SOLUBILIZATION OF THE ACETYLCHOLINE RECEPTOR PROTEIN FROM *LOLIGO OPALESCENS* WITHOUT DETERGENTS

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1. Introduction

We [1] recently showed that the acetylcholine (ACh) receptor isolated from the optic ganglia of squid *(Loligo opalescens)* is similar to the ACh nicotine receptor of electric organs of *Electrophorus electricus* [2], *Torpedo marmorata* [3] and of vertebrate skeletal muscle [4]. The assay for the receptor in crude extracts of optic ganglia was based on the ability of the receptor site to bind [^{14}C]decamethonium, [^{14}C]-d-tubocurarine and [^{125}I]α-bungarotoxin and for the capacity of the antagonists gallamine, d-tubocurarine and α-bungarotoxin to displace [^{14}C]decamethonium binding [1].

It is generally believed [5] that the ACh receptor is strongly membrane bound and that it can be solubilized only by treatment of membrane extracts containing the receptor with mild detergents such as Triton X-100 [6], sodium deoxycholate [7] or lubrol-WX [3,8]. Several laboratories [7,9–12] have succeeded in purifying the detergent solubilized receptor and have determined its molecular weight using different physical methods.

In our attempts to isolate membrane fragments rich in ACh receptors from optic ganglia of *Loligo opalescens* [1] we observed that the receptor protein could be solubilized without using detergents. In this paper we show that in salt solution (0.1 M NaCl, 10 mM phosphate buffer, pH 7.8) the [^{125}I]α-bungarotoxin labeled receptor protein of *Loligo opalescens* has a mol. wt. of 240 000±20 000 when determined by gel filtration on Bio-Gel A-5m or by sucrose gradient centrifugation. Sodium dodecyl sulphate disc gel electrophoresis indicates that the molecular weight of the smallest subunit of the receptor that binds [^{125}I]α-bungarotoxin is 40 000.

2. Methods and materials

2.1. *Preparation of the crude membrane fraction*

The procedure for the preparation of crude extracts from optic ganglia of squid has been described elsewhere [1] and is summarized below. The method yields membrane fragments rich in ACh nicotine receptors as well as solubilized receptor protein. *Loligo opalescens* were obtained frozen from the U.S. Freezer Co. (Monterey, California). Squid were decapitated and the optic ganglia were dissected from the head. 11 g of tissue which was routinely obtained from 50 squid was homogenized in 110 ml of 0.01 M phosphate buffer, pH 7.4 containing 0.1 M NaCl (this is hypo-osmotic to squid tissue) in a tissue homogenizer provided with a Teflon pestle (clearance 0.006–0.009 in). The pestle was set to rotate at 900 rev/min. for 2 min during which time the pestle was moved up and down. The homogenate was centrifuged at 27 000 *g* for 15 min. The supernatant was decanted and kept at 4°C and the residue was re-homogenized in 80 ml of the same buffer. The residue was discarded and the supernatants combined. The supernatant was fractionated with $(NH_4)_2SO_4$; the 20–40% $(NH_4)_2SO_4$ precipitate was taken up in 20 ml of 0.1 M NaCl adjusted to pH 7.8 with bicarbonate and was dialized for 4 hr against 8 litres of 0.1 M NaCl pH 7.8. The entire extraction procedure was carried out at 4°C.

This fraction was the richest in receptor material based on the binding of [^{125}I]α-bungarotoxin using

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the binding assay of Olsen et al. [13]. We have repeated some of the experiments described below using live squid *(Loligo paelii)* obtained from Woods Hole, Mass. USA with little or no difference in [^{125}I]α-bungarotoxin binding activity or physicochemical properties of the soluble ACh-receptor protein.

2.2. *Preparation of [^{125}I] α-bungarotoxin*

α-Bungarotoxin was isolated from the venom of *Bungarus multicinctus* (Miami Serpentarium) and was purified by gel filtration on Sephadex G-50 followed by chromatography on carboxymethyl (CM)-Sephadex (C-25). ^{125}I-labeled α-bungarotoxin was prepared by the method of Greenwood et al. [14] as modified by Berg et al. [15] and was determined on a Nuclear Chicago gamma ray spectrometer. Its specific activity was 12.8 × 10^6 cpm/μg protein.

2.3. *Sucrose gradient centrifugation*

Stock solutions containing 5% or 20% (w/v) sucrose in 0.1 M NaCl 10 mM phosphate pH 7.8 were prepared in water as the solvent. 50 μl of a 50% sucrose solution (w/v) was pipetted at the bottom of each centrifuge tube. The gradients (17–8% sucrose; 5.0 ml) were made with a Beckman density gradient former at room temperature and stored overnight at 4°C. The sample (0.18 ml) supplemented with 10 μl beef liver catalase (Sigma cat. # C–40) and 10 μl β-galactosidase (Sigma cat. # G-8504) to a final vol of 0.20 ml was layered on top of each 5 ml linear sucrose gradient. The tubes were centrifuged in a SW 50.1 Beckman rotor of a Beckman model L2-65B preparative ultracentrifuge at 49 000 rpm for 5 hr at 4°C. Fractions of approximately 0.1 ml were collected after perforation of the bottom of the tube.

After centrifugation the marker protein catalase was assayed by following the decrease in absorbancy of a solution containing enough H_2O_2 to give an absorption of 1.6 at 240 nm in 50 mM phosphate, pH 7.0. β-Galactosidase was assayed by following the hydrolysis of ortho-nitrophenyl-β-galactoside according to Horiuchi et al. [16].

Protein concentration was measured by the method of Lowry [17] with bovine serum albumin as a standard, AChE activity by measuring the rate of hydrolysis of acetylthiocholine [18].

2.4. *Gel Filtration on Bio-Gel A-5m*

The soluble ACh-receptor–[^{125}I] α-bungarotoxin complex obtained from the sucrose gradient was chromatographed on a column (1.5 × 100 cm) of Bio-Gel A-5m in 0.1 M NaCl, 10 mM phosphate buffer pH 7.8. The sample (2 ml) also contained 2 mg Dextran blue 2000 (Pharmacia), 120 μl β-galactosidase, and 120 μl catalase. 1 mg ovalbumin (Pharmacia) and 5 μl of [^{125}I] α-bungarotoxin (8 μg/ml) contained in a total vol. of 2 ml were also run through the same column but in a separate experiment. Two ml fractions were collected on a Gilson Mini Fractionator at 4°C at a constant flow rate of 10 ml/hr. β-Galactosidase and catalase were assayed as described in section 2.3; [^{125}I] α-bungarotoxin was measured by gamma counting and ovalbumin and dextran blue were measured by reading the optical density at 280 nm on a Cary 14 spectrophotometer.

2.4. *Sodium dodecyl sulphate gel electrophoresis*

Electrophoreses were performed in 7.5% polyacrylamide gels containing 0.1% SDS with a buffer of 0.1 M phosphate pH 8.5, 0.1% SDS. β-Galactosidase (Sigma cat. # G-8504) catalase (Sigma cat. # C-40), ovalbumin (Pharmacia) alcohol dehydrogenase (Sigma cat # A 7011) and cytochrome *c* (Nutritional Biochem. Corp.) were used for molecular weight calibration. SDS and β-mercaptoethanol were added to the protein samples at a final concentration of 1% before incubation for 1 min in a boiling water bath. The samples (100–200 μl) also contained sucrose (20%) and bromophenol blue as the tracking dye. Electrophoresis was done at 3 mA per tube for 2 hr. Gels were stained with 1% Coomassie Blue in 10% acetic acid–ethanol solution and were destained by shaking overnight in 10% acetic acid. The gels were cut up in 2.0 mm sections from the dye front with a scalpel blade and counted in a gamma counter.

3. Results

3.1. *Sucrose gradient centrifugation*

Suspensions of the crude membrane preparation in 0.1 M NaCl, 10 mM phosphate, pH 7.8 were layered on a linear sucrose gradient in the range 17–8% sucrose and centrifuged for 5 hr at 230 000 *g*. Fig. 1 shows the distribution of acetylcholinesterase and [^{125}I] α-bungarotoxin binding activities after centrifuga-

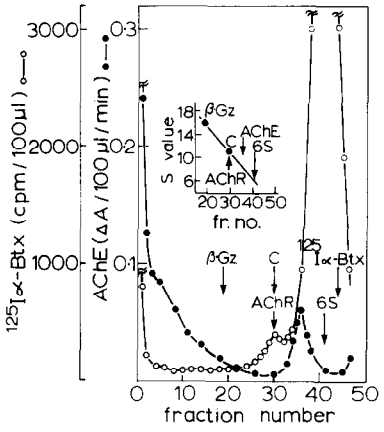

Fig. 1. Sucrose density gradient centrifugation of crude membrane fragments of squid optic ganglia. The conditions are given in the text. The temperature was 4°C. C is catalase, β-GZ is β-galactosidase, AChE is acetylcholinesterase and [^{125}]α-Btx is ^{125}I-labeled α-bungarotoxin. AChR is ^{125}Iα-Btx labeled ACh receptors. Fraction number 0 is bottom of tube (17% sucrose), number 47 is the top (8% sucrose). Centrifugation was carried out in a SW 50.1 rotor at 49 000 rev/min for 5 hr.

tion in the sucrose gradient. Two peaks of acetylcholinesterase and three peaks of [^{125}I]α-bungarotoxin binding activities are observed on this graph. The acetylcholinesterase and [^{125}I]α-bungarotoxin binding activities at the bottom of the tube are due to the presence of this enzyme and the ACh receptor in the membrane vesicles which pellet under the present conditions of centrifugation. This fraction (fraction MP) consists of closed membrane fragments of 0.1 µm average diameter [1].

The acetylcholinesterase in the soluble fraction has a sedimentation coefficient of 7 S (estimated by linear interpolation) assuming that the sedimentation coefficients of catalase and β-galactosidase are 11.4 and 16 S respectively. [^{125}I]α-bungarotoxin labeling is also found in the soluble fraction; this labeled material has an S value of 11 which is close to that of catalase (11.4 S) but is distinct from the soluble 7 S acetylcholinesterase. We refer to this material as [^{125}I]α-Btx–AChR for the [^{125}I]α-bungarotoxin labeled acetylcholine receptor. No membrane fragments are observed when this peak is examined under the electron microscope.

A third major peak of radioactivity, with a sedimentation coefficient of approximately 6 S appears near the top of the sucrose gradient. Free [^{125}I]α-bungarotoxin sediments near the top of the gradient (fig. 1).

3.2. *Gel filtration on Bio-Gel A-5m*

Fractions 26 to 33 obtained from 6 sucrose gradients were pooled, concentrated with Ficoll and applied to a Bio-Gel A-5m column. Fig. 2 shows the filtration profile of a Bio-Gel A-5m column of the [^{125}I]α-Btx–AChR peak. The radioactive peak coincided with that of catalase used as a marker. The second peak of radioactivity corresponds to that of free [^{125}I]α-bungarotoxin used as a marker.

Gel filtration gives indications on the Stokes radius of a macromolecule, not its molecular weight. The apparent molecular weight of the material present in the first peak was determined by using β-galactosidase, catalase, ovalbumin and [^{125}I]α-bungarotoxin as standards run in 0.1 M NaCl, 10 mM phosphate pH 7.8 at 4°C. Fig. 3 is a plot of the molecular weight of the four markers versus elution volume. The apparent Stokes radius of the receptor–toxin complex in 0.1 M NaCl, 10 mM phosphate, pH 7.8 at 4°C was always

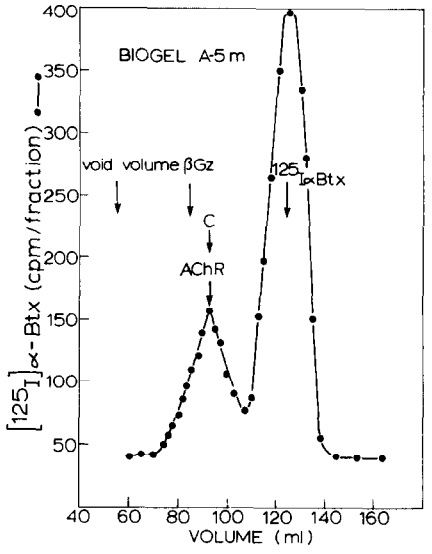

Fig. 2. Filtration on a Biogel A-5m column in 0.1 M NaCl, 10 mM phosphate buffer pH 7.8 at 4°C of [^{125}I]α-Btx–AChR peak obtained from sucrose gradient (fraction 26–33 fig. 1) β-Gz is β-galactosidase, C is catalase, AChR is ^{125}I-α-Btx labeled ACh receptor protein, ^{125}I-α-Btx is ^{125}I-labeled α-bungarotoxin.

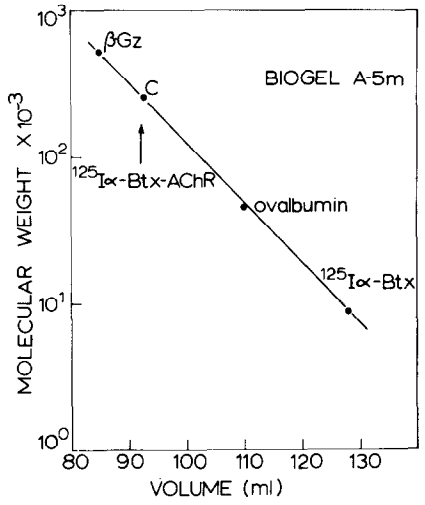

Fig. 3. Plot of the molecular weight of four markers and the [^{125}I]α-Btx–AChR complex versus fraction number. Filtration was done on a Biogel A-5 m column in 0.1 M NaCl 10 mM phosphate buffer pH 7.8 at 4°C. β-Gz is β-galactosidase, C is catalase, [^{125}I]α-Btx is ^{125}I-labeled α-bungarotoxin.

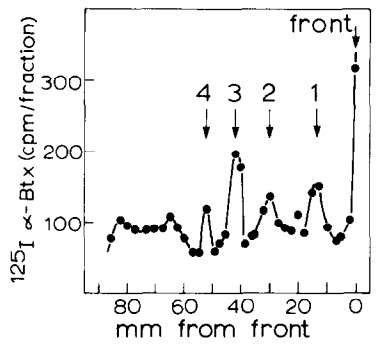

Fig. 4. Polyacrylamide gel electrophoresis of [^{125}I]α-Btx–AChR in 0.1% SDS. Sample of [^{125}I]α–Btx–AChR was obtained by sucrose gradient centrifugation followed by gel filtration on Biogel A-5m; sample was treated with 1% SDS in a boiling water bath for 60 sec. After electrophoresis was complete, gel was cut up in 2 mm segments and counted in a Nuclear Chicago gamma-ray spectrometer. The numbers 1, 2, 3 and 4 refer to the 4 peaks of radioactivity from the dye front.

the same as a globular protein of mol. wt. 240 000± 20 000 and is therefore similar to that of catalase and to the molecular weight of the receptor–toxin complex obtained by sucrose gradient centrifugation.

3.3. *Sodium dodecyl sulphate gel electrophoresis*

The acetylcholine receptor–toxin complex obtained from sucrose gradient centrifugation followed by gel filtration on Bio-Gel A-5m was concentrated to 200 μl with Ficoll and run on SDS disc gel electrophoresis with the hope that the [^{125}I]α-bungarotoxin would not dissociate from the receptor subunits under our experimental conditions. Fig. 4 shows the electrophoresis in 0.1% SDS of [^{125}I]α-Btx–AChR preincubated for 1 min in boiling water with 1% SDS. Electrophoresis in 0.1% SDS of the soluble receptor toxin complex yielded at least 4 peaks of radioactivity (1,2,3 and 4, fig. 4) between the markers of mol. wt. 40 000 to 280 000.

The molecular weight calibration of the gels is shown in fig. 5. Peaks of radioactivity had estimated mol. wt. of 1) 48 000, 2) 92 000, 3) 150 000 and 4) 280 000. It became apparent that the gels showed radioactive components running at approximately 2, 3, and 6 times the molecular weight of the smallest unit at

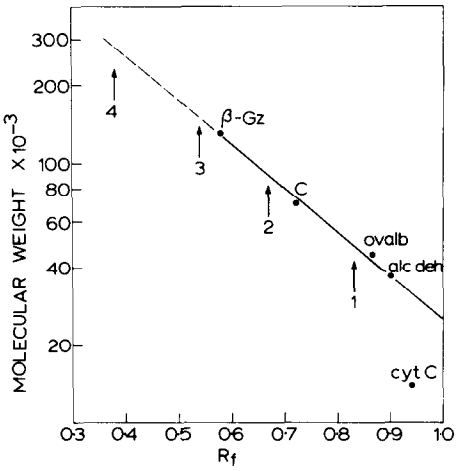

Fig. 5. Molecular weight determination of the subunits of the [^{125}I]α-Btx labeled ACh receptor protein. The numbers 1, 2, 3 and 4 correspond to the peaks of radioactivity shown in fig. 4 and may correspond to the smallest subunit, dimer trimer and native receptor protein, respectively (see discussion in the text). β-Gz in β-galactosidase, C is catalase, ovalb is ovalbumin, alc. deh. is alcohol dehydrogenase and cyt C is cytochrome *c*.

48 000. The intensity of the radioactivity in the bands was variable although the peak at mol. wt. 150 000 was the most intense.

4. Discussion

The results of this study show that in the optic ganglia of squid the ACh receptor protein can be solubilized without detergents. This property makes the squid receptor unique since mild detergents are required for the solubilization of the ACh receptor protein from electric organs of *Electrophorus electricus* or *Torpedo marmorata* [3,7].

There are several possible reasons for this difference in property between the two receptors. First, the two proteins may be embedded in the membrane structure differently. The eel receptor may be an integral protein, that of the squid a peripheral protein similar to eel acetylcholinesterase which can be solubilized from membranes rather easily [19]. Secondly, we used frozen tissue where solubilization may occur more readily than in fresh tissue. Finally, the conditions of extraction such as low ionic strength of the extraction medium followed by ammonium sulphate fractionation may also aid in solubilizing the receptor from the membranes. Whether or not the receptor protein from eel or *Torpedo* can be solubilized without detergents using the present or similar conditions is not known since the possibility has not been thoroughly investigated [5].

In some respects, however, the ACh receptor of squid is similar to that of eel or *Torpedo*. Both appear to be identical with the ACh nicotine receptor of vertebrate skeletal muscle on the basis of binding ACh agonists and antagonists [1–3]. The dissociation constant for cholinergic ligands and the amount of ligand bound per gram of protein are more or less similar [2,3] although not always identical [20,21]. Both receptors have an apparent mol. wt. of 240 000 [10] and the molecular weight of the smallest subunit in each case is about 40 000. Both are distinct from acetylcholinesterase [2].

It is interesting that the molecular weight of the ACh-receptor from squid optic ganglia is close to that obtained by Changeux [10] for the receptor from eel or by Raftery [22] for that from *Torpedo*. Meunier et al. [7] showed that the receptor protein exhibited unusual hydrodynamic properties in detergent solution. Gel filtration experiments indicated that the receptor had a Stokes radius close to that of β-galactosidase (mol. wt. = 550 000); sedimentation in sucrose density gradient suggested that the receptor had a molecular weight close to that of catalase (mol. wt. = 240 000). This behavior was attributed to the binding of detergent molecules to the protein or to the presence of a rod shaped complex [9]. The soluble [^{125}I]α-Btx–AChR from squid optic ganglia sediments with catalase on sucrose gradients and is eluted with catalase on a Bio-Gel A-5m column indicating an apparent mol. wt. of 240 000 for the receptor–toxin complex. It is likely that the estimates of the molecular weight of the receptor–toxin complex by the two methods are identical due to the absence of detergent which could alter its hydrodynamic properties. This finding confirms the idea that the unusual hydrodynamic properties of the ACh receptor–[^{3}H]α-toxin complex are due to the binding of detergent to the receptor molecule rather than to its rod-like shape [9].

Sodium dodecyl sulphate disc gel electrophoresis indicates that the molecular weight of the smallest subunit of the ACh-receptor–toxin complex is 48 000. It is possible that the receptor protein was not completely dissociated under present conditions of denaturation with SDS. If we assume that one toxin molecule (mol. wt. = 8000) binds per subunit and if the molecular weights observed are multiplets of the smallest subunit (48 000) then the molecular weights of the monomer, dimer, trimer and protomer would be 40 000, 76 000, 126 000 and 232 000 respectively.

A large portion (60%) of the total radioactivity applied to the sucrose gradient sedimented with a sedimentation coefficient of 6 S while a smaller portion (10%) was associated with the pellet and the 11 S peak (10%). The 6 S toxin labeled material is either a protein distinct from the ACh receptor or a degradation product of the receptor itself. We present further analysis of this material in a following paper [23].

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References

[1] Kato, G. and Tattrie, B. (1974) Proc. Israel Acad. Sci. (in the press).
[2] Changeux, J.-P., Meunier, J.-C. and Huchet, M. (1971) Mol. Pharmacol. 7, 538.
[3] Eldefrawi, M. E., Eldefrawi, A. T., Seifert, S. and O'Brien, R. D. (1972) Arch. Biochem. Biophys. 150, 210.
[4] Miledi, R. and Potter, L. T. (1971) Nature 233, 599.
[5] Meunier, J.-C., Sugiyama, H., Cartaud, J., Sealock, R. and Changeux, J.-P. (1973) *Brain Res.* 62, 307.
[6] Miledi, R., Molinoff, P. and Potter, L. (1971) Nature 229, 554.
[7] Meunier, J.-C., Olsen, R. W., Menez, A., Fromageot, P., Boquet, P. and Changeux, J.-P. (1972) Biochemistry 11, 1200.
[8] Carrol, R. C., Eldefrawi, M. E. and Edelstein, S. J. (1973) Biochem. Biophys. Res. Commun. 55, 864.
[9] Meunier, J.-C., Olsen, R. W. and Changeux, J.-P. (1972) FEBS Lett. 24, 63.
[10] Hucho, F., Changeux, J.-P. (1973) FEBS Lett. 38, 11.
[11] Eldefrawi, M. E. and Eldefrawi, A. T. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1776.
[12] Raftery, M. A. (1973) Arch. Biochem. Biophys. 154, 270.
[13] Olsen, R. W., Meunier, J.-C. and Changeux, J.-P. (1972) FEBS Lett. 28, 96.
[14] Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963) Biochem. J. 89, 114.
[15] Berg, D. K., Kelly, R. B., Sargent, P. B., Williamson, P. and Hall, Z. (1972) Proc. Natl. Acad. Sci. U.S., 69, 147.
[16] Horiuchi, T., Tamizawa, J. and Novick, A. (1962) Biochim. Biophys. Acta, 55, 152.
[17] Lowry, O. H., Rosbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[18] Ellman, K. P., Courtney, K. P., Andres, J. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88.
[19] Robaire, B. and Kato, G. (1973) FEBS Lett. 38, 83.
[20] Moody, T., Schmidt, J. and Raftery, M. A. (1973) Biochem. Biophys. Res. Commun. 53, 761.
[21] Martinez-Carrion, M. and Raftery, M. A. (1973) Biochem. Biophys. Res. Commun. 55, 1156.
[22] Raftery, M. A., Schmidt, J. and Clark, D. G. (1972) Arch. Biochem. Biophys. 152, 882.
[23] Kato, G. and Tattrie, B. (1974) FEBS Lett. (submitted).