 10 μL of 10% Triton X-100 and 10 μL of 2.3 μM [^{3}H]methyl-α-neurotoxin (to saturate 90% of available sites) were added. Aliquots of 100 μL were subjected to sucrose density gradient centrifugation as in Figure 1. The main figure shows representative distributions of receptor tagged with [^{3}H]methyl-α-neurotoxin, after reaction with the indicated concentrations of diamide and sucrose density gradient centrifugation. The inset shows the fraction of recovered ^{3}H activity in the monomer (○), dimer (□), trimer (△), and tetramer (◇) peaks plotted against the concentration of diamide. In the bottom panel, for example, the monomer peak was taken as tubes 11–17, dimer 18–24, trimer 24–27, and tetramer 28–31.

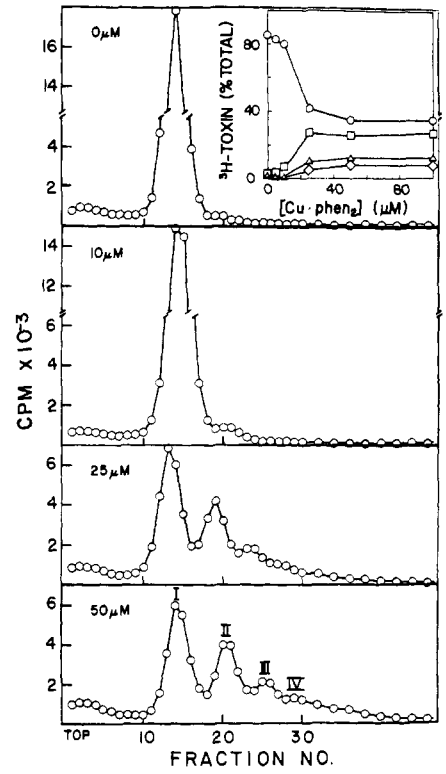

FIGURE 3: The conversion of reduced receptor monomer to oligomers by copper *o*-phenanthroline. Membrane was reacted with dithiothreitol and pelleted as in Figure 2. The pellets were resuspended in a total volume of 160 μL of NCM. To 25-μL aliquots was added 25 μL of $CuSO_4$–*o*-phenanthroline (1:2) in the same buffer, well oxygenated. After 3 min at 4 °C, the reaction was stopped and the receptor solubilized by the addition of 50 μL of 1% Triton X-100, 100 mM NaCl, 10 mM Mops, and 2 mM EDTA (pH 7.4). Twenty microliters of 1.3 μM [^{3}H]methyl-α-neurotoxin was added and the mixtures were subjected to sucrose density gradient sedimentation as in Figure 2. The main figure shows representative distributions of receptor in sucrose density gradients after reaction with the indicated concentration of Cu(*o*-phen)$_2$. The inset shows the fraction of recovered ^{3}H activity in the monomer (○), dimer (□), trimer (△), and tetramer (◇) peaks plotted against the concentration of Cu(*o*-phen)$_2$.

The receptor forms are identified in the sucrose density gradients by the radioactivity of the bound α-[^{3}H]neurotoxin. The sequential reduction and oxidation of the receptor results in no loss of α-neurotoxin binding activity. Furthermore, all α-neutrotoxin binding activity in the membrane remains soluble in Triton X-100 solution. Extraction of reduced monomer in Triton X-100 does not in itself result in oxidation to oligomers (Figure 2, top panel). In fact, addition of 100 μM diamide to a Triton X-100 extract of reduced receptor does not result in any conversion of monomer to oligomers (not shown); this conversion takes place only in the membrane. Thus, the distribution of bound α-neurotoxin found after sucrose density gradient centrifugation of Triton X-100 extracts of membrane treated as in Figure 2 represents the distribution of receptor forms present in the membrane.

The components labeled I and II have been isolated from identical gradients and positively identified by specific activity, polypeptide composition, and molecular weight as monomer and dimer (McNamee et al., 1975; Karlin et al., 1975; Reynolds & Karlin, 1978). The components labeled III and IV are, however, only tentatively identified as trimer and tetramer by their positions in the gradient. It should be noted that diamide generates approximately equal amounts of III and IV (Figure 2 inset).

Copper *o*-phenanthroline, which in the presence of oxygen oxidizes sulfhydryl groups to disulfides (Kobashi, 1968), also converts reduced receptor monomer in the membrane to oligomers (Figure 3). At 50 μM copper bis(*o*-phenanthroline) and 4 °C, 60% of reduced monomer is converted to oligomers, 50% of which is dimer (Figure 3 inset). There is somewhat greater production of III than of IV; also, copper phenanthroline produces more of III than does diamide. As with diamide, the reaction of copper phenanthroline with the reduced receptor results in no loss in α-neurotoxin binding activity compared with untreated membrane, and all receptor remains extractable in Triton X-100 solution. There is no conversion to oligomers when reduced receptor monomer in Triton X-100 is reacted with copper phenanthroline; as with diamide, the conversion occurs only in membrane.

The conversion of reduced monomer to oligomers is due to oxidation of sulfhydryl groups. This is shown by the effect of reacting the reduced receptor in the membrane with 5 mM *N*-ethylmaleimide before either diamide or copper phenanthroline; in these cases there is no conversion of monomer to oligomers. The question arises as to what extent the polymerization of reduced monomers by oxidation involves sulfhydryls present before reduction. This question was addressed by reacting receptor in the membrane first with *N*-ethylmaleimide, second with dithiothreitol, and finally with either diamide or copper phenanthroline (Figure 4). These reactions caused no loss in α-neutrotoxin binding. After *N*-ethylmaleimide, receptor in membrane is converted as before

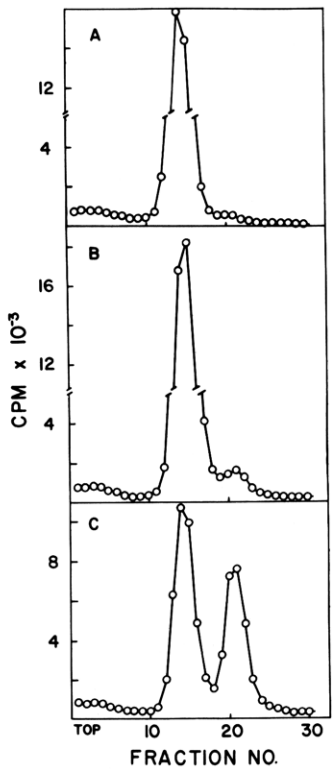

FIGURE 4: The effect of the alkylation with *N*-ethylmaleimide of sulfhydryl groups available before reduction of receptor with dithiothreitol on the subsequent conversion of reduced monomer to oligomers by diamide or copper *o*-phenanthroline. To 300 μL of membrane was added 300 μL of 10 mM *N*-ethylmaleimide in NP50. After 30 min at 4 °C, 100 μL of water was added, and the mixture was divided among four tubes and centrifuged in a Beckman Airfuge at 100 000 rpm for 3 min. Each pellet was resuspended in 175 μL of 2 mM dithiothreitol in 50 mM Tris buffer (pH 8.0). After 30 min at 25 °C, the mixtures were centrifuged as before. The pellets were resuspended in 175 μL of water, centrifuged, and finally resuspended in water and pooled in a total volume of 325 μL. (A) To 100 μL of the suspension was added 100 μL of NP50. After 30 min at 4 °C, 200 μL of 1% Triton X-100 in NP50 was added. (B) To 100 μL of the suspension was added 100 μL of 200 μM diamide in NP50. After 30 min at 4 °C, 200 μL of 1% Triton X-100 in NP50 was added. (C) To 100 μL of the suspension was added 100 μL of 100 μM Cu(*o*-phen)$_2$ in NCM. After 3 min at 4 °C, 200 μL of 1% Triton X-100 in NP50 was added. To 20-μL aliquots of mixtures A, B, and C was added 20 μL of 2.3 μM [^{3}H]methyl-α-neurotoxin. Duplicate 100-μL aliquots were subjected to sucrose density gradient centrifugation, fractionation, and counting as in Figure 1. The gradients were featureless beyond fraction 30.

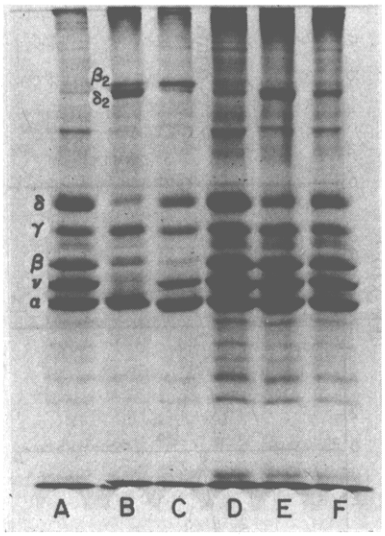

FIGURE 5: The effects on the polypeptide components of receptor-rich membrane of sequential alkylation, reduction, and oxidation, analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. To 175-μL aliquots of membrane, containing 1.5 nmol of α-neurotoxin binding sites and 0.9 mg of protein per mL, was added either (A, B, C) 5 μL of water or (D, E, F) 5 μL of 180 mM *N*-ethylmaleimide. After 30 min at 4 °C, the two mixtures were centrifuged in a Beckman Airfuge at 100 000 rpm for 3 min. The pellets were each resuspended in 175 mL of 2 mM dithiothreitol in 50 mM Tris buffer (pH 8.0). After 30 min at 25 °C, the mixtures were centrifuged as before and the pellets resuspended in 175 μL of water. This was repeated except each pellet was resuspended in 275 μL of water. To 85-μL aliquots of the suspensions was added (A, D) 85 μL of NP50, or (B, E) 85 μL of 100 μM Cu(*o*-phen)$_2$ in NCM100, or (C, F) 85 μL of 200 μM diamide in NP50. After either (A, C, D, F) 30 min at 4 °C or (B, E) 3 min at 4 °C, 5 μL of 175 mM *N*-ethylmaleimide was added to alkylate all remaining, accessible sulfhydryl groups. After 30 min at 4 °C, the mixtures were centrifuged as before, and the pellets were each resuspended in 100 μL of 2 mM *N*-ethylmaleimide–2% Triton X-100 in NP50. Twenty-five microliters of each mixture was tagged with [^{3}H]methyl-α-neurotoxin and analyzed by sucrose density gradient centrifugation, as in Figure 4. To each of the remaining 75-μL portions was added 750 μL of acetone, and after 20 min at 4 °C this mixture was centrifuged in a clinical centrifuge for 10 min. The pellets of precipitated protein were dried in vacuo, resuspended in 50 μL of sample buffer A (i.e., for the Laemmli system), and held at 50 °C for 1 h. Twenty-five microliters was added to wells in slabs of gel system A (no reducing agent). Electrophoresis was at 50 V for 1 h and 100 V for about 3 h. Slabs were stained according to Fairbanks et al. (1971). Receptor chains were identified by comparison with purified receptor coelectrophoresed.

to the monomer form by dithiothreitol (Figure 4A); diamide, however, even at 1 mM concentration, fails to convert more than a small fraction (~5%) of monomer to dimer (Figure 4B). In contrast, copper phenanthroline converts 45% of monomer to dimer but in this case generates no oligomers larger than dimer (Figure 4C). Dimerization of receptor by diamide apparently requires sulfhydryls existing before reduction by dithiothreitol; dimerization by copper phenanthroline does not.

Two other oxidizing agents resemble diamide and copper phenanthroline in their action on reduced receptor in membrane. One of them, 5,5′-dithiobis(2-nitrobenzoic acid), at the optimal concentration of 100 μM in 100 mM NaCl, 10 mM Mops buffer (pH 7.4), converts in 30 min at 4 °C about 40% of reduced monomer to oligomers, of which 80% is dimer (not shown). This conversion, like that by diamide, is blocked by reaction of the membrane with *N*-ethylmaleimide before reduction. The second oxidizing agent, potassium ferricyanide, at the optimal concentration of 1 mM and otherwise under the same conditions as 5,5′-dithiobis(2-nitrobenzoic acid), converts about 25% of reduced monomer to oligomers, 80% of which is dimer (not shown). This conversion is blocked only 50% by reaction of the membrane with *N*-ethylmaleimide prior to reduction and thus resembles somewhat that of copper phenanthroline. It should be noted that 5,5′-dithiobis(2-nitrobenzoic acid) and potassium ferricyanide completely reoxidize a disulfide close to the acetylcholine binding site under conditions which result in only partial conversion of reduced monomer to oligomers (Karlin, 1973).

Oxidative Cross-Linking of Polypeptide Components of Receptor. In untreated membrane, receptor occurs mainly as a dimer which is cross-linked by disulfide bonds between δ chains (Hamilton et al., 1977; Chang & Bock, 1977). These disulfide bonds are reduced by dithiothreitol in the formation of reduced receptor monomer. The four polypeptide components (α, β, γ, and δ) associated with purified receptor from *T. californica* (Weill et al., 1974) are major components of the reduced membrane (Figure 5A). There are in addition other polypeptide components of the membrane (one of which is labeled ν) which are not associated with purified receptor.

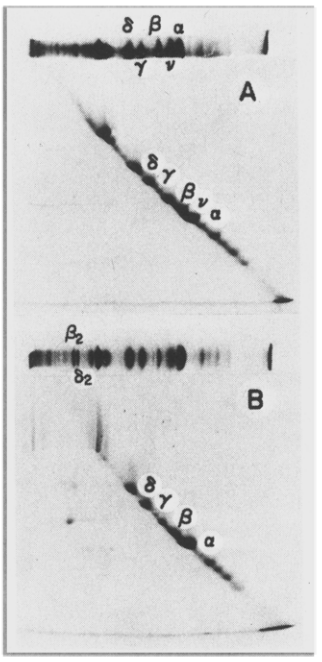

FIGURE 6: The effects of sequential reduction by dithiothreitol and oxidation by diamide on the polypeptide components of receptor-rich membrane analyzed by two-dimensional gel electrophoresis using the Laemmli system in both dimensions. In (A), membrane was reduced as in Figure 5A and in (B), membrane was reduced and treated with diamide as in Figure 5C. After electrophoresis of the samples in one direction as in Figure 5, the slab was sliced in columns and the resolving gel protion of each column was placed in hot 1% agarose containing 20 mM dithiothreitol in sample buffer A across the top of a second slab like the first. A duplicate slice of the first dimensional gel was stained and is shown above the second dimensional slab.

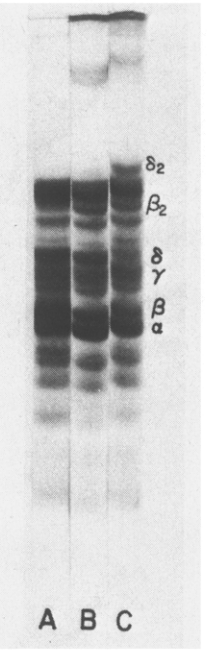

FIGURE 7: The effects of dithiothreitol (A) alone or followed by either (B) diamide or (C) copper phenanthroline on the polypeptide components of receptor-rich membrane analyzed by gel electrophoresis using a modified Fairbanks system (gel system B). The gels were 0.6 cm in diameter and 12-cm long. The membrane contained 0.5 nmol of α-neurotoxin binding sites per mg of protein. The reaction conditions for (A) were as in Figure 5A, (B) as in Figure 5C, and (C) as in Figure 5B. The samples were finally dissolved in sample buffer B.

The oxidation of reduced membrane with 50 μM copper phenanthroline generates two new components labeled (for reasons given below) δ_2 and β_2 and causes some loss of components β and δ (Figure 5B). More of δ_2 appears to be generated than of β_2 and more of δ is lost than of β. Oxidation of reduced membrane by 100 μM diamide also generates β_2 and δ_2, but more β_2 than δ_2, and more of β is lost than of δ (Figure 5C). Reaction of the membrane with 5 mM *N*-ethylmaleimide before reduction does not in itself alter the pattern of components (Figure 5D); neither copper phenanthroline (Figure 5E) nor diamide (Figure 5F), however, now generates β_2. Copper phenanthroline still generates considerably more δ_2 than does diamide.

The above results were obtained using the Laemmli gel system (system A; see Experimental Procedures). In this system, the apparent molecular weights of the components are α, 44 000; β, 52 000; γ, 59 000; δ, 66 000; δ_2, 170 000; and β_2, 192 000. The apparent molecular weights of the components α, β, γ, and δ differ by as much as 12% from those obtained in a modified Fairbanks system (gel system B; see Experimental Procedures; Weill et al., 1974). Furthermore, in gel system A the apparent molecular weight of β_2 is 3.6 times that of β and δ_2, 2.6 times that of δ.

The constituents of β_2 and δ_2 are revealed by two-dimensional gel electrophoresis in sodium dodecyl sulfate, in which the samples are not further reduced before the first-dimensional run (as in Figure 5) and are reduced completely prior to the second-dimensional run (Wang & Richards, 1974). All polypeptides with the same mobility in the first and second dimension will lie on a diagonal. Polypeptides which are part of a disulfide cross-linked aggregate during the first-dimensional run will lie to the left of the diagonal after the second-dimensional run. Since relative mobility is roughly proportional to logarithm of molecular weight, polypeptides which are in the form of disulfide cross-linked dimers in the first-dimensional run will lie on a line to the left of and parallel to the diagonal. Membrane samples were either reduced (Figure 6A) or reduced and reacted with diamide (Figure 6B). Both dimensions were run in gel system A. The components β_2 and δ_2, generated by diamide, give rise on reduction to β and δ, respectively (Figure 6B).

In gel system B (which gives more satisfactory results in cylindrical gels than in slabs), α, β, γ, and δ have apparent molecular weights of 39 000, 48 000, 58 000, and 64 000 (Weill et al., 1974). The components generated by reaction of reduced membrane with diamide (Figure 7B) or copper phenanthroline (Figure 7C), β_2 and δ_2, have apparent molecular weights in this system of 90 000 and 130 000, approximately twice the apparent molecular weights of β and δ, respectively. Two-dimensional gels, system B in the first dimension and system A in the second dimension, provide evidence again that β_2 is composed of two β's and δ_2 of two δ's (Figures 8 and 9); namely, a component with the same mobility as β in the second dimension is produced by reduction of a component, β_2, with twice the apparent molecular weight of β in the first dimension (Figures 8B and 9B) and similarly for δ and δ_2 (Figure 9B,C).

It is apparent from our analysis of the membrane using this two-dimensional system (B first, A second) that diamide generates more of β_2 than δ_2 (Figure 8B). The alkylation of sulfhydryls with *N*-ethylmaleimide prior to reduction blocks the generation by diamide of β_2, but not of the small quantity of δ_2 produced by diamide (Figure 8C). In the same two-dimensional system, it is seen that copper phenanthroline generates both β_2 and δ_2 in reduced membrane (Figure 9B) but only δ_2 in alkylated and reduced membrane (Figure 9C). (A dimer or trimer of ν gave rise to the spot of ν off the diagonal in Figure 9C.)

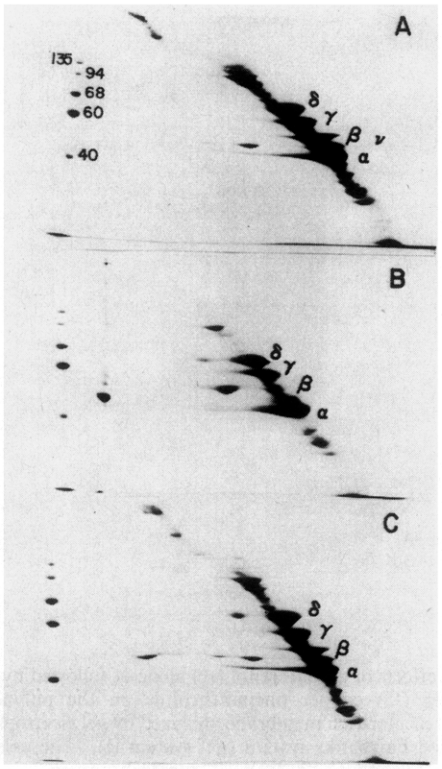

FIGURE 8: The effects on the polypeptide components of receptor-rich membrane of sequential alkylation with *N*-ethylmaleimide, reduction with dithiothreitol, and oxidation with diamide, analyzed by two-dimensional gel electrophoresis using gel system B in the first dimension and gel system A in the second. Aliquots of membrane containing 1.5 nmol of α-neurotoxin binding sites per mg of protein were treated as follows: (A) with dithiothreitol as in Figure 5A, (B) with dithiothreitol and diamide as in Figure 5C, and (C) with *N*-ethylmaleimide, dithiothreitol, and diamide as in Figure 5F. The membrane was pelleted as before and resuspended in 50-μL sample buffer B. After 1 h at 50 °C, 25-μL aliquots were layered on 3-mm diameter cylindrical polyacrylamide gels (system B) and electrophoresed at 0.5 mA/gel for 1 h and 2 mA/gel for 3 h. The gels were removed from the tubes and placed in a layer of hot 1% agarose containing sample buffer A with 20 mM dithiothreitol, over a 1.5-mm thick slab of polyacrylamide (system A). The second-dimension electrophoresis and staining were as in Figure 5. Protein standards were run at the left of the second-dimensional gel, and their molecular weights are indicated in (A). The first-dimensional gels are not shown.

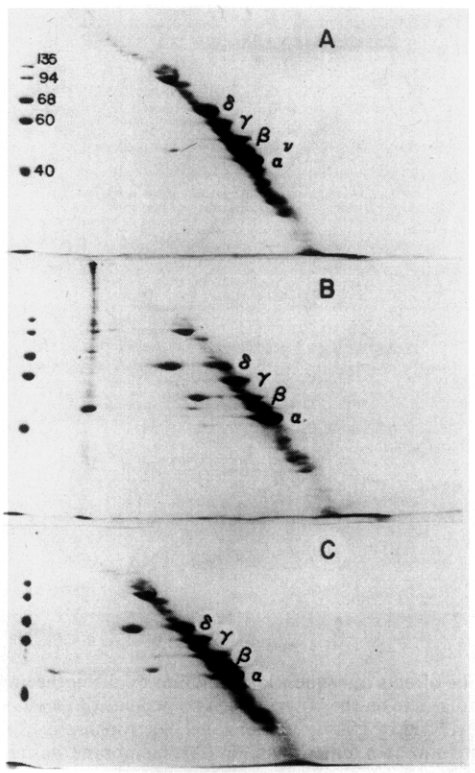

FIGURE 9: The effects on the polypeptide components of receptor-rich membrane of sequential alkylation, reduction, and oxidation with copper phenanthroline analyzed by two-dimensional gel electrophoresis. Membrane as in Figure 8 was treated as follows: (A) with dithiothreitol as in Figure 5A; (B) with dithiothreitol and copper phenanthroline as in Figure 5B; and (C) with *N*-ethylmaleimide, dithiothreitol, and copper phenanthroline as in Figure 5E. The copper phenanthroline reaction was stopped by the addition of 5 μL of 175 mM *N*-ethylmaleimide and 5 μL of 100 mM EDTA. The gels were prepared and run as in Figure 8. Molecular weight standards are at the left. The first-dimension gels are not shown.

It appears from the above that β contains free sulfhydryls prior to reduction of the membrane with dithiothreitol and that these sulfhydryls are accessible to and react with diamide and copper phenanthroline. This inference is supported by the effect of oxidation on the unreduced membrane. Both diamide and copper phenanthroline cause a partial conversion of both monomers and dimers present in untreated membrane to higher oligomeric forms. This conversion is accompanied in both cases by the generation of β_2. δ_2 is already present before oxidation and is not further polymerized (data not shown).

The sulfhydryl content of the membrane components can be demonstrated qualitatively by fluorographic analysis of the reaction with *N*-[^{3}H]ethylmaleimide. Reaction of unreduced membrane with *N*-[^{3}H]ethylmaleimide results in detectable labeling of all the receptor chains except α (Figure 10A). After reduction of the membrane with dithiothreitol, the labeling of all receptor chains increases, but α now labels most strongly (Figure 10B). There is no labeling at all if the membrane is reacted with nonradioactive *N*-ethylmaleimide first (Figure 10C); however, after *N*-ethylmaleimide first and dithiothreitol second, all receptor components react with *N*-[^{3}H]ethylmaleimide (Figure 10D). The block of the cross-linking of β's by *N*-ethylmaleimide treatment (Figures 4B, 5F, and 8C) must be due to the alkylation of a specific sulfhydryl group present before reduction. α chains, though rich in sulfhydryl groups after reduction, are not cross-linked under the conditions we have used.

It is apparent that the component designated ν is relatively rich in sulfhydryl groups (Figure 10A) and is probably identical with the "43 000 protein" observed by Sobel et al. (1978). Preparation of a membrane sample for gel electrophoresis even without the addition of oxidizing agents results in the polymerization of ν; this polymerization is largely blocked if, at some stage before the dissolution of the membrane in sodium dodecyl sulfate, an alkylating agent is added to the membrane. We have found that ν has the same electrophoretic mobility in gel system A as actin from striated muscle of torpedo and of rabbit.

Discussion

Receptor in plasma membrane fragments and presumably in the intact electroplax of *T. californica* electric tissue is cross-linked via disulfide bonds. The predominant form is dimer, and the cross-linking is almost exclusively between δ chains (Chang & Bock, 1977; Hamilton et al., 1977). The disulfide cross-links are readily reduced by dithiothreitol in membrane and in solution. The resulting reduced receptor monomers do not associate in solutions of Triton X-100 (Figure 1), Brij 58 (Reynolds & Karlin, 1978), or Ammonyx LO (not shown). Whether or not reduced monomers associate in the membrane is not clear. Reduced monomers in the membrane

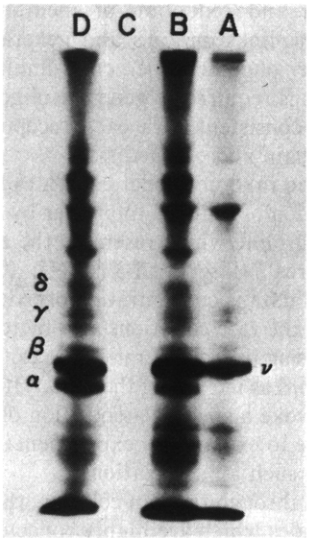

FIGURE 10: Fluorographic identification of polypeptide components of membrane reacting with *N*-[^{3}H]ethylmaleimide. Membrane containing 1.1 nmol of α-neurotoxin binding sites per mg of protein was reacted with (C, D) or without (A, B) 5 mM *N*-ethylmaleimide for 30 min at 4 °C. The membrane was pelleted as before and resuspended in either (A, C) 50 mM Tris buffer (pH 8.0) or (B, D) 2 mM dithiothreitol in Tris buffer. After 30 min at 4 °C (A, C) or 25 °C (B, D), the membrane was twice pelleted and resuspended in MEP. To 250 μL of suspension was added 5 μL of 13 mM *N*-[^{3}H]ethylmaleimide (25 mCi/mmol). After 30 min at 4 °C, the membrane was pelleted and resuspended in 100 μL of sample buffer A with 10 mM dithiothreitol and the suspension incubated at 50 °C for 1 h. Samples of 25 μL were layered on a slab gel (system A) and electrophoresed as before. The gel was stained as before and photographed. It was then prepared for fluorography by the procedure of Bonner & Laskey (1974). The film used was Kodak XR-5 and the exposure was for 24 h at −80 °C. (A) *N*-[^{3}H]Ethylmaleimide; (B) dithiothreitol and *N*-[^{3}H]ethylmaleimide; (C) *N*-ethylmaleimide and *N*-[^{3}H]ethylmaleimide; and (D) *N*-ethylmaleimide, dithiothreitol, and *N*-[^{3}H]ethylmaleimide.

are at least capable of collision, since disulfide cross-linking can be restored by reactions with oxidizing agents (Figures 2 and 3). Collisions between reduced monomers in detergent solution, however, are apparently not sufficiently frequent to lead to dimer formation with oxidizing agents. Either the concentrations of monomer in solution tested (~0.5 μM) are too low, or the detergent in the monomer–detergent complex prevents the juxtaposition of the appropriate sulfhydryls, or the orientation and/or configuration of monomer in the membrane which favors dimer formation cannot be duplicated in detergent solution. The question remains as to whether or not there is a specific association of reduced monomers in membrane which favors covalent dimerization. We will return to this question after consideration of the details of the disulfide cross-linking reactions.

It was surprising to discover that although native dimer is cross-linked via δ chains, oligomers formed by oxidation of reduced monomers were cross-linked via β as well as δ chains. Furthermore, β chains are cross-linked only to β chains and δ chains only to δ chains. Oxidation of reduced monomer generates one disulfide cross-linked polypeptide species composed of δ chains and one composed of β chains; i.e., in the two-dimensional gels, whether the first dimension is a Laemmli gel (Figure 6) or a Fairbanks gel (Figures 8 and 9), one species in the first dimension gives rise to almost all β off the diagonal in the second dimension, and similarly for δ. The apparent molecular weights of these two species, however, are different in the Laemmli and Fairbanks systems. In the latter system, the species which we call β_2 has an apparent molecular weight twice that of β and the species which we call δ_2 has an apparent molecular weight twice that of δ. In the Laemmli system δ_2 appears to have 2.6 times the apparent molecular weight of δ and β_2, 3.6 times the apparent molecular weight of β. Without further rationalization it may reasonably be concluded that δ_2 is a dimer of δ. In the case of β_2, we assume that the ratio of the apparent molecular weights of β_2 and β determined with the Fairbanks gel system are correct and that the apparent molecular weight of β_2 found with the Laemmli system is anomalous. Perhaps the β_2 dimer is running as a noncovalently associated dimer of dimers in the Laemmli system. Comparable dependence of apparent molecular weight on the gel system is seen, for example, with glycophorin (Silverberg & Marchesi, 1978).

The cross-linking of β to β_2 and of δ to δ_2 most likely involves disulfide bond formation between equivalent cysteinyl residues. An accessible cysteinyl residue on a δ chain in one monomer and the equivalent residue on a δ chain in a second monomer may be brought into juxtaposition by rotation and translation in the plane of the membrane and similarly for the β chain. (Similarly, an equivalent set of two or more cysteinyl residues on each monomer could be juxtaposed.) Given that the receptor in the membrane is not capable of translation normal to the plane of the membrane or rotation through the membrane, it would seem less probable that cysteinyl residues, other than pairs of equivalent residues (or pairs of equivalent sets of residues), could be brought into juxtaposition by translation and rotation restricted to the plane. Cross-linked receptor dimer, whether cross-linked through β's or δ's, is thus likely to have a twofold axis of symmetry, normal to the plane of the membrane, which passes through the disulfide bond.

It should be noted that in detergent solution oxidizing agents not only do not generate dimer from reduced monomer but also no β_2 or δ_2 are formed. β_2 and/or δ_2 are formed if and only if receptor dimers are formed. This is consistent with the proposed stoichiometry of the monomer of $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978); i.e., dimers of β or of δ can be formed only by intermonomer cross-linking. It should also be noted, however, that despite the proposed stoichiometry and a high sulfhydryl content following reduction (Figure 10), α chains are not oxidized to dimers either in solution or in membrane. The sulfhydryls on reduced α are apparently not positioned to form interchain or intermonomer disulfides; upon oxidation these sulfhydryls apparently re-form the original intrachain disulfides from which they were generated.

A second surprising discovery is that different oxidizing agents acting on reduced monomer in the membrane prefer different chains. Diamide oxidizes β to β_2 to a much greater extent than it oxidizes δ to δ_2. Copper phenanthroline oxidizes δ to δ_2 to a greater extent than it oxidizes β to β_2; the difference, however, is not as marked as it is with diamide (Figures 5, 8, and 9). The differentiation of the pathways of oxidation is clarified by the effects of alkylation with *N*-ethylmaleimide before reduction. Following reduction of alkylated receptor in membrane, diamide generates about 5% as much dimer as it does in the absence of alkylation (cf. Figures 2 and 4), and as expected no β_2 is generated (Figures 5 and 8C). Copper phenanthroline under the same conditions generates dimer (Figure 4) through δ_2 but not through β_2 (Figures 5 and 9C). The explanation for the differences in the preferred sites of reaction of diamide and copper phenanthroline is not obvious. Differential accessibility of the reagents due to steric factors or to differences in lipophilicity could be involved. It should be noted that the membrane used in these experiments is largely in the form of closed vesicles with the extracellular surface out; namely, these vesicles have

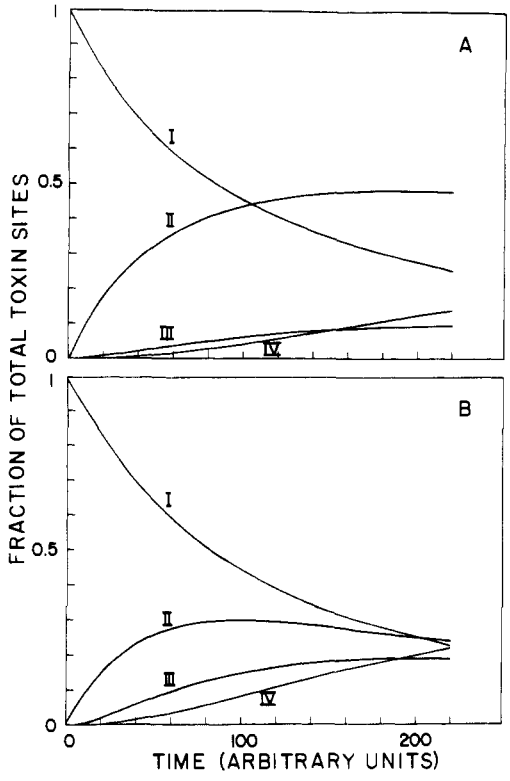

FIGURE 11: The theoretical time dependence of the relative concentrations of monomer (I), dimer (II), trimer (III), and tetramer (IV). The concentrations are expressed in terms of the fraction of total α-neurotoxin binding sites which are contained in each species. (A) The time course of the reaction with diamide, assuming diamide cross-links β chains 10-fold faster than δ chains. (B) The time course of the reaction with copper phenanthroline, assuming equal rates of cross-linking β chains and δ chains. See Appendix for equations.

contained volume for Na^+, and there is equal binding of the impermeable α-neurotoxin to the vesicles and to the vesicles solubilized in detergent (unpublished observations). The vesicles also appear closed in electron micrographs (Karlin et al., 1978).

In unreduced receptor in membrane, δ is mostly in the form of δ_2. Under these circumstances both diamide and copper phenanthroline generate higher oligomers by cross-linking β's. Monomers and dimers in unreduced receptor are either in association or are mobile and capable of collision.

In trying to decide whether the course of the formation of oligomers might imply that reduced monomers were associated in the membrane, we analyzed a model of the reactions assuming random collisions. It is assumed that β cross-links only to β and δ to δ and that the rates of these reactions are independent of oligomer size. In the case of diamide, it is assumed that the rate of cross-linking β's is 10-fold the rate of cross-linking δ's (Figure 11A). In the case of copper phenanthroline, it is assumed that the rate of cross-linking β's is equal to the rate of cross-linking δ's (Figure 11B). The rate equations (Appendix) were solved numerically, and the results are expressed in terms of the fraction of the total number of α-neurotoxin binding sites associated with each form. In fact, each even-numbered oligomer consists of two subspecies, one having two uncross-linked β's and the other having two uncross-linked δ's. These are kinetically indistinguishable in reactions with copper phenanthroline, but diamide reacts much more slowly with the subspecies having free δ's than that having free β's. The result is that diamide generates relatively more even-numbered oligomers than does copper phenanthroline, almost all of which are subspecies with free δ's.

After alkylation and reduction of membrane, copper phenanthroline cross-links only δ's and generates receptor dimer and no higher oligomers; i.e., cross-linking of both β chains and δ chains is required to generate oligomers higher than dimer, again consistent with each receptor monomer containing one β chain and one δ chain.

It is clear that the random model generates time courses for the polymerization of reduced monomer by diamide and by copper phenanthroline which resemble the experimental curves seen in Figures 2 (inset) and 3 (inset). (The ordinate of the experimental data is concentration of oxidizing agent, but this enters into the rate equations as a factor in the rate constant, and increasing the concentration has exactly the same effect on the equations as increasing the time.) It is, therefore, not necessary to invoke a specific association of reduced receptor in membrane to explain our experimental results, but we cannot rule out such an association.

If cross-linking is through random collision, these collisions are between molecules which are highly concentrated in the membrane. There are of the order of 10^4 toxin binding sites/μm^2 (Karlin et al. 1978; reviewed by Heidmann & Changeux, 1978), and the average distance between receptor monomers, if not associated, would be of the order of the diameter of a monomer.

The functional significance of cross-linked dimer in native membrane is not obvious. Evaluating the functional effects of the reduction of dimer to monomer is complicated by the concomitant reduction of a disulfide near an acetylcholine binding site on the α chain (Weill et al., 1974). Reduction of receptor in membrane under conditions which should lead to complete conversion to monomer as well as to reduction of the α-chain disulfide leads to a decrease in the permeability response of torpedo membrane vesicles (Hazelbauer & Changeux, 1974) to receptor activators; however, this inhibition can be ascribed to the decreased binding of the activator and not to an inhibition of the cation translocation function of the receptor (Schiebler et al., 1977). This same conclusion was drawn concerning the effect of dithiothreitol on the response of the electroplax of electrophorus electric tissue (Karlin, 1969). Furthermore, in the case of torpedo membrane, the inhibition of the permeability response due to reduction of the receptor with 5 mM dithiothreitol is completely reversed by 5 μM dithiobischoline, which does not generate dimer (Wise & Karlin, unpublished results). We conclude that the acetylcholine-induced increase in cation permeability in torpedo membrane is not dependent on disulfide cross-links between receptor monomers. Because we have not excluded the possibility that even after reduction receptor monomers might associate noncovalently, we can not exclude the possibility that dimer might be involved in the permeability response. In electrophorus membrane there is no detectable cross-linked dimer, and treatment with dithiothreitol followed by either diamide or copper phenanthroline does not generate dimer.

The cross-linking of reduced receptor monomer in membrane through β chains and/or through δ chains is consistent with the status of the β and δ chains as integral stoichiometric components of the receptor both in the membrane and in detergent solution. The arrangement of these chains in the membrane and their contributions to the overall function of receptor are still to be determined.

Acknowledgments

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Appendix

Assume the stoichiometry of monomer is $\alpha_2\beta\gamma\delta$. Assume cross-linking involves formation of either β_2 or δ_2. Each monomer is divalent: it can react through one β and one δ. Monomer and odd oligomers therefore have one unreacted β and one unreacted δ, and even oligomers have either two unreacted β's or two unreacted δ's. Let the concentration of monomer be r_1, dimer r_2, trimer r_3, and tetramer r_4. Let b_2 and b_4 be the concentrations of dimer and tetramer, respectively, with two free β chains, and d_2 and d_4, the concentrations of dimer and tetramer with two free δ chains. By definition

$$r_2 = b_2 + d_2$$

$$r_4 = b_4 + d_4$$

If k_B is the rate of cross-linking of two β chains and k_D is the rate of cross-linking of two δ chains, the rate equations, ignoring species larger than tetramer, are

$$r_1' = -k_B r_1 \Sigma_B - k_D r_1 \Sigma_D$$

$$b_2' = \tfrac{1}{2} k_D r_1^2 - 2k_B b_2 \Sigma_B$$

$$d_2' = \tfrac{1}{2} k_B r_1^2 - 2k_D d_2 \Sigma_D$$

$$r_3' = 2k_B r_1 b_2 + 2k_D r_1 d_2 - k_B r_3 \Sigma_B - k_D r_3 \Sigma_D$$

$$b_4' = 2k_B b_2^2 + k_D r_1 r_3 - 2k_B b_4 \Sigma_B$$

$$d_4' = 2k_D d_2^2 + k_B r_1 r_3 - 2k_D d_4 \Sigma_D$$

where $\Sigma_B = r_1 + 2b_2 + r_3 + 2b_4$, $\Sigma_D = r_1 + 2d_2 + r_3 + 2d_4$, and prime indicates the derivative with respect to time. The corresponding difference equations were solved numerically, taking the initial value of $r_1 = 1$ and of $r_2 = r_3 = r_4 = 0$ and taking $k_B \Delta t = 0.01$ and $k_D \Delta t = 0.001$ for reaction with diamide (Figure 11A) and $k_B \Delta t = k_D \Delta t = 0.005$ for reaction with copper phenanthroline (Figure 11B). The relative concentration of α-neurotoxin binding sites is given by r_1, $2r_2$, $3r_3$, and $4r_4$, which are the quantities plotted in Figure 11.

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