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Rapid preparation of the nicotinic acetylcholine receptor for crystallization in detergent solution

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A novel rapid purification method for the nicotinic acetylcholine receptor from *Torpedo* electric tissue was developed. It allows preparation of 10 mg quantities of pure and stabile receptor protein within 2 days. This protein is used for crystallization attempts. Conditions are described which reproducibly yield crystals.

Nicotinic acetylcholine receptor; Large-scale purification; Crystallization

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) has become the model for many membrane molecules of its kind. After its first isolation from electric tissue of the *Electrophorus electricus* ray by affinity chromatography in 1972 [1] it became available in large quantities, and many principles of signal transduction through excitable membranes were first investigated with this molecule. For excellent reviews covering its biophysics [2,3], regulatory aspects [4,5], immunology [6], molecular and cell biology [7,8] and general biochemistry [8–11], the reader is referred to the respective literature. Despite this wealth of information, many basic questions concerning its mechanism of action and its structure-function relationships are still unanswered. Many answers require knowledge of the secondary and tertiary structure of the receptor protein which would be supplied most comprehensively by X-ray analysis of AChR crystals. Crystallization requires large amounts of pure, structurally homogeneous and stabile protein, which is not easily obtainable with a membrane protein. In the case of AChR purification, especially classical affinity chromatography [1,12] may turn out not to be the ideal method. Here we describe a novel, large-scale purification of AChR from *Torpedo californica* electric tissue which seems favourable for crystallization attempts.

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2. MATERIALS

T. californica electric tissue was purchased frozen in liquid N_2 from Pacific Bio-Marine Laboratories, Inc. (Venice, CA). It was stored at −75°C. β-Octyl-β-D-glucopyranoside was from Bachem, Bubendorf, Switzerland, and lithium 3,5-diiodosalicylate was a product from Sigma (St. Louis, MO). 2-Methyl-2,4-pentanediol (MPD) was purchased from Aldrich Chemie, Steinheim, FRG; 1,2,3-heptanetriol from Sigma; and morpholinoethanesulfonic acid (Mops) from Serva, Heidelberg. PMSF, NaCl, EDTA, electrophoresis materials, sucrose and all other chemicals were of the highest purity commercially available. SDS-polyacrylamide gel electrophoresis was performed according to [13]. Protein determination was by the Lowry method [14], with bovine serum albumin as standard.

3. RESULTS

3.1. *Purification*

Prerequisite for successful crystallization attempts is a rich source of receptor protein and a convenient large-scale purification. The following procedure seems to yield reproducibly stable AChR in large quantities. The optimal source of

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AChR, even in these days of recombinant DNA techniques, is the electric tissue of *Torpedo* sp., introduced decades ago by David Nachmansohn [15]. Starting from this tissue we (i) prepare receptor-rich membranes by sucrose gradient centrifugation; (ii) extract the peripheral membrane proteins with the chaotropic salt lithium diiodosalicylate; (iii) extract the integral membrane proteins including the AChR with the mild detergent β-octylglucoside (β-OG); and (iv) remove most of the contaminant proteins by chromatography on a Blue Sepharose column from Pharmacia. This procedure yields about 10 mg AChR from 100 g electric tissue. The individual steps are now described in more detail.

Receptor-rich membranes are prepared basically as described previously [16]. 100 g frozen electric tissue from *Torpedo californica* are homogenized for 3 min in a Waring Blendor at maximum speed with 200 ml of 20 mM sodium phosphate buffer, pH 7.4, containing 2 mM EDTA, 0.1 mM PMSF and 400 mM NaCl. The homogenate is centrifuged for 90 min at 27000 × *g* (4°C). The pellet is resuspended in 100 ml of the same medium, but without sodium chloride. This time the Waring Blendor is used for homogenization at minimum speed for 3 min. The homogenate is centrifuged for 90 min at 39000 × *g* (4°C). This step is repeated once. The soft upper part of the pellet is removed and homogenized by pushing it through a syringe. This homogenate is centrifuged for 10 min at 2900 × *g* (4°C). The pellet is discarded and the supernatant which contains the receptor-rich membranes is layered in portions of approx. 5 ml each on three centrifuge tubes, each containing a sucrose gradient (25–50%, w/v, in H_2O, with 0.02% NaN_3). Density-gradient centrifugation is performed overnight (about 16 h) at 22000 × *g* (4°C).

On the second day of this preparation the gradient is fractionated in 2 ml fractions. Fractions 5–15 (from the bottom) contain most of the receptor-rich membranes and are pooled for further processing. These membranes already contain more than 60% AChR (fig.1, track 1).

To extract the peripheral membrane proteins, mainly the 43 kDa protein (ν_1, α'), the membranes are collected after 20-fold dilution with water containing 1 mM EGTA by 30 min centrifugation at 39000 × *g* (4°C). The pellet is resuspended in 15 ml of 20 mM Tris-HCl buffer, pH 8.1, containing 1 mM EGTA and 10 mM lithium diiodosalicylate. The suspension is gently shaken for 1 h at room temperature and subsequently centrifuged for 15 min at 100000 × *g* (4°C). The supernatant contains most of the 43 kDa proteins (fig.1, track 2); the pellet shows predominantly the characteristic $\alpha,\beta,\gamma,\delta$-band pattern of the AChR (fig.1, track 3). The pellet is extracted with 5 ml of 50 mM Tris-HCl, pH 7.4, containing 2% β-octylglucoside and 2 mM EGTA. After 1 h extraction at room temperature, the mixture is centrifuged for 15 min at 100000 × *g* (4°C). An SDS-polyacrylamide gel electrophoresis of the supernatant is shown in fig.1, track 4.

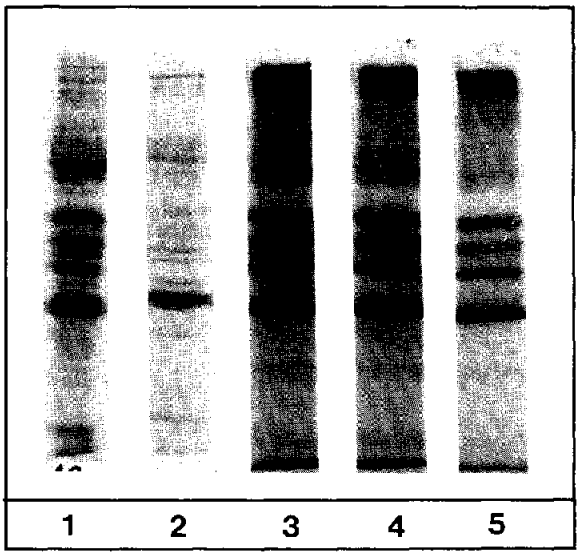

Fig.1. SDS-polyacrylamide gel electrophoresis documenting the purity of the AChR at different purification steps. Lanes: 1, receptor-rich membranes after density gradient centrifugation; 2, peripheral membrane proteins extracted with lithium diiodosalicylate. The predominant band represents the 43 kDa protein; 3, the remaining integral membrane proteins; 4, proteins extracted with 2% β-octylglucoside; 5, protein after Cibachrome Blue Sepharose chromatography. 10% polyacrylamide gels after Coomassie blue staining.

The detergent solubilized receptor is further purified by chromatography on a Cibacrone Blue Sepharose column (3.8 × 1 cm). The elution buffer is 50 mM Tris-HCl, pH 7.4, containing 2% β-octylglucoside, 1 mM EGTA, and 0.02% NaN_3. More than 90% of the AChR elutes from this column with a purity shown in fig.1, track 5.

The overall yield of the purification described

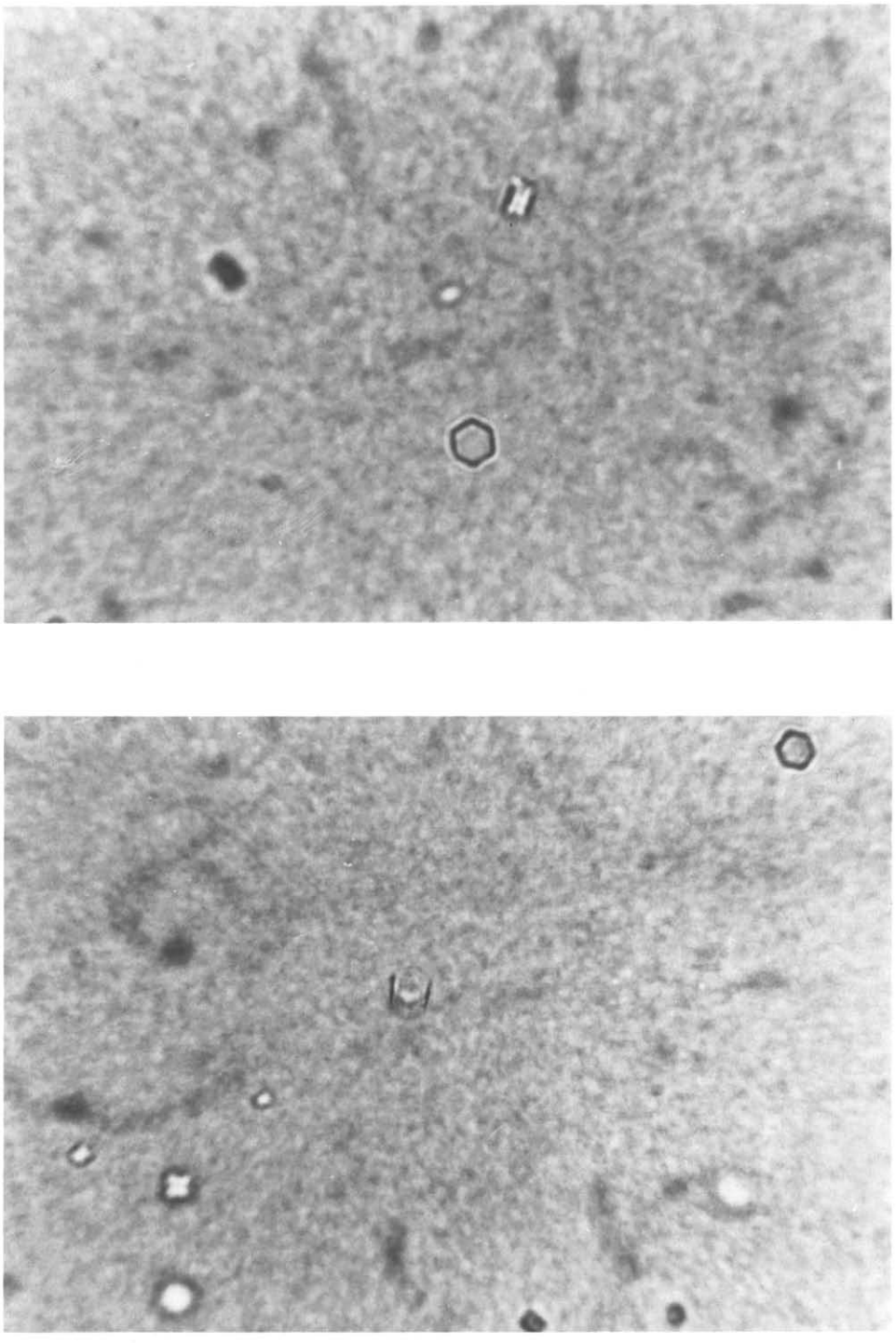

Fig.2. Crystals obtained as described in the text. In the top frame a side view and a view from the top of apparently hexagonal crystals are shown. In the center of the lower frame a hexagonal crystal, slightly tilted and viewed from the top, gives an impression of the shape of these crystals. Diameter of the hexagons: about 15 μm.

here is of the order of 50%. Since the starting material, the receptor-rich membranes obtained by sucrose density gradient centrifugation is already more than 60% pure, the purification factor obtained by the further steps is not dramatic. The specific activity of the pure AChR was about 7 nmol ^{125}I-α-bungarotoxin-binding sites/mg protein.

The protein was concentrated (if necessary) by centrifugation through Centricon-30 microconcentrators, from the Amicon Division of W.R. Grace Co., Danvers, MA.

3.2. *Crystallization*

Crystallization was attempted by the 'hanging drop method' [17]. 5 μl protein solution were mixed on a coverslide with 5 μl of buffer containing the precipitating agent (see below). The coverslide was placed upside down on a tissue culture tray well. The trays were stored at 8°C. After 3–10 days, crystals (fig.2) were observed under the following conditions: the optimal protein concentration was 8–10 mg/ml in 50 mM Mops (or Tris-HCl) buffer, containing 1% β-octylglucoside. The pH range was 6.6–7.5. Precipitation and crystal formation were accomplished by 34–45% MPD or 24–45% MPD with the addition of 3–8% heptanetriol. All buffers contained 100 mM NaCl, 10 mM EGTA, and 0.02% NaN_3. Crystals of up to 10–20 μm length with a hexagonal cross-section formed, embedded in amorphous precipitate. Controls (without protein) were negative.

4. DISCUSSION

The AChR preparation described here is different from others described in the literature in several respects: it is rapid (two days), avoids tortuous steps like elution from affinity columns with high concentrations of desensitizing ligands, yields large amounts of relatively pure protein which is not separated from its membrane lipids. This latter point may be of special importance since lipid-protein interactions are of utmost importance for stability and activity of AChR ([8] pp.147–157).

The crystals obtained are still too small for X-ray or even synchrotron radiation investigations. But the ease and reproducibility with which they are obtained make further experiments rather promising.

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