PRESENCE OF A LATTICE STRUCTURE IN MEMBRANE FRAGMENTS RICH IN NICOTINIC RECEPTOR PROTEIN FROM THE ELECTRIC ORGAN OF *TORPEDO MARMORATA*

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Received 31 March 1973

1. Introduction

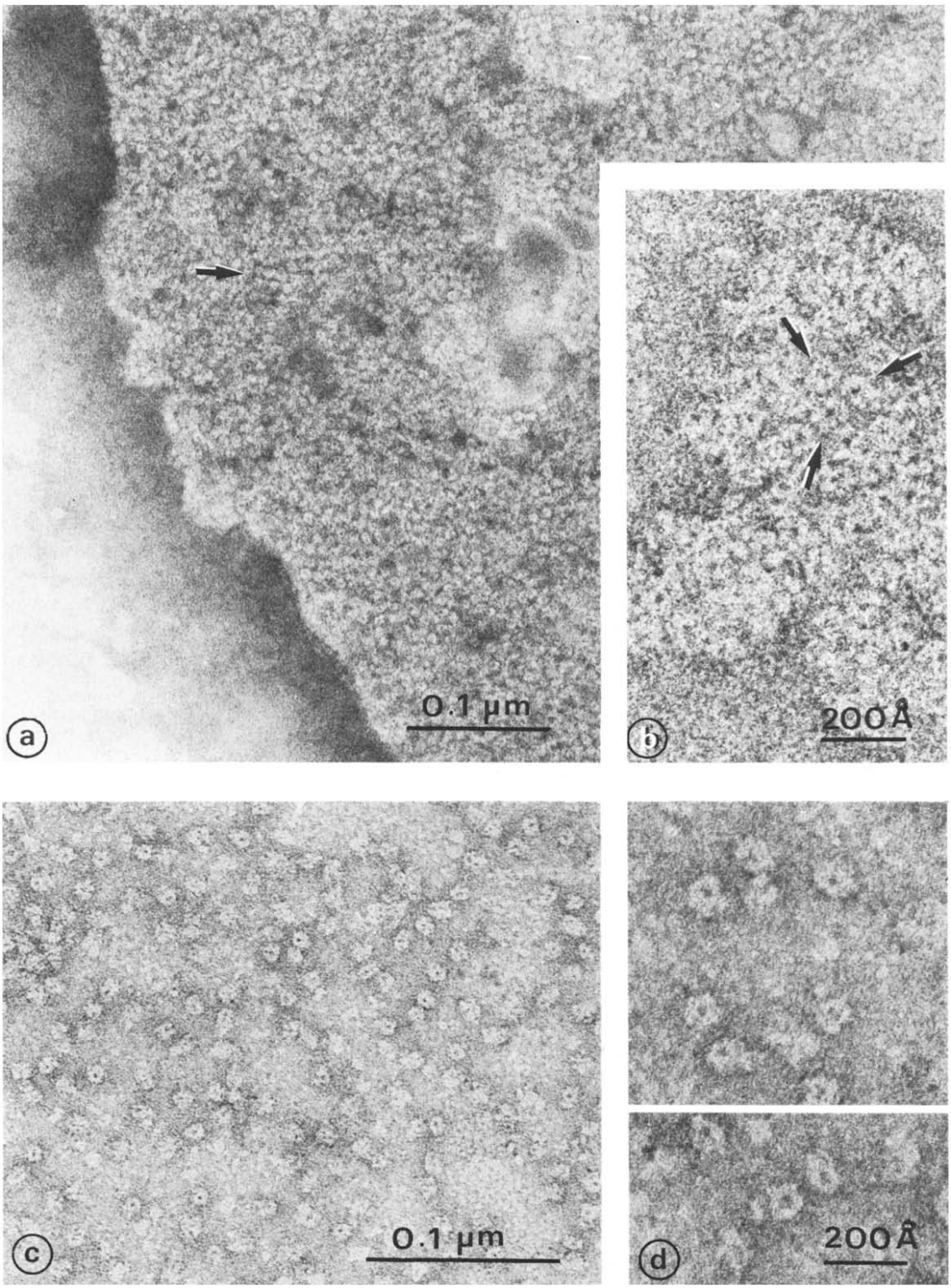
Recently Cohen et al. [1] have developed a fractionation method of *Torpedo* electric tissue which yields membrane fragments particularly rich in cholinergic (nicotinic) receptor protein. Observation of this purified fraction by *both* freeze-etching and negative staining reveals a lattice organization of particles. Similar particles are seen in negatively stained preparations of highly purified receptor protein from *Electrophorus* and most probably correspond to the cholinergic receptor protein.

2. Materials and methods

Membrane fragments from *Torpedo* electric tissue were prepared by the technique of Cohen et al. [1]. The specific activity of the preparations ranged between 1,000 and 2,000 nmoles *Naja nigricollis* [^{3}H] α-toxin binding sites per g protein. Two methods were used to prepare specimens for electron microscopy:

1) For negative staining, a drop of a suspension of membrane fragments (0.5 mg protein per ml) was deposited on a carbon-coated grid. The grid was blotted with filter paper and washed with a few drops of 0.1% ammonium acetate, before the sample was negatively stained with an aqueous solution of 0.5% uranyl acetate or 1% sodium phosphotungstate. No difference was seen when the membrane fragments were fixed with 1% glutaraldehyde before staining.
2) For freeze-etching, the membrane suspension (2–3 mg proteins) was diluted two-fold and centrifuged (Spinco rotor 30; 27,000 rpm; 1 hr); the pellet was then resuspended in its volume of distilled water. Small drops of the concentrated suspension were mounted on cardboard disks and rapidly frozen in liquid Freon R 22. All samples were freeze-etched in a Balzers apparatus. The etching-time was 1.5 min at $-100°$C. Replicas were obtained by evaporation of a carbon-platinum alloy. During the etching-time, specimen contamination was prevented by leaving the cooled knife above the fractured surface.

The receptor protein from *Electrophorus* was purified by affinity chromatography in the presence of 1% Triton X-100 according to Olsen et al. [2]. The specific activity of the preparation was (5.9 ± 0.5) μmoles of [^{3}H] α-toxin binding sites (measured by our Millipore filtration assay) per g protein (estimated by the method of Lowry). For negative staining, improvement of spreading properties of the solution was achieved by adding small quantities of Bacitracin (final conc. 20 μg/ml) [3] to the solution (in 1% Triton X-100) of receptor protein (0.4 mg protein/ml). Negative staining was performed in the usual manner with 0.5% aqueous uranyl acetate.


0.1 µm
a
200 Å
b
0.1 µm
c
200 Å
d

Electron microscopy was performed with a Philips EM 300 or a Siemens Elmiskop 101 using 80 kV accelerating voltage, a double condenser illumination system, and a 50 μm objective aperture. The anticontamination cooling device was used routinely.

3. Results

3.1. Membrane fragments from Torpedo

3.1.1. Negative staining

A large fraction (up to 50%) of the membrane fragments shows by negative staining particles having an average diameter of 80–90 Å which are locally organized in a regular structure with a center to center distance of 90–100 Å (fig. 1a).

At high magnification (fig. 1b) the stained particles show a central hole or pit of 15 Å in diameter filled by the electron dense stain. Occasionally a pattern of 3 to 6 subunits is found. The preparations often show very small membrane fragments (fig. 1b) which may result from disruption of larger fragments during the staining procedure.

3.1.2. Freeze-etching

The preparation was carried out without glycerol; therefore both fractured and etched surfaces should be visible. It is now established that the fracture plane splits the membrane within its hydrophobic core [4] and that deep-etching reveals the real surface of the membrane [5, 6].

Fig. 2a shows a membrane surface which presumably results from deep-etching rather than from fracture. The surface of the fragment is characterized by an array of repeating particles forming hexagonal lattices with a center-to-center distance of approx. 90–100 Å. The structure of the particles visualized by freeze-etching appears very similar to that observed by negative staining. Each particle shows a central pit, and a subunit organization is seen in the best replicated particles.

The membrane fragment shown in fig.2b presents two different surfaces: one exposed by deep-etching, the other by fracture. The etched surface (E) is again characterized by the closely packed particles while the fracture plane (F) contains randomly distributed particles and smooth areas. The closely packed particles are therefore exposed to the surface of the membrane fragments.

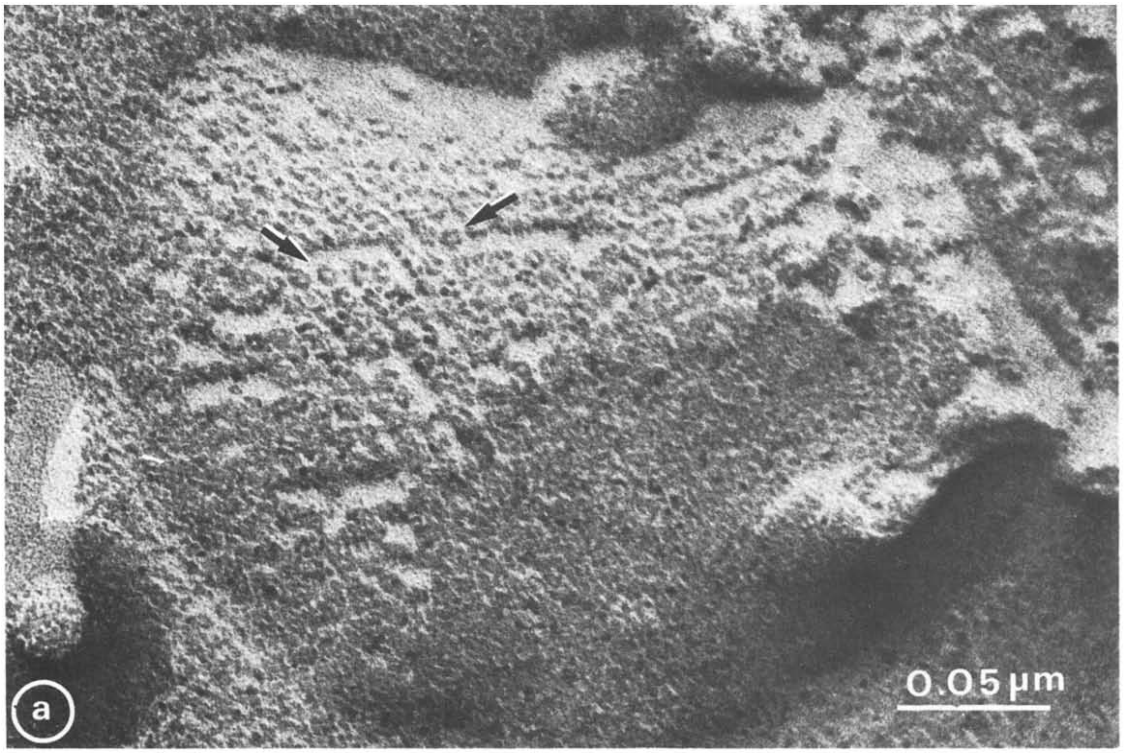
3.2. Purified receptor protein from Electrophorus

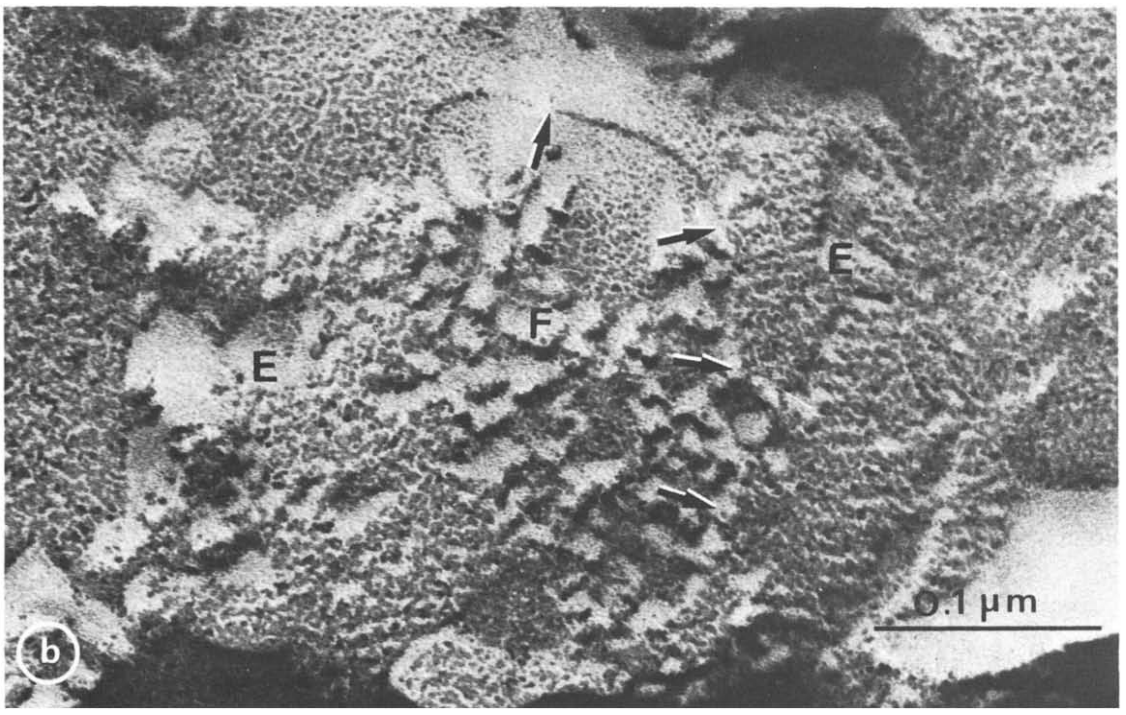
In order to identify the particles seen by both negative staining and freeze etching in *Torpedo* membrane fragments, a preparation of highly purified receptor protein from *Electrophorus* was negatively stained by uranyl acetate. Fig.1c shows an homogeneous population of particles with an average diameter of 80–90 Å. At high resolution these particles show the characteristic central pit and a subunit pattern. The number of subunits per particle, although difficult to ascertain, is 5 or 6. Each subunit has a diameter of approx. 30–40 Å.

4. Discussion

One of the most characteristic features of the receptor rich membrane fragments from *Torpedo* is that they show by freeze-etching a lattice organization of particles. Locally organized patterns of particles are also observed by negative staining. In both cases the repeating units have the same size, central pit and subunit pattern as the particles present in a soluble preparation of cholinergic receptor protein purified from *Electrophorus* electric organ and, therefore, are interpreted as cholinergic receptor oligomers. This interpretation is supported by the fact that 1) the size of the repeating unit of 80–90 Å is consistent with the physical data presently available on the receptor oligomer (Stokes radius 70 Å, apparent M.W. 315–360,000) [7]. 2) The repeating units

Fig.1. *Torpedo:* a) Negatively stained (1% sodium phosphotungstate) membrane fraction rich in cholinergic receptor. The arrow points to regular arrays of particles. × 240,000.
b) Higher magnification of the same preparation negatively stained with 0.5% uranyl acetate. Individual particles (arrows) show an electron dense center. × 650,000.
Electrophorus:
c) Preparation of highly purified receptor protein negatively stained by uranyl acetate. The homogeneity of the preparation is evident. × 300,000.
d) High magnification of c). The electron dense core and subunit pattern are seen in most individual particles. × 650,000.


a
0.05 µm


E
F
E
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are exposed to the membrane surface as expected for a membrane bound pharmacological receptor.

From the freeze etched preparation, one estimates a density of 12–15,000 repeating units per μm^2 of membrane surface. This number is in the same range, although smaller, than that of *Naja nigricollis* [^{3}H] α-toxin binding sites counted by autoradiography in *Electrophorus* subsynaptic membrane [8].

An eventual relation of the observed lattice structure to membrane cooperative effects [9] is not clear. It is worth mentioning, however, that acetylcholine binding to the same membrane follows an S-shaped curve [10].

Acknowledgements

We thank Monique Huchet, Michel Recouvreur and Jean-Marie Gouezou for their technical assistance, Louise Mazzoni for photography and Michèle Cornu for typewriting.

This work was supported by funds from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Collège de France, the Commissariat à l'Energie Atomique and the National Institutes of Health. J.B. Cohen was a recipient of N.I.H. Fellowship 1F02 EY 52,916-01 of the National Eye Institute.

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Fig. 2. a) Freeze-etching of a preparation of membrane fragments rich in cholinergic receptor showing a surface revealed by deep-etching Note the hexagonal array of particles. It is possible to observe the substructure of some well resolved particles (arrows). × 400,000. b) Micrograph showing both fractured face (F) with some randomly distributed globular particles and etched surface (E) with closely packed particles with a central hole. The arrows indicate the ridge between the fracture face and the etched surface. × 300,000.