Purification and Characterization of the Cholinergic Receptor Protein in its Membrane-Bound and Detergent-Soluble Forms from the Electric Organ of Torpedo marmorata

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The cholinergic receptor protein has been purified from electric organs of the electric fishes Electrophorus electricus and Torpedo by affinity chromatography through a column made up of a cholinergic ligand covalently bound to Sepharose [for review see Karlin (1974) and Changeux (1975)]. However, it has been shown that a prolonged incubation of the solubilized cholinergic receptor with a cholinergic agonist (Weber et al., 1975), as well as the passage through an affinity column (Sugiyama & Changeux, 1975), may change the molecular properties of the receptor protein. In the present communication, we describe a simple and rapid method for the purification of large amounts of acetylcholine-receptor-rich membranes as well as of acetylcholine-receptor protein, without the use of an affinity-chromatography step.

It has been shown previously (Cohen et al., 1972; Nickel & Potter, 1973; Duguid & Raftery, 1973a,b), that the fresh Torpedo electric organ can be fractionated after homogenization by a differential centrifugation followed by an ultracentrifugation in a sucrose gradient, in order to yield membrane fragments particularly rich in the cholinergic-receptor protein. These membranes have a specific radioactivity of 2–3 μmol of 3H-labelled α-toxin-binding sites/g of protein and most likely represent pieces of subsynaptic membrane. The original fractionation method of Cohen et al. (1972) has been improved in order to obtain larger quantities of completely pure subsynaptic membranes.

All the purification steps were carried out in the presence of 0.1 mM-phenylmethanesulphonyl fluoride as a proteinase inhibitor and 0.02% NaN₃ to prevent bacterial growth. The freshly dissected electric organ from Torpedo marmorata was homogenized in distilled water containing NaN₃ and phenylmethanesulphonyl fluoride, and then submitted to successive differential centrifugations. A first low-speed centrifugation (7000g, 10 min) of the homogenate gave a supernatant S₁ and a pellet P₁, which was homogenized again and re-centrifuged in the same conditions to yield the S₂ supernatant. Supernatants S₁ and S₂ were mixed to form supernatant S₁₂, which was centrifuged at 10000g for 2 h to pellet the membranes (P₂). P₃ was resuspended in 32% sucrose and the resulting membrane suspension (E₁) was layered on top of a discontinuous sucrose gradient (34–37.5–41.5%) and centrifuged at 100000g for 6 h. The band at 37.5% sucrose was collected, diluted and centrifuged to pellet the membranes, which were again resuspended in 32% sucrose (E₂). Suspension E₂ was layered on top of a continuous (35–43%) sucrose gradient and centrifuged at 100000g for 6 h. The cholinergic-receptor-
Table 1. Results of the purification of receptor-rich membrane fragments (M) and Triton X-100-extracted receptor protein (R) from Torpedo marmorata electric organ (A. Sobel & J. P. Changeux, unpublished work)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Acetylcholine-receptor protein (nmol)</th>
<th>Sp. activity (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{1,2}$</td>
<td>12400</td>
<td>1240</td>
<td>100</td>
</tr>
<tr>
<td>E$_1$</td>
<td>2400</td>
<td>860</td>
<td>370</td>
</tr>
<tr>
<td>E$_2$</td>
<td>250</td>
<td>620</td>
<td>2500</td>
</tr>
<tr>
<td>M</td>
<td>140</td>
<td>560</td>
<td>4000</td>
</tr>
<tr>
<td>R</td>
<td>50</td>
<td>350</td>
<td>7000</td>
</tr>
</tbody>
</table>

rich fractions were pooled and constituted the pure subsynaptic membrane preparation (M).

Table 1 summarizes the results of this purification: 1 kg of fresh electric organ yields membranes containing 140 mg of protein with a specific radioactivity higher than 4000 nmol of $^3$H-labelled $\alpha$-toxin-binding sites/g of protein. Such a high specific radioactivity means that the cholinergic-receptor protein represents about 50% of the proteins in these membranes, whereas the concentration of acetylcholinesterase catalytic sites is more than 100 times smaller. The purified membrane fragments form a band at 38% sucrose after equilibration on a sucrose density gradient; this corresponds to a significantly higher density than that of a regular cytoplasmic membrane, and may be explained by the high protein/lipid ratio (1.2–1.5) in these membranes.

Electron-microscopic observations of negatively stained purified membrane fragments reveal that nearly all of them (more than 100 were photographed) show a dense packing (approx. 10 000–15 000 per $\mu$m$^2$) of rosette-like structures, 8 nm in diameter, similar to those first observed by Cartaud et al. (1973) and Nickel & Potter (1973). These particles, which are assumed to be cholinergic-receptor molecules, may locally form regular arrays with an hexagonal symmetry.

Analysis of the polypeptide composition of the purified membranes by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate reveals only four main bands corresponding to mol. wts. of 40 000, 43 000, 50 000 and 66 000 (Fig. 1). If the membranes are labelled with 4-$[^3$H](N-maleimido)phenyltrimethylammonium only the 40 000-mol. wt. component is radioactive, as expected from previous work (Karlin et al., 1975). Thus only this polypeptide chain bears the acetylcholine-receptor site of the cholinergic-receptor protein.

These receptor-rich membranes constitute a very suitable preparation for the study of the functional properties of the cholinergic-receptor protein in its membrane environment (Changeux et al., 1975). They are also a very convenient starting material for the purification of the receptor protein.

The first step of this purification was the dissolution of the membrane proteins with 4% Triton X-100 in the presence of 10 mM-Tris/HCl, pH 7.4, 100 mM-NaCl, 0.1 mM-phenylmethylsulphonyl fluoride and 0.02% NaN$_3$. Subsequent centrifugation on a 5–20% sucrose gradient in the same medium, except that 0.1% Triton X-100 instead of 4% was present, gave several peaks of $^3$H-labelled $\alpha$-toxin-binding activity, in agreement with previous findings (review by Changeux, 1975); the two major ones had
Fig. 1. Densitometric scans of various preparations of (a) purified receptor-rich membrane fragments and (b) Triton X-100-extracted purified receptor protein after electrophoresis in sodium dodecyl sulphate/polyacrylamide gel

The migration front is indicated by a vertical arrow. A, B, C and D indicate respectively the protein species with apparent mol. wts. 40000, 50000, 66000 and 43000. Only band A is labelled with $4^{[3H]}(N$-maleimido)phenyltrimethylammonium. The numbers in parentheses give the relative ratio of the area of each band to that of band A.

sedimentation coefficients of 9S and 12S. However, if the extraction and the centrifugation steps were carried out in the presence of 10 mM-2-mercaptoethanol, only one symmetrical receptor peak was obtained, with a sedimentation coefficient of 9S. The fractions of this simple peak were pooled to give the purified cholinergic-receptor protein. Starting from 1 kg of electric organ, one can obtain up to 50 mg of protein with a specific activity of 7-9 pmol of $\alpha$-toxin-binding sites/g of protein (Table 1). A similar protein band was obtained when the receptor was submitted to polyacrylamide-gel electrophoresis in non-denaturing conditions, in the presence of 0.1% Triton X-100. By isoelectric focusing, the purified receptor protein focused as a single band at pH 5.3, even in the presence of 1 M- or 2 M-urea.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate still revealed several protein bands in the purified receptor fraction (Fig. 1). This is in agreement with observations of Karlin et al. (1975) and Raftrey et al. (1975), who have found four protein bands with purified receptor from Torpedo californica. Here, however, only three bands were present: a major one of apparent mol. wt. 40000, which is labelled with $4^{[3H]}(N$-maleimido)phenyltrimethylammonium, and two minor ones of apparent mol. wts. 50000 and 66000. The 43000-mol. wt. band present in the receptor-rich membranes has therefore been eliminated by the sucrose-gradient centrifugation step in the presence of 2-mercaptoethanol.

There is concrete evidence that the 40000-mol. wt. band labelled by radioactive 4-(N-maleimido)phenyltrimethylammonium belongs to the cholinergic-receptor protein,
but are the other two bands also integral parts of it? They cannot be separated from the major band in non-denaturing conditions by classical separation methods such as DEAE-cellulose chromatography or isoelectric focusing in the presence of Triton X-100 and 0.5M or 2M-urea. In these experiments, all the fractions containing ^3H-labelled α-toxin-binding activity were tested by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate; they always contained the three bands with little quantitative variation in their relative ratios. However, from one preparation of purified receptor protein to the other, the relative ratios of the different bands vary, as determined by densitometric recordings after staining with Coomassie Blue. Also, membrane fractions of increasing purity contained increasing amounts of the 40000-mol.wt. polypeptide as compared with the amounts of the other peptides present (Fig. 1). This variability indicates that the peptides of apparent mol.wts. 50000 and 66000 may be contaminants. Unfortunately, any attempt to identify the nature of these possible contaminants has failed up to now. On the other hand, it is possible that the 40000-mol.wt. band is composed of two distinct, but unresolved polypeptides of identical apparent molecular weights.

These results may be compared with early studies on purified receptor protein from Electrophorus electricus electric organ, where two or three bands were present [reviews by Changeux (1975) and Karlin (1974)]; only one of them, of apparent mol.wt. 40000, was labelled with 4-[^3H](N-maleimido)phenyltrimethylammonium. In conclusion, the cholinergic-receptor protein most likely contains only one major component of mol.wt. about 40000. The present preparation makes it possible to obtain large amounts of a highly purified protein and should therefore enable us to clarify the detailed structure of the cholinergic-receptor protein.

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The Metabolism of Phosphatidylinositol in Excitable Membranes

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Phosphatidylinositol accounts for about 5% of the total phospholipids of mammalian cell membranes. In the plasma membrane, if the erythrocyte is any guide, it is located entirely in the inner monolayer, where it is accessible to cytoplasmic enzymes (Buckley & Hawthorne, 1972; Garrett & Redman, 1975). One such enzyme, which is