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AFFINITY DIRECTED CROSSLINKING OF ACETYLCHOLINE RECEPTOR POLYPEPTIDE COMPONENTS IN POST-SYNAPTIC MEMBRANES

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SUMMARY: A photolabile derivative of α-bungarotoxin which binds specifically to Torpedo californica acetylcholine receptor has been used to investigate the topography of the membrane associated protein. It is shown that the toxin can be crosslinked to a polypeptide of 40,000 daltons, to which it is known to bind, and in addition to another polypeptide of 65,000 daltons which is a major constituent of the membrane. The results substantiate the notion that this nicotinic acetylcholine receptor is composed of different polypeptides and that some of these interact with each other or are in close proximity on the exterior surface of the post-synaptic membrane.

INTRODUCTION

The use of snake venom α-toxin (1) for development of specific assay procedures has aided the isolation and purification of nicotinic acetylcholine receptor (AcChR) from the electric organs of fish such as Torpedo, Narcine or electric eel, which provide ideal starting materials because of their relatively high concentration of AcChR. Biophysical studies using Torpedo californica-AcChR have indicated a monomeric molecular weight of about 250,000 daltons (2,3,4). For this solubilized, purified protein four different polypeptide chains of apparent molecular weights 40,000, 50,000, 60,000 and 65,000 have been found upon SDS-polyacrylamide gel electrophoresis (5,6). The same four subunits are present in AcChR-enriched membrane fragments (6,7,8) in addition to a few other polypeptides (see also Figure 1). Using affinity labeling

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Abbreviations: AcChR, acetylcholine receptor; α-BuTx, α-Bungarotoxin; SDS, sodium dodecylsulfate; DEAE, diethylaminoethyl; $RSSR'N_3$, crosslinking reagent (see Figure 2); CBB, Coomassie Brilliant Blue.

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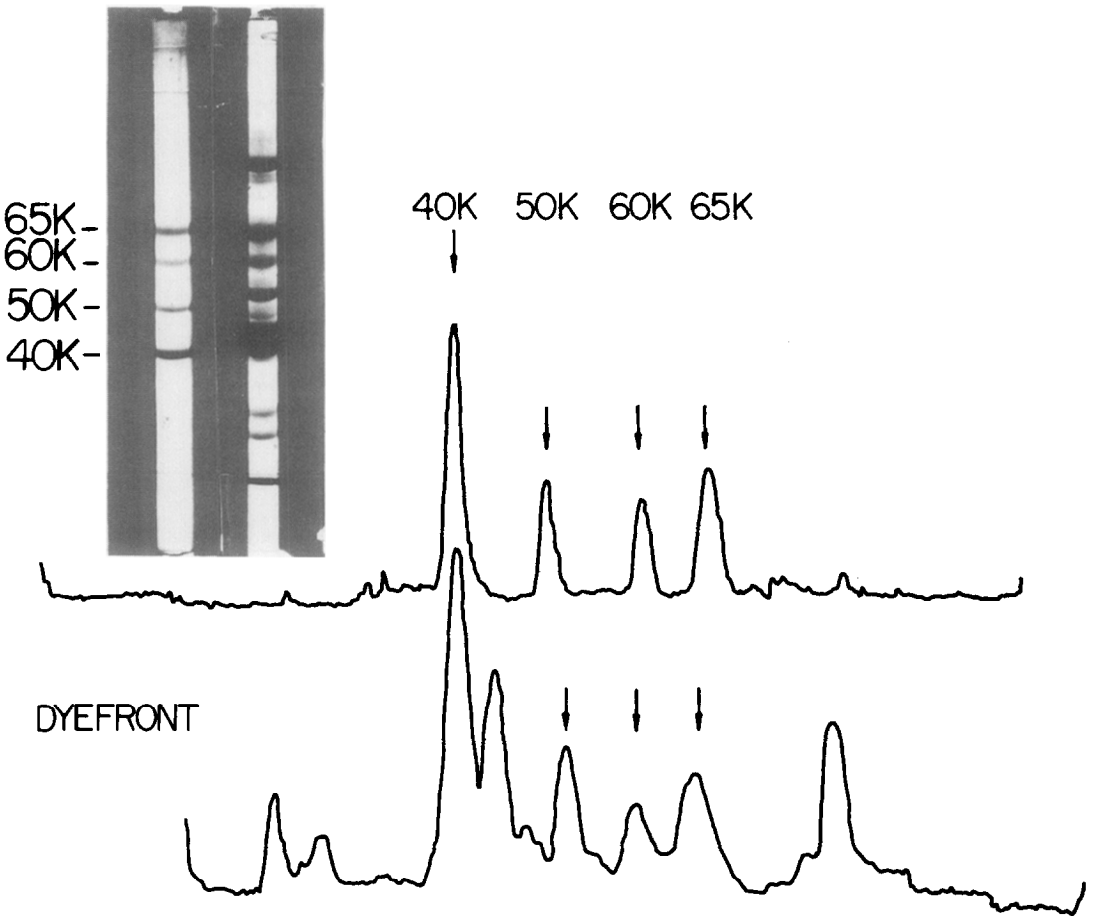

FIGURE 1: SDS-polyacrylamide gel electrophoresis under reducing conditions of solubilized and purified AcChR (gel on the left) and membrane-bound AcChR (gel on the right) after Coomassie Brilliant Blue staining. The gel scans at 550 nm are shown: solubilized purified AcChR on top and membrane-bound AcChR on the bottom. The ratio of the four AcChR-polypeptides (40,000; 50,000; 60,000; 65,000 dalton) according to their staining intensities are 1:0.4:0.3:0.5 in the case of the purified AcChR and 1:0.4:0.4:0.6 for the membrane-bound AcChR.

it was found that the 40,000 dalton subunit carries binding sites for small ligands (5,9,10,11) and for α-bungarotoxin (BuTx) (5,12,13).

In this communication we describe an approach designed to determine which polypeptide constituents of the AcChR-enriched membranes are close to or in contact with the 40,000 dalton polypeptide. An azido-containing reagent was first attached covalently to [^{125}I] α-BuTx. Following binding of this toxin to the receptor crosslinking was then achieved by photolysis and resulted in

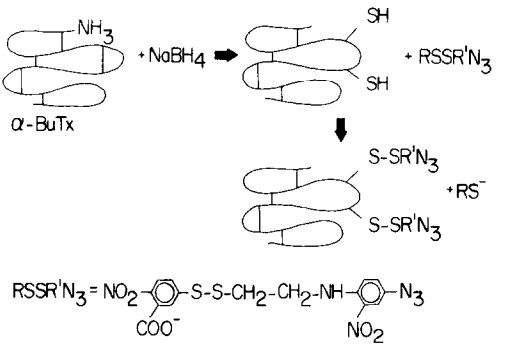

FIGURE 2: Reduction and alkylation of α-BuTx. α-BuTx is drawn schematically Cys_{30} and Cys_{34} form a disulfide bridge which can be reduced rapidly and selectively. Reaction with the mixed disulfide $RSSR'N_3$ then affords the desired modified toxin.

the formation of radiolabeled polypeptides. The results show that α-BuTx is crosslinked to the 40,000 dalton and in addition to the 65,000 dalton subunit, which strongly supports the notion that these two polypeptides are closely associated in their membrane-bound environment.

MATERIALS AND METHODS

AcChR-rich membrane fragments were prepared from electric organs of Torpedo californica using procedures developed in this laboratory (6,14). α-BuTx was purified from crude venom of Bungarus multicinctus (Sigma Chemical Co.) and labeled with [^{125}I] as described by Clark et al (15). Reagents used in SDS-polyacrylamide gel electrophoresis were purchased from BioRAD Laboratories. All other chemicals used were of the purest grade commercially available. The synthesis and purification of the crosslinking reagent ($R\text{-}SS\text{-}R'N_3$), see Figure 2, will be published elsewhere (Dr. D. Muchmore, unpublished). SDS-polyacrylamide gel electrophoresis was carried out according to (16). [^{125}I] α-BuTx-AcChR complexes were determined according to (17). For spectrophotometric measurements a Cary Model 118C was used and radioactivity was determined in a Beckman Gamma 4000.

Modification of [^{125}I] α-BuTx:

To 100 µl of [^{125}I] α-BuTx (0.75 mg/ml) in 5 mM sodium phosphate buffer pH 7.5, $NaBH_4$ was added to a final concentration of 0.1 M following the procedure described in (18). The reduced toxin was diluted ~3 fold into 0.1 M sodium phosphate buffer pH 8.0 containing the crosslinking reagent $RSSR'N_3$ ($4.8 \cdot 10^{-5}$M). Reaction between the SH-groups of the toxin and the crosslinking reagent resulted in covalent incorporation of the azido residue and formation of thionitrobenzoic acid (RS^-) which could be followed spectrophotometrically by the increase of absorbance at 412 nm as when using Ellman's reagent (19).

Crosslinking Experiments:

AcChR-rich membrane fragments (0.7 µM in α-BuTx binding sites) were incubated with modified [^{125}I] α-BuTx-$SSR'N_3$ (0.7 µM) for 4 hours at room temperature in 10 mM sodium phosphate, pH 7.5. The toxin-AcChR complex was ir-

radiated for 1 hour at 4°C. The light source used was the long wavelength range (>300 nm) of a UV-lamp (UVSL•25, Ultra-Violet Products, San Gabriel, Ca. USA), which was placed at a distance of 2 cm from the sample. The membrane fragments were sedimented for 15 minutes in an Eppendorf centrifuge. The supernatant was discarded and the pellet dissolved, denatured in 3% SDS without reducing agents and submitted to polyacrylamide gel electrophoresis. The Coomassie Blue stained gels were sliced into 1 mm pieces (gel slicer SL 280 Hoefer Scientific Instruments) and counted in a Beckman Gamma 4000.

RESULTS AND DISCUSSION

All long neurotoxins (71-74 amino acids with 5 disulfide bridges) have one disulfide bridge, Cys 30 - Cys 34, which appears to be exposed since it can be reduced rapidly and selectively by $NaBH_4$ (18). Reduction of this disulfide bridge followed by alkylation with iodoacetamide had little effect on toxicity (18,20).

We have utilized the same reduction conditions using α-BuTx and have modified the reduced toxin with a reagent designed to introduce a photolabile group into the toxin by means of a disulfide-sulfhydryl exchange reaction (Figure 2). The resulting product contained about 3 moles of reagent per toxin molecule based on the increased absorbance at 412 nm due to the formation of RS^- (Figure 2). This indicated that on the average slightly more than one disulfide bridge was reduced. The resultant modified α-BuTx was still capable of binding to the AcChR as judged by DEAE filter disc assay (17). Binding of [^{125}I] α-BuTx-SSR'N_3 was completely prevented when the membrane fragments were preincubated with unlabeled α-BuTx (4 to 5 fold excess over toxin binding sites). In addition photolysis resulted in no significant incorporation of radioactivity into the AcChR as determined by the DEAE filter disc assay. This result justified the assumption that both α-BuTx and [^{125}I] α-BuTx-SSR'N_3 interact with identical sites. For crosslinking membrane-bound AcChR was first incubated with modified [^{125}I] α-BuTx-SS-R'N_3 to saturate toxin binding sites and then the complex was irradiated. SDS-polyacrylamide gel electrophoresis of such treated membrane fragments was carried out in the absence of reducing agents, since the crosslinker was attached to the toxin via disulfide bridges. Following staining with Coomassie Brilliant Blue no

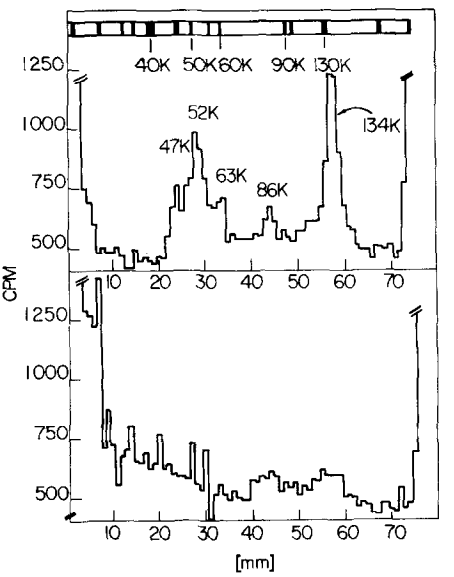

FIGURE 3: TOP: SDS-gel electrophoresis of AcChR-rich membrane fragments after crosslinking with [^{125}I] α-BuTx-SSR'. The insert on top shows schematically the polypeptide pattern after Coomassie Blue staining. The diagram shows the distribution of [^{125}I]. The apparent molecular weights of modified polypeptides are indicated. BOTTOM: SDS-gel electrophoresis of AcChR-rich membrane fragments which were treated with [^{125}I] α-BuTx-SSR' but not irradiated.

major differences were observed in the polypeptide pattern compared to that obtained using membrane fragments not submitted to the crosslinking procedure. Irradiation of membrane fragments in the absence of added toxin also did not cause any detectable changes. Figure 3 shows the distribution of [^{125}I] across a gel of crosslinked [^{125}I] α-BuTx-SSR'-AcChR. The radioactivity migrating at the dyefront was due to [^{125}I] α-BuTx-SSR' which was not covalently bound. In addition several components containing significant amounts of radioactivity were separated with apparent molecular weights of 47,000, 52,000 and 134,000 daltons in addition to a few minor components. It is likely that the crosslinked component of 47,000 daltons results from crosslinking to the 40,000 dalton component. Since two to three azido groups were bound per toxin molecule (see above) several other reaction products might be expected, due to (1) two toxin molecules reacting with the same 40,000 dalton subunit to yield

a 52,000 dalton component, (2) one toxin molecule crosslinking two different 40,000 dalton subunits to form an 85,000 dalton component, (3) toxin molecules crosslinking polypeptides other than the 40,000 dalton subunit, such as the 43,000, 50,000, 60,000, 65,000 or 90,000 dalton polypeptides, (4) crosslinking of two different polypeptides.

The fact that a major peak of radioactivity was associated with a component of $M.W._{apparent}$ 134,000 daltons indicated that toxin could possibly be crosslinked to polypeptides other than the 40,000 dalton subunit, namely the dimeric form of the 65,000 dalton polypeptide of the AcChR complex. It has been shown earlier that Torpedo californica AcChR in the membrane-bound state exists predominantly as a dimer formed by a disulfide linkage(s) between its 65,000 dalton subunits (7,3,8,21). To exclude the possibility that this band represented crosslinked products of polypeptides other than the 65,000 dalton subunit, membrane fragments were reduced by incubation for 1 hour at room temperature with 1 mM dithiothreitol to convert AcChR dimers to monomers. Excess reducing agent and free SH-groups were then alkylated with iodoacetamide. After washing and homogenization, the membrane fragments were submitted to the crosslinking procedure as described above. Figure 4 shows that most of the dimeric form, the 130,000 dalton component, was now reduced to a 65,000 dalton polypeptide (CBB staining). The distribution of radioactivity again showed two bands at 47,000 and 53,000 daltons but little or no labeled component corresponding to 134,000 daltons. Instead some radioactivity migrated as a component of 73,000 daltons, which most likely represents a 65,000 dalton polypeptide crosslinked to a [^{125}I] α-BuTx-SSR'N_3 molecule. Control experiments were conducted to show the specificity of the crosslinking reactions. It was demonstrated that none of the polypeptides which were labeled after irradiation were labeled without irradiation (Figure 3). Further, when membrane fragments were protected by unlabeled α-BuTx no crosslinked components were detected.

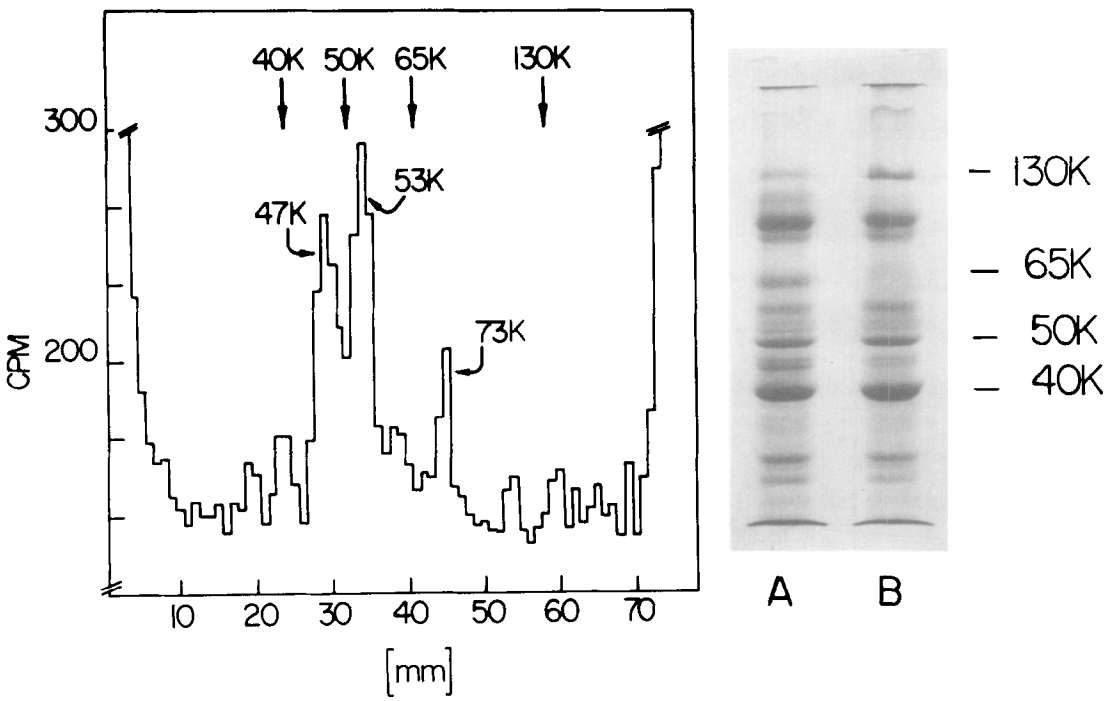

FIGURE 4: SDS-polyacrylamide gel electrophoresis without reducing agents of membrane-bound AcChR. GEL A: The membrane-bound AcChR was treated with dithiothreitol and then alkylated with iodoacetamide (see Results and Discussion) prior to gel electrophoresis. GEL B: membrane-bound AcChR was treated with iodoacetamide without prior exposure to reducing agents. Membrane-bound AcChR, treated as in GEL A was submitted to the crosslinking procedure as described in Methods. The diagram shows the distribution of [^{125}I] after SDS-gel electrophoresis. Arrows on top indicate the position of unmodified AcChR-polypeptides. 47,000, 53,000 and 73,000 daltons are the apparent molecular weights of polypeptides crosslinked to [^{125}I] α-BuTx-SSR'.

There are several implications resulting from the crosslinking results. First, the specificity of the reaction of the modified toxin precludes crosslinking to membrane components other than those that occur at or close to the toxin binding site, i.e., the 40,000 dalton AcChR subunit. Secondly, the results imply that the 65,000 dalton polypeptide is a component of the receptor complex. This was suggested earlier on the basis of the composition of detergent extracted Torpedo californica AcChR following purification by affinity chromatographic methods under conditions where calcium activated proteolysis

was eliminated (22,23,11) by inclusion of EDTA rather than PMSF. Recently the validity of this conclusion has been questioned (24) since membranes from Torpedo marmorata were prepared that contained, in selected fractions from a sucrose gradient, only 40,000 and 43,000 dalton polypeptides. Following detergent treatment of these fractions a solubilized component that bound α-toxin was obtained that contained only one polypeptide of M.W. 40,000 daltons. Sonication was used in preparation of membranes with possible degradation due to cavitation and in addition PMSF rather than EDTA was added to limit proteolysis. The 9S value reported agrees with that for the monomeric form of Torpedo californica AcChR (25), however, the specific activity (7,000 nmoles of α-toxin sites/gm protein) is considerably lower than that reported for Torpedo californica with its full complement of subunits (10,000 nmoles/gm protein) and significantly lower than that expected (~25,000 nmoles/gm protein) if the subunits of 50,000, 60,000 and 65,000 daltons were mere contaminants.

That the purified AcChR of 9S monomeric form and 13S dimeric form (see below) have subunits other than one of 40,000 daltons in association with each other is strongly supported by crosslinking experiments on purified receptor preparations (3,11). It is important, however, to demonstrate whether such association exists in membrane preparations. We have previously demonstrated (9), using the cholinergic antagonist analogue bis-(3-azidopyridinium) 1, 10-decane perchlorate, that in membranes the 50,000 dalton polypeptide is in close proximity to the 40,000 dalton species that binds cholinergic ligands. In addition we have more recently shown (13) that conformational transitions initiated at the 40,000 dalton subunit upon interaction with cholinergic agonists or antagonists are transmitted to polypeptides of M.W. 50,000 and 65,000. These studies are consonant with the results we report here based on a structural approach, namely affinity directed crosslinking of subunits within the AcChR polypeptide complex in a membrane environment.

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REFERENCES

1. Lee, C.Y. and Chang, C.C. (1966) Memb. Inst. Butantum. Symp. Internac. 33, 555.
2. Martinez-Carrion, M., Sator, V. and Raftery, M.A. (1975) Biochem. Biophys. Res. Commun. 65, 129.
3. Hucho, F., Bandini, G. and Suarez-Isla , B.A. (1978) Eur. of Biochem. 83, 335.
4. Reynolds, J.A. and Karlin, A. (1978) Biochemistry 17, 2035.
5. Weill, C.L., McNamee, M.G. and Karlin, A. (1974) Biochem. Biophys. Res. Commun. 61, 997.
6. Reed, K., Vandlen, R.L., Bode, J., Duguid, J.K. and Raftery, M.A. (1975) Arch. Biochem. Biophys. 167, 138.
7. Hamilton, S.L., McLaughlin, M. and Karlin, A. (1977) Biochem. Biophys. Res. Commun. 79, 692.
8. Witzemann, V. and Raftery, M.A. (1978) Biochem. Biophys. Res. Commun. 81, 1025.
9. Witzemann, V. and Raftery, M.A. (1978) Biochemistry 16, 5862.
10. Moore, H.P. Hsu and Raftery, M.A. (1978) Submitted.
11. Chang, R.S.L., Potter, L.T. and Smith, D.S. (1977) Tissue and Cell 9, 623.
12. Hucho, F., Layer, P., Kiefer, H.R. and Bandini, G. (1976) Proc. Natl. Acad. Sci. USA 73, 2624.
13. Witzemann, V. and Raftery, M.A. (1978) Biochemistry, in press.
14. Duguid, J.K. and Raftery, M.A. (1973) Biochemistry 12, 3693.
15. Clark, D.G., Macmurchie, D.D., Elliot, E., Wolcott, R.G., Landel, A.M. and Raftery, M.A. (1972) Biochemistry 11, 1663.
16. Laemmli, J.K. (1970) Nature 227, 680.
17. Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349.
18. Chicheportiche, R., Vincent, J.-P., Kopeyan, C., Schweitz, H. and Lazdunski, M. (1975) Biochemistry 14, 2081.
19. Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70.
20. Botes, D.P. (1974) Biochem. Biophys. Acta 359, 242.
21. Chang, H.W. and Bock, E. (1977) Biochemistry 16, 4513.
22. Raftery, M.A., Vandlen, R.L., Reed, K.L. and Lee, T. (1975) Cold Spring Harbor Symp. Quant. Biol. 40, 193.
23. Karlin, A., Weill, C.L., McNamee, M.G. and Valderrama, R. (1975) Cold Spring Harbor Symp. Quant. Biol. 40, 203.
24. Sobel, A., Weber, M. and Changeux, J.-P. (1977) Eur. J. Biochem. 80, 215.
25. Raftery, M.A., Schmidt, J. and Clark, D.G. (1972) Arch. Biochem. Biophys. 152, 882.