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Affinity-Directed Cross-Linking of Membrane-Bound Acetylcholine Receptor Polypeptides with Photolabile α-Bungarotoxin Derivatives†

Veit Witzemann,‡ D. Muchmore,§ and Michael A. Raftery*

ABSTRACT: Photolabile derivatives of [^{125}I]-α-bungarotoxin that retain specific binding to *Torpedo californica* acetylcholine receptor have been utilized as structural probes of the receptor complex of polypeptide components in its membrane-associated form. The derivatized toxins contained aryl azide side chains poised to form covalent cross-links to both associated and adjacent polypeptides following toxin–receptor complex formation. The results demonstrate that, depending on the possible radius of extension of the photoactivated group from the parent toxin, either (1) both the polypeptide to which the toxin derivative binds and an adjacent polypeptide can be derivatized upon photolysis or (2) only the adjacent polypeptide is labeled. The results lend strong support to the notion that the nicotinic receptor from *T. california* is composed of a complex of different polypeptides.

Torpedo californica electric tissue has been one of the main sources of both highly enriched membrane preparations of AcChR[1] (Duguid & Raftery, 1973) in addition to providing starting material for the purification of detergent-solubilized receptor preparations (Raftery, 1973; Schmidt & Raftery, 1973a). Purified *T. californica* receptor is considered to consist of four types of polypeptide chains (Raftery et al., 1974, 1975; Weill et al., 1974; Karlin et al., 1975; Vandlen et al., 1976, 1979; Flanagan et al., 1975; Hamilton et al., 1977; Chang & Bock, 1977; Chang et al., 1977; Hucho et al., 1978; Lindstrom et al., 1978). The same polypeptides occurring in the same apparent ratios, as defined by Coomassie Blue stained bands in $NaDodSO_4$ gel electrophoresis, are present in enriched membrane preparations (Duguid & Raftery, 1973; Raftery et al., 1974; Reed et al., 1975; Witzemann & Raftery, 1977, 1978a,b). In addition, these membranes prepared by centrifugation procedures or by additional steps such as affinity partitioning (Flanagan et al., 1975) still contain a few additional polypeptides, notably those of molecular weight 43 000 and 90 000. It has recently been shown that highly enriched membranes can be depleted of polypeptides except those characteristic of purified AcChR by brief treatment at pH 11 under conditions of low ionic strength (Neubig et al., 1979; Elliott et al., 1979) following the procedures of Steck & Yu (1973) for selective extraction of red blood cell membrane proteins. Despite the identical subunit composition of detergent-extracted *T. californica* receptor purified by affinity chromatographic procedures and the membrane preparations, the question still remains as to whether these polypeptides are in actual physical contact within the postsynaptic membrane and naturally whether more than one of these is involved in synaptic transmission at the molecular level. We have adopted two main approaches in an attempt to answer this question. The first of these involves detection of conformational changes

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering (Contribution No. 5988), California Institute of Technology, Pasadena, California 91125. *Received May 3, 1979.* Supported by U.S. Public Health Service Grant NS 10294, by a grant from the Muscular Dystrophy Association of America, by a grant from the American Heart Foundation, and by a grant from the Deutscheforschungsgemeinschaft (to V.W.). A preliminary account of part of this research has been given [see Witzemann & Raftery (1978a)].

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[1] Abbreviations used: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; Me_2SO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; $IAcNH_2$, iodoacetamide; DEAE, diethylaminoethyl; Carb, carbamylcholine.

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in the constituent polypeptides of enriched membranes upon addition of a cholinergic ligand such as carbamylcholine. The probe used for these studies was [^{3}H]bis(azidoethidium bromide), and in the absence of cholinergic ligand this photoaffinity reagent labeled only the 40 000-dalton polypeptide of the enriched membranes (Witzemann & Raftery, 1978a). Following preincubation with Carb, which induces changes in AcChR binding affinity over a period of seconds to minutes, photolabeling resulted in a substantial increase in the amount of incorporation into the 40 000 polypeptide in addition to significant labeling of two of the other polypeptides (of molecular weight 50 000 and 65 000, respectively). We interpret this result as compelling evidence for conformational changes generated in the 40 000-dalton subunit, to which Carb binds, being communicated to the other two polypeptides. On the basis of this evidence, we therefore consider that at least these polypeptides (M_r 40 × 10^3, 50 × 10^3, and 65 × 10^3) form a structural complex within the postsynaptic membrane.

The second approach which we describe here is based on the known specificity of α-toxins for the cholinergic receptor and specifically for the 40 000-dalton polypeptide of *Torpedo* AcChR (Hucho et al., 1976; Witzemann & Raftery, 1977, 1978a,b). We have made use of selective reduction of one of the five disulfide bonds in α-BuTx following the methodology of Chicheportiche et al. (1975) to introduce covalently, via mixed disulfide bond formation, side chains of varying lengths containing photolabile groups having the potential of cross-linking the modified α-toxin to neighboring polypeptides upon photoactivation. The results show that the modified α-toxin containing a short photolabile side chain (~14 Å) can be cross-linked to the 40 000-dalton polypeptide in addition to some cross-linking to another polypeptide of 65 000 daltons which we have previously claimed to be part of the AcChR complex. Use of a longer (~33 Å) side chain resulted in selective cross-linking of the modified α-toxin to the 65 000-dalton polypeptide only. We interpret these results as further compelling support for the notion that the acetylcholine receptor is composed "in sensu stricto" of a complex of subunits of differing molecular weights in its native membrane environment.

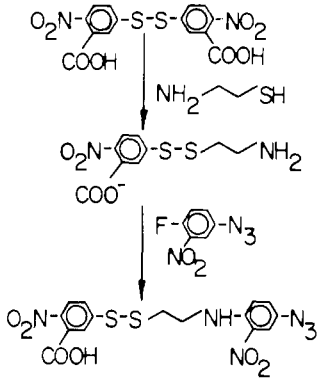
Experimental Section

Materials. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. ω-Aminododecanoic acid was from K & K Laboratories. 4-Fluoro-5-nitrophenyl azide was obtained from Pierce Chemical Co. 2-Aminoethanethiol was from Eastman Kodak Co. α-BuTx was purified from crude fractions of *Bungarus multicinctus* venom, obtained from Miami Serpentarium, as described by Clark et al. (1972). [^{125}I]-α-BuTx was prepared and assayed as described by Blanchard et al. (1979).

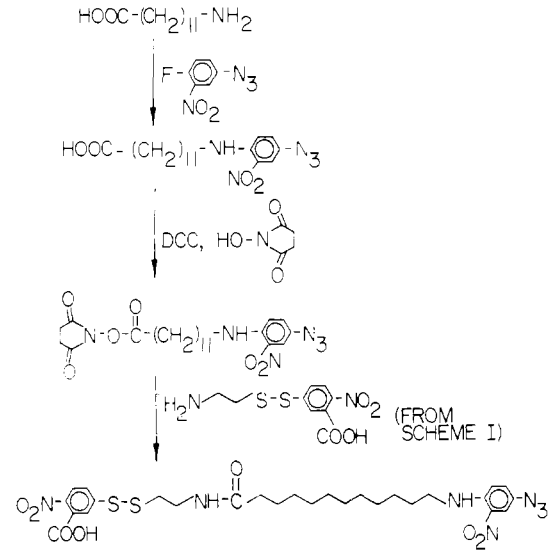
AcChR-enriched membrane preparations were obtained from *T. californica* electroplax by methods adapted from Duguid & Raftery (1973) and Reed et al. (1975) as described by J. Elliott, S. Blanchard, W. Wu, J. Miller, C. Strader, P. Hartig, J. Racs, and M. A. Raftery (unpublished experiments). The specific activity of these preparations ranged from 1.5 to 2.5 nmol of [^{125}I]-α-BuTx bound per mg of protein.

Synthesis of 2-[(4-Azido-2-nitrophenyl)amino]ethyl 3-Carboxy-4-nitrophenyl Disulfide. This reagent was synthesized according to Scheme I. 5,5′-Dithiobis(2-nitrobenzoic acid) (600 mg, 0.0015 mol) was dissolved in 30 mL of absolute ethanol and treated with 480 mg (0.0048 mol) of triethylamine. This mixture was stirred as 160 mg (0.0014 mol) of cysteamine hydrochloride dissolved in 10 mL of ethanol was added dropwise. Upon completion of the addition, triethylamine was added until the pH of an aliquot diluted 1:1 in water reached a value of 6.1. The solution was allowed to stand for 2 h, and then 263 mg of tan crystals was collected.

Scheme I



Scheme II

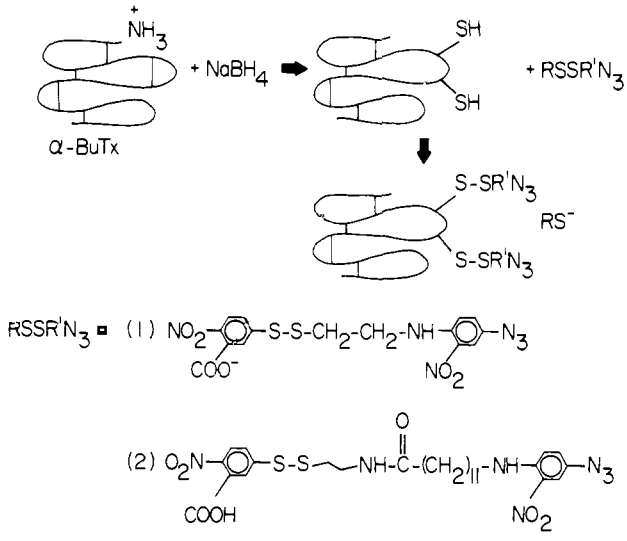


This material (10 mg, 0.00037 mol) was added to a solution of 200 mg (0.0011 mol) of 4-fluoro-3-nitrophenyl azide and 50 mg (0.0005 mol) of triethylamine in 3 mL of Me_2SO. This suspension was stirred under a nitrogen atmosphere at 45 °C for 4.5 h, at which time the solids had disappeared. The mixture was diluted with water, rinsed with several portions of ether, and allowed to dry on a watch glass. The residue was taken up in 0.5 mL of 2:1 ethanol–Me_2SO and filtered through a 2.5 × 96 cm Sephadex LH-20 column. The initial orange-colored fractions were pooled and dried in vacuo to yield 40 mg of product: NMR (Me_2SO-d_6) δ 8.3 (t, J = 6 Hz, 1 H), 8.7 (m, 6 H), 2.7 (q, J = 6 Hz, 2 H), 2.3 (t, J = 6 Hz, 2 H). On TLC (silica) the orange spot turned yellow upon treatment of the plate with ethanethiol in triethylamine. No other yellow spots appeared on the plate. λ_{max} (EtOH) = 331 and 484 nm.

Synthesis of 2-[[[(4-Azido-2-nitrophenyl)amino]dodecanoyl]amino]ethyl 3-Carboxy-4-nitrophenyl Disulfide (II). This reagent was synthesized according to Scheme II.

Synthesis of IV. 3-Nitro-4-fluorophenyl azide (1.70 g) and ω-aminododecanoic acid (2.15 g) were dissolved in a mixture of 40 mL of dry dimethylformamide, 100 mL of chloroform, and 50 mL of triethylamine and heated under reflux for 3 h. The mixture was poured into 300 mL of water and allowed to cool. It was then extracted 3 times with ether (3 × 100 mL). After acidification to pH 2, the aqueous layer was again

Scheme III



extracted with ether (3 × 100 mL). This ether solution was dried by shaking with brine and with anhydrous $CaCl_2$ and was then evaporated to dryness. The product was redissolved in THF and reevaporated. The product was dried in vacuo over drierite. Yield was 2.2 g.

Synthesis of V. IV (1.88 g) was dissolved in dry THF, 0.57 g of *N*-hydroxysuccinimide was added, and the mixture was stirred until it dissolved. Then 1.0 g of dicyclohexylcarbodiimide was added with stirring. Precipitation of dicyclohexylurea began after 2 min. The mixture was allowed to stand for 16 h after which the urea was filtered off. Upon evaporation of solvent the product crystallized.

Synthesis of VI. V (0.47 g), I (0.26 g) (see Scheme I), and triethylamine (0.1 g) were dissolved in 50 mL of dry Me_2SO and allowed to react at room temperature for 40 h with stirring in the dark. The mixture was then poured into 300 mL of water and extracted with ether (3 × 100 mL). The aqueous layer was recovered and acidified with HCl to pH ~2 and reextracted with chloroform. The chloroform layer was dried with anhydrous $CaCl_2$ and evaporated to dryness. Preparative thin-layer chromatography (silica gel; Merck) was used each time prior to modification experiments to ascertain that the cross-linking reagents were free of decomposition products arising upon storage. (The long-chain cross-linker was less stable than the short-chain cross-linker.) If necessary, the chromatographic purification as repeated several times. The developing solvent was ethyl acetate–2-propanol (1:1); R_f = 0.38 (reagent I) or 0.52 (reagent II). The dark yellow to orange product (which turned brown upon irradiation with ultraviolet light as well as when exposed to daylight) was scraped off the TLC plates, and methanol was used to elute the product from the silica gel.

Synthesis of Affinity Labeling Reagents Derived from [^{125}I]-α-BuTx. The photoaffinity reagents were synthesized from [^{125}I]-α-BuTx according to Scheme III. $NaBH_4$ was added to 50 or 100 μL of [^{125}I]-α-BuTx (0.75 mg/mL) in 5 μM sodium phosphate buffer, pH 7.5, to a final concentration of 0.1 M according to the procedure used by Chicheportieche et al. (1975) for reduction of *Naja nivea* α-toxin. Reduction by $NaBH_4$ was allowed to proceed for 6 min. The reaction was then stopped by adjusting the pH to ~3 with 0.1 M HCl. On the average, about one disulfide bridge had been reduced as determined by measuring the concentration of free SH groups with Ellman's reagent (DTNB) (Ellman, 1959). The reduced [^{125}I]-α-BuTx was diluted approximately three- to fourfold into 0.1 M sodium phosphate buffer, pH 8.0, containing reagent I or II at a concentration of (4.8–6.6) × 10^{-5} M. Mixed disulfide formation between the reduced [^{125}I]-α-BuTx and the reagents was followed by estimation of released thionitrobenzoate ion at 412 nm in buffer of pH 7.0. This stock solution of the affinity labeling reagent could be used directly after an ~20-fold dilution. The concentration of free reagent I or II was then so low that it had no effect on the polypeptide pattern of membrane fragments observed upon $NaDodSO_4$–polyacrylamide gel electrophoresis. The stock solution was used within 2–3 days to avoid difficulties due to possible decomposition of the affinity labeling reagent upon prolonged storage.

Photoaffinity-Directed Cross-Linking. (1) Using AcChR Dimer Preparations. We have previously shown that treatment of AcChR-enriched membrane preparations with alkylating agents, such as iodoacetamide, resulted in stabilization of the receptor as a 13.4S form (Raftery et al., 1972) and that this resulted from disulfide bond formation between 65 000-dalton subunits to yield a species of 130 000 daltons on $NaDodSO_4$–polyacrylamide gels (Witzemann & Raftery, 1978b). Such alkylated membranes (0.3–0.7 μM in α-BuTx binding sites) were incubated with [^{125}I]-α-BuTx-SSR′N_3 or [^{125}I]-α-BuTx-SSR″N_3 (0.25–0.7 μM) for 3–4 h at room temperature in 10 mM sodium phosphate, pH 7.5, for complex formation. The toxin–receptor complexes were irradiated for 1 h at 4 °C by using as a light source the long-wavelength range of a UV lamp (UVSL. 25, Ultra Violet Products, San Gabriel, CA) placed at a distance of 2 cm from the sample. The irradiated membranes were then sedimented for 15 min in an Eppendorf centrifuge. The pellet was dissolved and denatured in 3% $NaDodSO_4$ and used for $NaDodSO_4$–polyacrylamide gel electrophoresis essentially according to Laemmli (1970) using 8.75% acrylamide–0.23% methylenebis(acrylamide) gels. The main difference was that no reducing agents were used during denaturation since this would result in cleavage of the cross-linked products. Following staining with Coomassie Blue to locate the protein bands, the gels were sliced into 1-mm segments with a Hoefer Scientific Instruments gel slicer (Model SL 280), and these were counted for ^{125}I content in a Beckman γ 4000 counter.

(2) Using AcChR Monomer Preparations. Membrane preparations (ca. 0.6 μM in [^{125}I]-α-BuTx binding sites) were reduced by incubation with DTT (1 mM) for 1 h at room temperature. Free SH groups and excess reducing agent were then reacted with $IAcNH_2$ (6 mM) for 10 min at room temperature. The membranes were sedimented in an Eppendorf centrifuge for 15 min, resuspended 0.1 M sodium phosphate buffer, pH 8.0, and homogenized for 30 s in a Virtis 23 at full speed. $NaDodSO_4$ gel electrophoresis of these preparations (in the absence of mercaptoethanol) showed that all of the 65 000 polypeptide migrated with that apparent molecular weight and no 134 000 component (Witzemann & Raftery, 1978a,b), representing S–S-linked dimers of this subunit, was observed. Labeling of this reduced, alkylated preparation was conducted exactly as described for AcChR dimer preparations. $NaDodSO_4$ gel electrophoresis also was performed as described for the AcChR dimer preparations.

Analytical Methods. [^{125}I]-α-BuTx–AcChR complex formation was determined by the method of Schmidt & Raftery (1973b) using DEAE-cellulose disks (DE-81). Protein was determined by the method of Lowry et al. (1951). Spectrophotometric measurements were carried out by a Cary Model 118C spectrophotometer, and radioactivity measurements were made by using a Beckman γ 4000 spectrometer. $NaDodSO_4$

electrophoresis gels were scanned, following staining with Coomassie Brilliant Blue, in a Gilford linear transport unit attached to a Gilford Model 240 spectrophotometer. Thin-layer chromatography was performed on Eastman Kodak silica gel G plastic-coated plates. The solvents used were ethyl acetate–2-propanol (1:1 v/v).

Results

Modified α-Bungarotoxin Derivatives. (1) Preparation. The degree of modification of [^{125}I]-α-BuTx was determined spectrophotometrically by using two cuvettes, one of which contained identical solutions of reagent I or II in 0.1 M sodium phosphate buffer, pH 8.0. The reduced [^{125}I]-α-BuTx was added to the sample cuvette and the same volume of buffer was added to the reference cuvette and increasing absorbance at 412 nm due to the formation of thionitrobenzoate ions was recorded. The value reached after ca. 2 h (no further significant changes were observed) was then used to calculate the concentration of reaction products, by using $\epsilon = 1.36 \times 10^4$ cm^{-1} M^{-1} (Ellman, 1959). Since one thionitrobenzoate ion is released upon modification of one SH residue, the number of potential cross-linking groups covalently attached to the reduced [^{125}I]-α-BuTx disulfide bonds could be determined. Control experiments showed that the cross-linking reagents I and II, when used in a two- to threefold excess over free SH residues, would react to at least 90% with the number of SH residues per toxin molecule as was found by using DTNB. Values determined for the toxin preparations used in the cross-linking experiments described below were as follows: in the case of reagent I, 2.6 ± 0.3 and, for reagent II, 2.4 ± 0.7 SH residues per toxin molecule were modified by the cross-linking reagents. These results indicated that on the average about 2–3 potential cross-linking groups had been covalently introduced into the toxin and that, therefore, slightly more than one disulfide bridge per toxin molecule had been reduced.

(2) Binding to the AcChR. The binding properties of the modified toxins were tested by using the DEAE filter disk method (Schmidt & Raftery, 1973b). The apparent rates of reaction of the modified toxin preparations with membrane-bound AcChR were lower than values obtained in parallel experiments with [^{125}I]-α-BuTx and appeared to reach only about 70% of that measured in the control. The amount of toxin bound at equilibrium was also about 30% lower than that in the control, again by using the DEAE assay. When, however, the total bound toxin was determined by centrifugation assay (airfuge; Beckman Instruments), the difference was only 10–15%. From these experiments it was not possible to decide conclusively whether part of the modified toxin preparations was inactivated due to the reduction and labeling procedure or whether the affinity for AcChR had decreased to such an extent that the determination of total toxin bound using the DEAE filter disk assay resulted in values which underestimated the actual number of toxin molecules bound. Results reported by Chicheportiche et al. (1975) favor the second possibility. Neurotoxin III from *Naja haje*, after reduction of one disulfide bridge and following carboxyamidoethylation, exhibited a decreased affinity for receptor (by a factor of 15) due to a decrease of the rate of association (as observed in our case) but also to an increase of the rate of dissociation (not determined for our toxin derivatives). These changes were not accompanied, however, by changes in toxicity as determined by LD_{50} values (Chicheportiche et al., 1975; Botes, 1974), indicating that the biological activity of such treated α-neurotoxins could be retained to ~100%. Protection experiments clearly demonstrated that the binding of the toxin derivatives could be completely prevented when all specific binding sites

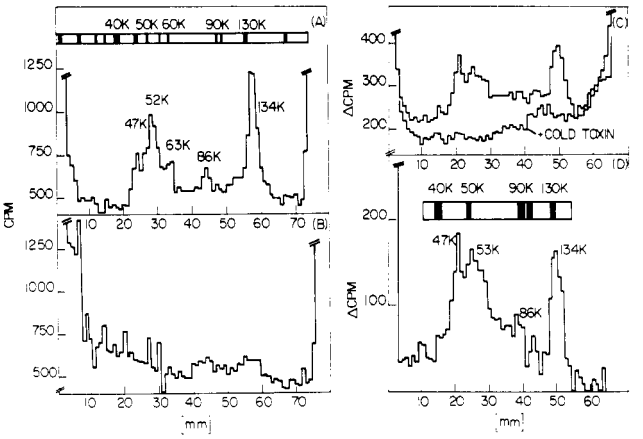


FIGURE 1: (A) $NaDodSO_4$ gel electrophoresis of AcChR-enriched membrane fragments following cross-linking with [^{125}I]-α-BuTx-SSR′N_3. The insert schematically shows the polypeptide pattern following Coomassie Blue staining. The diagram shows the distribution of ^{125}I following cross-linking and $NaDodSO_4$ gel electrophoresis. The apparent molecular weights of cross-linked polypeptides are indicated. (B) $NaDodSO_4$ gel electrophoresis of AcChR-enriched membrane fragments which were treated with [^{125}I]-α-BuTx-SSR′N_3 but which were not irradiated prior to electrophoresis. (C) Membrane fragments were treated with [^{125}I]-α-BuTx-SSR′N_3 as described in (A), and the distribution of radioactivity upon $NaDodSO_4$–polyacrylamide gel electrophoresis is shown in the upper graph. The control (lower curve) shows the distribution of radioactivity following gel electrophoresis of membranes which were submitted to the identical cross-linking procedure but which had previously been treated with unlabeled α-BuTx. (D) The profile shows the difference of radioactivity bound to membrane fragments after cross-linking in presence or absence of unlabeled α-BuTx as analyzed in (C). The insert schematically shows the polypeptide pattern, indicating apparent molecular weights, following Coomassie Blue staining.

were blocked by unlabeled α-BuTx. The DEAE assay method and centrifugation experiments yielded the same results.

Labeling of Membranes with the Short Cross-Linker [^{125}I]-α-BuTx-SSR′N_3. (1) Using Disulfide Bonded AcChR Preparations. As shown in Figure 1B no significant amounts of radioactivity were associated with any components of defined molecular weight in $NaDodSO_4$ gel electrophoresis of mixtures of [^{125}I]-α-BuTx-SSR′N_3 and AcChR in the absence of photolysis. However, when the complex of receptor and modified toxin was irradiated and then subjected to $NaDodSO_4$ gel electrophoresis, several distinct components were labeled (Figure 1A,D). These appeared to have molecular weights of 47×10^3, 52×10^3, 63×10^3, 86×10^3, and 134×10^3. Generation of these labeled components was prevented by pretreatment of the AcChR-enriched membrane with unlabeled α-BuTx before addition of [^{125}I]-α-BuTx-SSR′N_3 (Figure 1C). It is most likely that the cross-linked component of 47 000 daltons resulted from cross-linking of the modified α-BuTx to the receptor subunit of 40 000 daltons to which it is considered to bind (Hucho et al., 1976; Witzemann & Raftery, 1977, 1978a). Due to the fact that between 2 and 3 azido-containing groups were covalently bound to the [^{125}I]-α-toxin, several other reaction products could have arisen as a result of (1) two toxin molecules reacting with the same 40 000 polypeptide to yield the component of molecular weight ~52 000. (2) one toxin molecule cross-linked to two 40 000 dalton to yield an ~86 000 component, (3) toxin molecules cross-linking polypeptides other than the 40 000 subunit such as that of molecular weight 43 000, or (4) cross-linking of polypeptides of differing molecular weight such as 40 000 and 43 000 components.

In all cases where labeling was conducted, a major peak of radioactivity was associated with a component of molecular

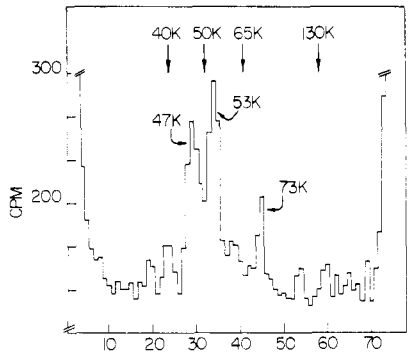


FIGURE 2: $NaDodSO_4$ polyacrylamide gel electrophoresis, without reducing agent, of membrane-bound AcChR. Membrane-bound AcChR was treated with DTT and $IAcNH_2$ prior to labeling to convert all AcChR to its 9S form. The diagram shows the distribution of cross-linked [^{125}I]-α-BuTx-SSR′N_3 after $NaDodSO_4$ gel electrophoresis. Arrows at the top indicate from Coomassie Blue staining the positions of unmodified AcChR polypeptides; lower arrows indicate the apparent molecular weights of radiolabeled products.

weight of apparently 134 000, indicating that the toxin could possibly be cross-linked to polypeptides other than the 40 000 component and especially to the dimeric form of the 65 000 polypeptide shown (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978b) to exist in AcChR dimers (Raftery et al., 1972) of *T. californica* receptor. In order to test this possibility, we conducted the experiments described below.

(2) Using Reduced AcChR Preparations. Following reduction and alkylation with $IAcNH_2$, the labeling experiments were repeated by using [^{125}I]-α-BuTx-SSR′N_3 followed by $NaDodSO_4$ gel electrophoresis in the absence of reducing agents to prevent cleavage of cross-linked products. The pattern of radioactivity observed in this case is shown in Figure 2. The major labeled components of apparent molecular weight 47×10^3 and 53×10^3 were again observed with the 47 000 component most likely representing the toxin cross-linked to the 40 000 subunit of the AcChR and the 53 000 subunit perhaps representing two toxin molecules associated with a single 40 000 polypeptide or even perhaps labeling of the 50 000 component by a single toxin molecule. The major new feature, however, was the almost total lack of a labeled component at 134 000 and generation of a new cross-linked species of apparent molecular weight 73 000. Thus, we consider it most likely that this new species represents the 65 000-dalton polypeptide cross-linked to the modified α-BuTx since the only difference between these experiments and those shown in Figure 1 was the conversion of the AcChR to the monomeric form by generation of the 65 000-dalton polypeptide from its dimeric form of 130 000 daltons. The data therefore can most readily be interpreted to indicate that the toxin bound to the 40 000 component of the receptor can become cross-linked upon photolysis to the membrane polypeptide of molecular weight 65 000.

Various control experiments were conducted. No labeled components were observed when the membranes were pretreated with unlabeled α-BuTx prior to addition of the modified toxin, followed by photolysis. In addition, no cross-linking occurred without irradiation of the photolabile toxin derivative (see Figure 1).

Labeling of Membranes with the Long Cross-Linker [^{125}I]-α-BuTx-SSR″N_3. (1) Using Reduced AcChR Preparations. Following photolysis of membrane preparations in which the AcChR had been reduced with DTT and alkylated with $IAcNH_2$, $NaDodSO_4$ gel electrophoretic profiles demonstrated the unique labeling of a component of apparent molecular weight slightly greater than 65 000 (Figure 3A). In this case only minimal labeling of components of molecular weight smaller than this was observed. Control experiments in which the membrane fragments were first treated with unlabeled α-BuTx prior to addition of [^{125}I]-α-BuTx-SSR″N_3, followed by photolysis and $NaDodSO_4$ gel electrophoresis, showed that no cross-linked products were formed. It therefore appeared that with the longer cross-linking reagent attached to [^{125}I]-α-BuTx the polypeptide to which the toxin is considered to bind, i.e., the 40 000-dalton species, was no longer labeled but that only a polypeptide of 65 000 molecular weight was labeled instead.

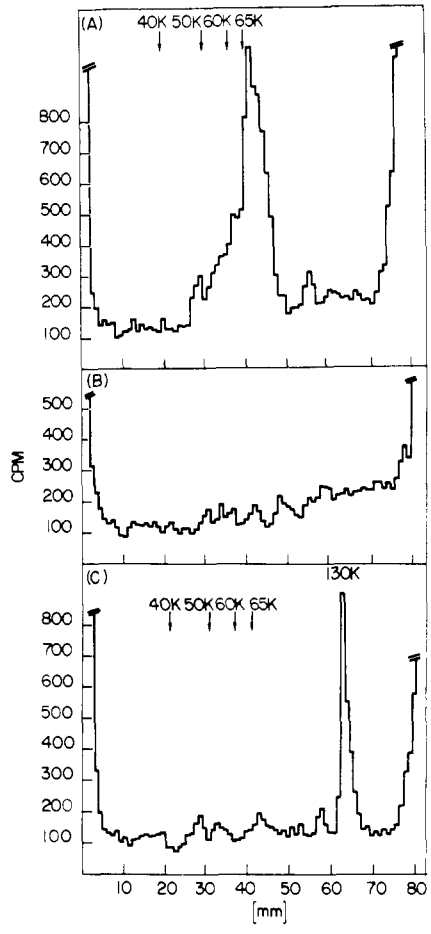


FIGURE 3: $NaDodSO_4$ gel electrophoresis of products formed upon irradiation of long cross-linker [^{125}I]-α-BuTx-SSR″N_3 complexes with AcChR. (A) Distribution of radioactivity following $NaDodSO_4$ gel electrophoresis of membrane fragments that were reduced with DTT and alkylated with $IAcNH_2$ during preparation to convert the AcChR to its 9S form, followed by binding of [^{125}I]-α-BuTx-SSR″N_3 and photolysis prior to electrophoresis. (B) Same as (A) except membrane fragments were protected with unmodified α-BuTx prior to addition of the cross-linking agent and photolysis. (C) $NaDodSO_4$ gel electrophoresis of membrane fragments prepared in the presence of $IAcNH_2$ to ensure stability of AcChR dimers. The pattern shows the distribution of radioactivity following photolysis in the presence of [^{125}I]-α-BuTx-SSR″N_3. The arrows in each panel indicate the positions of the AcChR constituent polypeptides corresponding to molecular weights of 40×10^3, 50×10^3, 60×10^3, and 65×10^3, respectively.

(2) Using Disulfide Bonded AcChR Preparations. Highly purified membrane fragments containing AcChR dimers, stabilized by treatment of the membranes with $IAcNH_2$ during homogenization and isolation, were treated with the long cross-linking reagent, [^{125}I]-α-BuTx-SSR″N_3. Following $NaDodSO_4$ gel electrophoresis in the absence of a reducing agent, a unique radioactive product of apparent molecular weight 130×10^3 was obtained (Figure 3C) with little indication of

cross-linking of the labeled toxin to any other membrane components. It was especially striking that the component of molecular weight greater than 65 000 observed with AcChR monomer preparations was essentially absent. This distribution of radioactivity, yielding radiolabeled components of apparent molecular weight ~71 000 for reduced AcChR preparations and 130 000 for disulfide bonded AcChR preparations, can best be interpreted as indicating that the toxin derivative was uniquely cross-linked to the AcChR subunit of 65 000.

Discussion

Affinity-Directed Cross-Linking. The experiments described in this manuscript represent a structural approach designed to determine whether polypeptides other than the species of molecular weight 40 000, which is thought to bind cholinergic ligands and α-toxins, are constituent polypeptides of the entity known as the acetylcholine receptor. Toward this end we have made functional derivatives of α-bungarotoxin, one of the most specific ligands known to associate with the AcChR. These derivatives contain photolabile groups attached to spacers and can be bound covalently to the α-toxin. Photolysis of these modified toxin derivatives generates nitrenes which can react with many types of adjacent bonds. The high affinity binding of α-BuTx greatly aides this type of approach since all of the reagent introduced can be bound to the AcChR. Furthermore, the number of photolabile groups introduced into the preferentially reduced α-toxin can readily be determined by virtue of the spectroscopic properties of the leaving group generated upon reaction with reagents I and II.

The results we have obtained show that the modified toxin derivatives [^{125}I]-α-BuTx-SSR′N_3 and [^{125}I]-α-BuTx-SSR″N_3 still bind with high affinity to membrane-bound AcChR and complex formation could be followed by using either the DEAE assay procedure of Schmidt & Raftery (1973b) or by sedimentation of the membranes. The toxin derivatives appear to have slightly altered binding properties in that the rate of complex formation with AcChR was slightly decreased. This is in agreement with studies where α-neurotoxin from *N. haje* was reduced and alkylated by iodoacetamide (Chicheportiche et al., 1975). This had, however, no effect on the toxicity of such treated toxin preparations. Demonstration of the specificity of [^{125}I]-α-BuTx-SSR′N_3 and [^{125}I]-α-BuTx-SSR″N_3 for AcChR resulted from protection experiments using native α-BuTx, and therefore only toxin derivatives which were competitive with α-BuTx for the same specific binding sites would result in cross-linked products following irradiation. To ascertain the identity of the specific polypeptides in the membrane preparations which were labeled by virtue of their proximity to the irreversibly bound toxin derivatives, we have used $NaDodSO_4$ gel electrophoresis in the absence of reducing agents. Inclusion of such agents would have resulted in cleavage of the cross-linked product from the α-toxin since the spacer containing the photolabile group was attached to the toxin via a disulfide bond. Not surprisingly, a product was obtained of apparent molecular weight 47 000. This can be interpreted to represent cross-linking of the modified α-toxin to the 40 000-dalton polypeptide to which the toxin is thought to bind. The other products that are formed bear more importantly on the question that we set out to answer; i.e., what other, if any, polypeptides are sufficiently close to the bound toxin to be cross-linked upon photoactivation of the modified toxin–receptor system? One other major product formed had a molecular weight of 53 000. The interpretation of how this product was formed is not unambiguous. It could have arisen from cross-linking of more than one toxin molecule to a single 40 000-dalton polypeptide. This is possible since the 9S form of the AcChR of molecular weight 270 000 ± 30 000 (Martinez-Carrion et al., 1975) is thought to contain more than one copy of the 40 000 polypeptide (Vandlen et al., 1979). However, this product could also arise from cross-linking of the α-toxin derivatives to a species of 50 000 since it is not necessary that the apparent molecular weight observed upon $NaDodSO_4$ gel electrophoresis be a linear combination of the α-toxin molecular weight (8000) and a given polypeptide molecular weight to which it is cross-linked.

The second clear result obtained (Figure 2) in these studies was that the bound α-toxin derivative [^{125}I]-α-BuTx-SSR′N_3 was cross-linked to a polypeptide of apparent molecular weight 65 000 when the receptor was isolated in the monomeric 9S form (Raftery et al., 1972) and to a polypeptide of apparent molecular weight 130 000 (Figure 1) in the 13.2S AcChR form (Raftery et al., 1972). This dimeric form of the receptor appears to be the major naturally occurring form in the membrane-bound environment. The occurrence of a specific disulfide bridge between two 65 000-dalton polypeptides in the dimeric form has been demonstrated in several laboratories (Suarez-Isla et al., 1977; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978b). The labeling of this polypeptide by using [^{125}I]-α-BuTx-SSR′N_3 was clearly demonstrated (see Figure 1A,D). The more dramatic result, however, was obtained with the longer spacer arm attached to [^{125}I]-α-BuTx-SSR″N_3. In this case only the 65 000-dalton polypeptide in isolated AcChR monomeric form and its dimer of 130 000 daltons in the isolated AcChR dimeric form were cross-linked upon photolysis of the bound toxin. Thus, these results, based upon a structural approach, strongly imply that a polypeptide of 65 000 daltons is a constituent part of the AcChR in its membrane-bound environment.

One further implication arises from the results. Since we have shown that at least 95% of the α-BuTx binding sites occur on the synaptic face of the postsynaptic membrane (Hartig & Raftery, 1979), it follows that the vast majority of the 40 000-dalton polypeptides are most likely exposed on this same face [see also Hartig & Raftery (1977)]. The cross-linking results reported here further imply that the polypeptide of molecular weight 65 000 also has some exposure on the synaptic face of the membrane.

Comparison with Results from Cholinergic Ligand Binding Studies. These results are in direct agreement with those we have obtained by using a completely different approach. Photolabeling of AcChR-enriched membranes from *T. californica* with [^{3}H]bis(azidoethidium bromide) shows that in the absence of added cholinergic ligands only the polypeptide of 40 000 molecular weight was labeled. However, in the presence of added cholinergic agonists that are known to convert *T. californica* AcChR to a state of higher ligand affinity (Lee et al., 1977), labeling was extended to additional polypeptides of 50 000 and 65 000 daltons, respectively (Witzemann & Raftery, 1978a). We interpreted this result as being indicative of conformation changes being transmitted from the ligand binding site on the 40 000-dalton polypeptide for cholinergic agonists (Moore & Raftery, 1979) to the other polypeptides of 50 000 and 65 000 daltons.

A third line of evidence which we have obtained to implicate polypeptides other than the 40 000-dalton species as part of the AcChR complex involved the use of a photolabile cholinergic antagonist 1,10-decane[^{3}H]bis(3-azidopyridinium) diiodide (Witzemann & Raftery, 1977). This radiolabeled photoactivated antagonist derivative was used to label *T. californica* AcChR both in a Triton-solubilized, highly purified, state and in the membrane-bound state. In both cases the

reagent labeled the 40 000-dalton polypeptide of the preparation. In the case of the purified receptor preparation, a polypeptide of 60 000 daltons was also labeled while in the membrane-bound receptor a polypeptide of 50 000 daltons was labeled. These results were interpreted (Witzemann & Raftery, 1977) as indicative that the binding site for the antagonist was located on the 40 000 polypeptide and the additional labeling of other polypeptides, in solution and in the membrane-bound state, was interpreted as evidence of their proximity to the ligand binding site. The labeling was considered to arise possibly due to long-lived nitrene species. An alternative explanation could be that the toxin (and antagonist) actually binds to these other polypeptides to putative sites, as if the polypeptides were homologous with the 40 000-dalton species.

Relationship of Cross-Linking Results to Studies of Purified AcChR Subunit Composition. Purification of detergent-solubilized AcChR from *T. californica* electroplaque membranes was initially achieved in our laboratory (Schmidt & Raftery, 1973a; Raftery, 1973) using affinity chromatographic procedures developed for *Narcine entemedor* AcChR (Schmidt & Raftery, 1972). In these early preparations of purified AcChR, some degradation of polypeptides occurred due to lack of inhibition of proteolytic enzymes present in the preparation. Following purification of highly enriched AcChR membranes from *T. californica* (Duguid & Raftery, 1973), it was possible to ascertain which membrane-bound polypeptides were constituents of the purified macromolecular complex by comparison of the peptides present in these highly enriched fractions with AcChR purified from these same fractions. Raftery et al. (1974) showed that four polypeptides (M_r 40 × 10^3, 50 × 10^3, 60 × 10^3, and 65 × 10^3) which were present in the membrane fractions copurified on affinity chromatography of detergent extracts of the membranes. However, receptor purified by the same methods from crude membrane fractions appeared to have the 40 000 and 50 000 components but lesser amounts of the higher molecular weight polypeptides of apparent molecular weight 60 000 and 65 000. Weill et al. (1974) found a similar polypeptide composition for purified AcChR also from *T. californica*. The use of EDTA for inhibition of calcium-activated proteases allowed purification of the solubilized AcChR from crude membrane preparations in an intact state (Raftery et al., 1975; Karlin et al., 1975). Due to the complexity of this polypeptide pattern the question arises as to whether polypeptides present in membrane fractions but not in association with each other might, upon solubilization in detergent micelles, associate by hydrophobic interactions. In this respect Sobel et al. (1977) claim to have purified membranes from *Torpedo marmorata* electroplax that contained, in selected fractions from a sucrose gradient, only polypeptides of 40 000 and 43 000 daltons. Following detergent treatment of these fractions to yield a solubilized component that bound α-toxin, only one polypeptide of molecular weight 40 000 was observed upon $NaDodSO_4$ gel electrophoresis. The major component of this preparation had a value of 9 S determined from sucrose density gradient centrifugation, in agreement with the value obtained for the monomeric form of solubilized *T. californica* AcChR (Raftery et al., 1972). The similar S values of the preparations could conceivably be reconciled if indeed the receptor was composed only of the 40 000 polypeptide complex and the other polypeptides of molecular weight 50 000, 60 000, and 65 000 were "mere contaminants". Two arguments can be raised against this possibility. The first is that the receptor can be maintained in a 13.2S form in addition to the 9S form (Raftery et al., 1972) by treatment of the membranes with alkylating agents to prevent disulfide interchange, and it can then be shown that this appears to be entirely due to disulfide bridge formation between 65 000-dalton polypeptides based on $NaDodSO_4$ gel electrophoretic analysis (Suarez-Isla & Hucho, 1977; Chang & Bock, 1977; Hamilton et al., 1977; Witzemann & Raftery, 1978b). This dimeric form has been shown to have the identical polypeptide composition attributed to the 9S form (Vandlen et al., 1976). Thus, it appears that all the polypeptides form a complex in detergent solution. The second argument rests on the specific activities of the preparations described in the literature. Sobel et al. (1977) report that a specific activity of 7000 nmol of α-toxin binding sites per g of protein. This is considerably lower than the values reported for *T. californica* AcChR containing four polypeptides, i.e., specific activities of 8000–10 000 nmol of α-toxin binding sites per g of protein. On the basis of the apparent relative amounts of the four polypeptides present in *T. californica* AcChR-purified preparations, the specific activity of the preparation of Sobel et al. should be approximately 25 000 nmol of α-toxin binding sites per g of protein if only the 40 000-dalton polypeptide species represents the AcChR macromolecular complex.

A further consideration is that when the AcChR is solubilized and purified under identical conditions from *T. californica*, *T. marmorata*, *Torpedo nobiliana*, and *Narcine braziliensis* (Deutsch & Raftery, 1979), $NaDodSO_4$ gel electrophoretic analysis reveals that all of these preparations contain the four polypeptide components discussed above. These results with *N. braziliensis* agree with those of Chang et al. (1977). Additionally, we have described earlier (Claudio & Raftery, 1977) studies showing that the four types of polypeptides do not appear to have common antigenic determinants and thus are not degradation products of each other. Immunological studies, by Lindstrom et al. (1978), have showed that antibodies made to the four *T. californica* AcChR subunits each caused, following injection, a diminution in the number of synaptic receptors present in rat muscle, a condition symptomatic of experimental autoimmune disease (Patrick & Lindstrom, 1973).

The results which we have discussed above with respect to the use of a photolabile derivative of the cholinergic antagonist and a photolabeling reagent derived from ethidium bromide which can be used to differentiate between conformational states of the AcChR in the presence and absence of cholinergic ligands constitute strong evidence that the membrane-bound AcChR complex involves polypeptides in addition to the 40 000-dalton species on which cholinergic ligand binding sites are located. The structural approach which we have described in this manuscript lends further strong evidence in support of this notion.

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Interaction of Calf Brain Tubulin with Poly(ethylene glycols)†

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ABSTRACT: The effects of poly(ethylene glycol) (PEG) on the solution properties of calf brain tubulin were investigated at pH 7.0. In vitro reconstitution is promoted by PEG with the polymers of higher molecular weight being more efficient in lowering the free energy of the propagation step of microtubule formation. The dependence of the apparent association constant of microtubule formation on PEG concentration was analyzed by the linked-function theory of Wyman [Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–286], leading to a conclusion that the thermodynamic instability of the system is reduced by formation of microtubules. Such conclusion was substantiated by the results of investigation of the preferential solvent interaction of PEG with tubulin by density measurements. Application of multicomponent thermodynamic theory shows that tubulin is preferentially hydrated in all PEG solutions, leading to an increase in the chemical potential of PEG. This unfavorable thermodynamic interaction leads to phase separation as evidenced by the precipitation of tubulin at higher PEG concentrations. Concomitant monitoring of the conformation of tubulin by comparing the accessibility of sulfhydryl groups and circular dichroic spectra at pH 7.0 indicates that PEG does not induce observable structural changes in tubulin. The results of spectrophotometric titration of tyrosine residues are consistent with that of circular dichroic spectroscopic study that PEG prevents the protein from unfolding at pH 10.

The self-assembly process of tubulin to microtubules has been the subject of intensive investigation since the initial report by Weisenberg (1972) on the conditions of in vitro reconstitution of microtubules. One of the areas of major interest is the role of proteins which copurify with tubulin. Tubulin dimers (5.8 S) purified by the cycle method, followed by gel column chromatography, have been described repeatedly as being incapable of polymerization into microtubules by themselves (Kuriyama, 1975; Kirschner & Williams, 1974; Keates & Hall, 1975). Contrary to these reports, however, Timasheff

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0006-2960/79/0418-5518$01.00/0