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SPECIFIC MOLECULAR AGGREGATES OR TORPEDO CALIFORNICA ACETYLCHOLINE RECEPTOR

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SUMMARY Acetylcholine receptor-enriched membrane fragments prepared from the electric tissue of Torpedo californica showed altered electrophoretic patterns depending on whether polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out in the presence or absence of reducing agent. It was demonstrated that disulfide bonds exist between the 65,000 dalton subunits of the acetylcholine receptor, resulting in formation of dimers and trimers of this subunit. No such specific intermolecular disulfide bonded complexes were observed for the other three acetylcholine receptor subunit types.

INTRODUCTION

Acetylcholine receptor (AcChR) from the electric tissue of Torpedo californica has been purified in detergent solubilized form (see References in 1). Upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate in the presence of reducing reagents, four polypeptides were found with apparent molecular weights of 40,000, 50,000, 60,000 and 65,000 daltons. The same four subunits appear as the most prominent Coomassie Blue staining bands upon similar SDS gel electrophoresis of membrane fragments highly enriched in AcChR (2). A specific function has been assigned only to the 40,000 dalton subunit, which apparently contains binding sites for small ligands (3-5) and α-neurotoxins (3,4,6).

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AcChR, acetylcholine receptor; DTT, dithiothreitol; SDS, sodium dodecylsulfate.

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We have recently shown that conformational changes induced by ligand binding to the 40,000 dalton subunit are communicated to the subunits of molecular weight 50,000 and 65,000 (7). This suggests that at least these three polypeptides (M.W. 40,000, 50,000 and 65,000) are functional subunits of the AcChR complex.

The molecular weight of the receptor has been estimated by several methods (see References in 1) and it has been shown to occur as a monomer and dimer upon solubilization (11) and purification (18). We present evidence in this communication that specific disulfide bonds between 65,000 dalton subunits lead to the formation of AcChR dimers and higher molecular weight complexes in the membranes with which the AcChR is associated.

MATERIALS AND METHODS

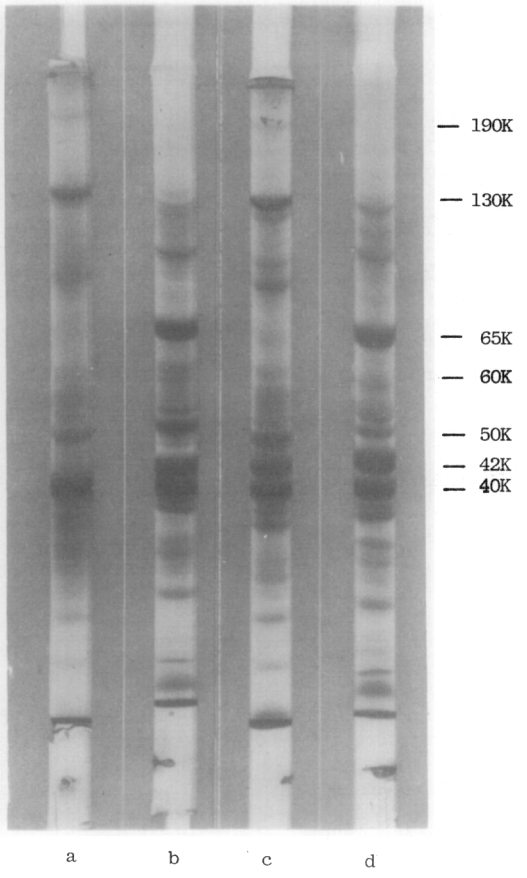
Reagents used in SDS-polyacrylamide gel electrophoresis were purchased from BIO-RAD Laboratories. All other chemicals used were of the purest grade commercially available. Electric organs of Torpedo californica (fresh or stored at -90°C) were used for the preparation of AcChR-enriched membrane fragments as described in (2,9). The buffer used during the preparation of the crude membrane fragments was Torpedo Ringers. Free sulfhydryl groups of membrane fragment preparations (ca 4 mg protein/ml) were modified at pH 7.4 and room temperature by adding fresh aqueous solutions of N-ethylmaleimide or iodoacetamide to final concentrations of 10 mM. After 1 hour incubation samples were submitted to SDS-polyacrylamide gel electrophoresis following the procedure described by Laemmli (11), leading to final concentrations of N-ethylmaleimide and iodoacetamide of 5 mM since rate constants for solvolysis of these compounds far exceed the one hour incubation time (15-17). Additional experiments were conducted by adding fresh N-ethylmaleimide and iodoacetamide (10 mM) to the denaturing solutions just before addition to the samples. Denaturation in SDS was carried out in the presence or absence of 2.5% β-mercaptoethanol. Gels were scanned at 550 nm using a Gilford linear transport unit.

For preparation of membrane fragments in the presence of iodoacetamide electric tissue was homogenized in Torpedo Ringers containing 5 mM iodoacetamide and all purification steps were carried out in the presence of iodoacetamide (10 mM) except the sucrose zonal centrifugation step.

RESULTS

Membrane fragments from electric tissue of Torpedo californica which contained high levels of cholinergic receptor (20-50% of total protein) were denatured in the presence of β-mercaptoethanol (or DTT) and the resulting polypeptide pattern after polyacrylamide gel electrophoresis demonstrated the

enrichment of AcChR protein over other membrane proteins: the most abundant polypeptide components had apparent molecular weights of 40,000, 42,000, 50,000 and 65,000 (Figure 1). The relative amount of the 42,000 dalton component varied in different membrane fragment preparations (compare Figures 1 and 2) and this polypeptide was not a major component of the purified AcChR. Compared to the staining pattern of purified AcChR, the 60,000 dalton component of membrane fragments prepared in Torpedo Ringers stained less intensely. If SDS-polyacrylamide gel electrophoresis of membrane bound AcChR was conducted in the absence of reducing agents an altered electrophoretic pattern was observed. The band corresponding to the 40,000 dalton polypeptide remained constant and the amounts of 50,000 and 60,000 dalton components were decreased, whereas the 42,000 and the 65,000 dalton species disappeared almost completely. Two new bands arose instead at positions corresponding to molecular weights of about 130,000 and 190,000 (Figure 1). To determine the origin of these two high molecular weight components membrane fragments were submitted to two-dimensional SDS-polyacrylamide gel electrophoresis, the first dimension being run in the absence of reducing agents. This gel was then incubated for 30 minutes at room temperature in β-mercaptoethanol-containing buffer before separation of the polypeptides in the second dimension (Figure 2). Both the 130,000 and the 190,000 dalton components migrated as 65,000 dalton polypeptides and no major changes occurred with respect to the distribution of the other three AcChR subunits. These all appeared on the diagonal in the second dimension, indicating that their electrophoretic behavior was essentially the same in the presence or absence of reducing agents. Protein which did not enter the separation gel or even the stacking gel contained all the polypeptides seen under reducing conditions (not visible on the photograph). This could be due to incomplete denaturation. To exclude artifactual cross-linking by formation of disulfide bridges *in vitro*, membrane fragments were reacted with N-ethylmaleimide or iodoacetamide prior to and during SDS-denaturation to block all free sulfhydryl groups. Little 65,000 dalton subunit was ob-



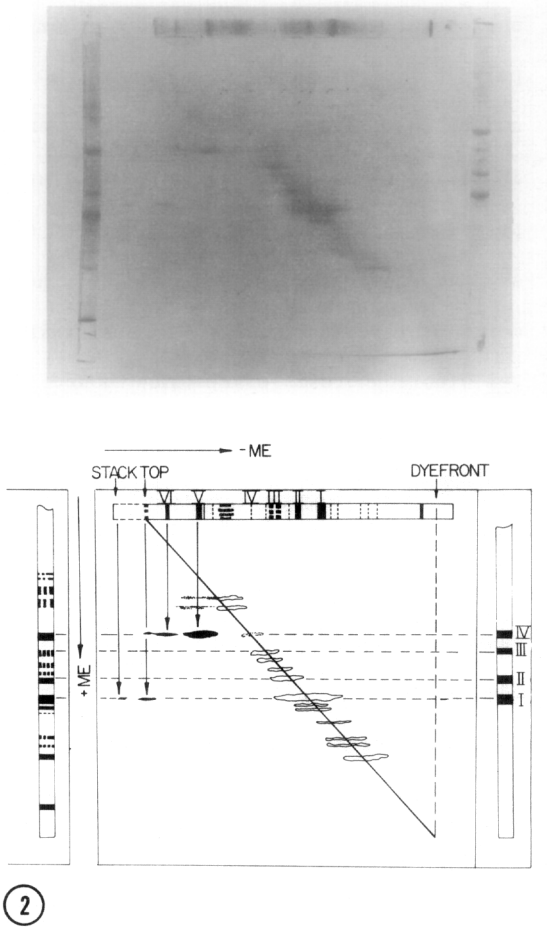


FIGURE 1: SDS-Polyacrylamide Gel Electrophoresis of Membrane Fragments: AcChR-rich membrane fragments (200 µg protein) were treated with 2% SDS (W/V), a) in the absence of β-mercaptoethanol or, b) in the presence of β-mercaptoethanol (2.5%), or the membrane fragments were reacted with iodoacetamide 10 mM and SDS-polyacrylamide gel electrophoresis was performed, c) in the absence of β-mercaptoethanol, d) in the presence of β-mercaptoethanol (2.5%). The numbers indicate the apparent molecular weights.

FIGURE 2: Two dimensional Analysis of Aggregates Cross-linked by Disulfide Bonds: The slab gel shows the spots generated by cleaving the cross-linked components with β-mercaptoethanol. The tube gel on top of the slab gel shows membrane fragments after SDS-polyacrylamide gel electrophoresis without β-mercaptoethanol (= first dimension). On the left side: membrane fragments after SDS-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol; right side: solubilized purified AcChR after SDS-polyacrylamide gel electrophoresis in the presence of β mercaptoethanol. Schematic drawing: blank spots represent the polypeptides which migrate identically in the presence or absence of β-mercaptoethanol; off diagonal spots derived from cross-linked products are drawn in black. I corresponds to an apparent molecular weight of 40,000 daltons, II = 50,000, III = 60,000, IV = 65,000, V = 130,000 and VI = 190,000 daltons, respectively.

TABLE 1: RELATIVE RATIOS OF AcChR SUBUNITS IN THE PRESENCE OR ABSENCE OF β-MERCAPTOETHANOL

CONDITIONS: AcChR-RICH MEMBRANE FRAGMENTS IN TORPEDO RINGERS BUFFER	MW	APPARENT RATIOS OF AcChR SUBUNITS*					
		40K	50K	60K	65K	130K	190K
SH-GROUPS NOT MODIFIED	+RSH**	1	0.43	0.28	0.5	----	----
	-RSH	1	0.39	0.25	---	0.26	0.02
SH-GROUPS REACTED WITH iodoacetamide	+RSH	1	0.31	0.31	0.56	----	----
	-RSH	1	0.27	0.27	----	0.36	0.08
SH-GROUPS REACTED WITH N-ethyl-maleimide	+RSH	1	0.24	0.24	0.61	----	----
	-RSH	1	0.26	0.26	----	0.53	0.08

* Apparent ratios determined by scanning Coomassie Blue Stained gels.

** RSH denotes β-mercaptoethanol

served but in its place polypeptides of apparent M.W. 130,000 and 190,000 daltons were seen (Figure 1). These specific effects were further confirmed when electric tissue was homogenized and worked up in the presence of iodoacetamide. Under these conditions artifactual reactions between free sulfhydryl groups should be excluded throughout the purification procedure as well as during denaturation. SDS-polyacrylamide gel electrophoresis with or without β-mercaptoethanol led to the same results as before with crude membrane fragments containing almost no 65,000 dalton component in the absence of β-mercaptoethanol (not shown). Sucrose velocity centrifugation experiments of detergent extracts also showed increased levels of AcChR dimers following treatment of membranes with iodoacetamide (J. Miller and M. Raftery, unpublished). In contrast to these specific intermolecular disulfide bonds between 65,000 dalton subunits artifactual cross-linking could be demonstrated for the 42,000 dalton component associated with the membranes. Without blocking the free sulfhydryl groups this polypeptide was detectable only in the presence of β-mercaptoethanol and membrane fragments

treated with N-ethylmaleimide or iodoacetamide showed the 42,000 dalton component whether reducing agent was present or absent during denaturation (Figure 1). Scans of Coomassie Blue stained gels were used to estimate the relative amounts of each subunit. With or without β-mercaptoethanol the ratios of the 50,000 and 60,000 dalton subunits stayed rather constant when compared to the corresponding 40,000 dalton subunit. There were no indications of specific intermolecular disulfide bonds between these three subunits (Table 1).

DISCUSSION

One of the four AcChR subunits, the 65,000 dalton polypeptide was found to exist mainly in dimeric form and to some extent in trimeric form in membranes if denaturation by SDS, prior to SDS-polyacrylamide gel electrophoresis, was carried out in the absence of reducing agents. This result was obtained both with and without alkylating agents being present during and after denaturation. In the presence of β-mercaptoethanol all such cross-linked polypeptides were dissociated and migrated as 65,000 dalton components. (Dimers of the 65,000 dalton subunit were also observed with freshly prepared purified AcChR (Vandlen and Raftery, 1978). The quantitative differences observed with the other AcChR subunits (especially the 50,000 dalton subunit following chemical modification) could be due to incomplete denaturation by SDS and this point needs further investigation, since no 50,000 polypeptide was observed off the diagonal in the second dimension. The specific cross-linking by disulfide bridges of 65,000 dalton subunits indicates a possible structural function for AcChR dimers or higher oligomeric forms. Previously, dimeric forms and possibly higher aggregates of native receptor were shown to exist by fractionation on Sepharose 6B (11, 18, 19), by sucrose density centrifugation (8,12) by sedimentation equilibrium (13) and on non-denaturing polyacrylamide gel systems (8). Preliminary results demonstrated that the dimeric AcChR can be transformed into its monomers by addition of dithiothreitol (8), indicative of disulfide bonds being involved in forming oligo-

mers of AcChR. However, it was not clear that such covalent bonds were formed in a specific manner since without alkylation of free sulfhydryls purified AcChR undergoes changes in dimer: monomer ratios as a function of time (to be published). During preparation of this manuscript similar observations with respect to the dimerization of a 68,000 dalton subunit in membranes were described by Suarez and Hucho (15) whereas the formation of higher molecular weight disulfide cross-linked complexes was not observed. The observation of the occurrence of disulfide-linked trimers of the 65,000 dalton polypeptide of the membrane-bound AcChR complex requires that at least some of these are linked by more than one disulfide bond.

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