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FRACTIONATION OF PROTEIN COMPONENTS OF PLASMA MEMBRANES FROM THE ELECTRIC ORGAN OF *TORPEDO MARMORATA*

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A procedure has been developed for the separation of intrinsic proteins of plasma membranes from the electric organ of *Torpedo marmorata*. ($Na^+ + K^+$)-ATPase, nicotinic acetylcholine receptor and acetylcholinesterase remained active after solubilization with the nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$). These components could be separated by ion exchange chromatography on DEAE-Sephadex A-25. Fractions enriched in ouabain-sensitive K^+-phosphatase or ($Na^+ + K^+$)-ATPase activity showed two bands in sodium dodecyl sulphate polyacrylamide gel electrophoresis corresponding to the α- and β-subunits. The ($Na^+ + K^+$)-ATPase was shown to have immunological determinants in common with a 93 kDa polypeptide which copurified with the nicotinic acetylcholine receptor, also after solubilization in Triton X-100 and chromatography on *Naja naja siamensis* α-toxin-Sepharose columns. The data suggest that the α-subunit of ($Na^+ + K^+$)-ATPase associates with the acetylcholine receptor in the membranes of the electric organ.

Introduction

Neuronal information transfer in vertebrate skeletal muscle and in the electric organ of certain fishes; e.g. *Torpedo spp* and *Electrophorus*, is mediated by acetylcholine. The transmitter is crucially involved in the control of ion transport through synaptic membranes. Three proteins which are essential for the cholinergic control of ion flows are present: the nicotinic acetylcholine receptor (nAChR) complex, which translates the binding of acetylcholine into permeability changes, the enzyme acetylcholinesterase (EC 3.1.1.7) which limits the duration of the acetylcholine action by hydrolytic removal of the transmitter from the membrane environment, and the enzyme ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), which controls ion levels at the synapse. The synaptic acetylcholinesterase is mainly a peripheral membrane protein [1], while the nicotinic acetylcholine receptor and the ($Na^+ + K^+$)-ATPase are intrinsic membrane proteins requiring detergents for solubilization [2,3].

The nicotinic acetylcholine receptors have been studied both in highly receptor-enriched membrane preparations [4–6] and in the purified state after detergent solubilization and affinity chromatography [7–9]. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of purified nicotinic acetylcholine receptor revealed four polypeptides with apparent molecular weights of about 41 000, 47 000, 55 000 and 64 000 [10–14]. In addition to these a high M_r polypeptide (about 93 000) often becomes visible. Various suggestions have been made about the nature of this latter polypeptide, such as it being an aggregation product of receptor polypeptides [11] or a subunit of ($Na^+ + K^+$)-ATPase [13,14].

The purpose of this work has been to examine if the 93 kDa polypeptide band, obtained together with the nicotinic acetylcholine receptor poly-

peptides purified from *Torpedo marmorata* electric organ, might contain parts of the electroplax ($Na^+ + K^+$)-ATPase. We have fractionated the protein components of the plasma membranes of *Torpedo* electric tissue by gel exclusion and ion exchange chromatography techniques after solubilization with the nonionic detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$) [15]. The nature of the 93 kDa polypeptide band was examined by comparing its apparent molecular weight (SDS-polyacrylamide gel electrophoresis) with the polypeptide bands obtained from the ($Na^+ + K^+$)-ATPase fraction. Further, immunological cross-reactivity between the purified ATPase and antibodies to the 93 kDa polypeptide was studied.

Materials and Methods

Electric organ of *Torpedo marmorata* was kindly supplied by Station Biologique d'Arcachon, France, and was kept deep frozen at −90°C until use. Membrane-bound proteins were extracted with the nonionic detergent $C_{12}E_8$ (The Kouyoh Trading Co, Tokyo, Japan) [15]. Fractionation was performed on Sepharose 6-B and DEAE-Sephadex A-25 gels (Pharmacia Fine Chemicals, Uppsala, Sweden).

Crude membrane fractions (CMF) were prepared according to a modification of Ref. 16. 100 g of electric tissue were thawed and homogenized in 2 vol. of 50 mM Tris buffer (pH 7.5), 1 mM EDTA and Na_2ATP, 250 mM sucrose, 0.1 mM phenylmethylsulphonyl fluoride and 0.02% NaN_3 in a Braun homogenizer at 4°C followed by a teflon-glass homogenizer. The homogenate was centrifuged at 4000 × *g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 48000 × *g* for 30 min. The supernatant was discarded and the pellet was resuspended in a final volume of 20 ml Tris buffer (excluding Na_2ATP) and stored at −90°C until use. The yield of crude membrane fractions was 230 mg protein/100 mg electroplax, containing 45% of the K^+-phosphatase activity of the original homogenate.

Extraction of the crude membrane fraction was performed essentially as described in Refs. 15 and 17. The crude membrane fraction was pelleted at 100000 × *g* for 20 min and rehomogenized in 20 mM Tris buffer (pH 7.5), 2 mM EDTA and dithiothreitol, 300 mM KCl, 40% glycerol, and an equal volume of $C_{12}E_8$ in water was added. The highest yield of K^+-phosphatase activity was found after solubilization with a final concentration of 10 mg $C_{12}E_8$/ml : 5 mg protein/ml. At higher concentrations of detergent the enzyme was inactivated [17]. After incubation for 30 min at room temperature the sample was centrifuged at 100000 × *g* for 60 min. The crude membrane fraction extract (CMF-E) contained 60% of the protein, 50–70% of the K^+-phosphatase activity and 60% of the α-bungarotoxin binding capacity present in the crude membrane fraction.

Fractionation on Sepharose 6-B. Aliquots of 2 ml containing 5 mg solubilized protein (CMF-E) without dithiothreitol were applied to a Sepharose 6-B column [17,18] (1.5 × 90 cm) and eluted with a flow rate of 5–7 ml/h at 15°C with 10 mM Tris buffer (pH 7.5), 1 mM EDTA, 150 mg $C_{12}E_8$/litre.

Fractionation on DEAE-Sephadex A-25 column. The gel was equilibrated in 10 mM Tris buffer (pH 7.5), 1 mM EDTA, 20% glycerol, 0.5 g $C_{12}E_8$/litre, 0.02% NaN_3. The crude membrane fraction extract sample (20 mg) was loaded on the column (2.5 × 10 cm), operated at a rate of 60 ml/h at 8 or 15°C. Elution was performed with equilibration buffer containing increasing concentrations of KCl [17] (Fig. 2a) and fractions of 2.5 ml were collected.

Peaks obtained from gel exclusion and DEAE-Sephadex chromatography were tested for K^+-phosphatase activity with *p*-nitrophenylphosphate as substrate at 20°C. The ($Na^+ + K^+$)-ATPase activity was measured essentially as described in [17,19] and modified for samples containing $C_{12}E_8$. 250 μl of assay medium (260 mM NaCl, 40 mM KCl, 6 mM $MgCl_2$, 60 mM histidine, 3.6 mg bovine serum albumin/ml, pH 7.4) were mixed with 50–200 μl of fractions (H_2O was added to a total volume of 200 μl) in the presence or absence of 50 μl of 10 mM ouabain. The reaction was started with 50 μl of 30 mM ATP and terminated after 5–15 min at 20°C with 1 ml ice-cold reagent (0.5 M HCl containing 3 g ascorbic acid, 0.5 g ammonium heptamolybdate and 2.5 ml Triton X-114 per 100 ml). For colour development 1.5 ml AC-reagent (containing per 100 ml 2 g sodium meta-arsenite, 2 g sodium citrate, 2 ml acetic acid, 6 ml Triton X-100 and 0.3 g SDS) was added, the

tubes warmed to 37°C for 10 min and the absorbance read at 850 nm.

Amounts of nicotinic acetylcholine receptor were measured by the ability of the receptor to bind α-[^{125}I]bungarotoxin using the DEAE-filter method described by Schmidt and Raftery [20]. The acetylcholinesterase activity was measured according to Ellman et al. [21] with acetylthiocholine iodide as substrate. Protein was determined by the method of Lowry after precipitation with 5% trichloroacetic acid and with bovine serum albumin as standard. The homogeneity of the isolated fractions was examined by 9% SDS-polyacrylamide gel electrophoresis [22]. Protein (10–25 μg) from the eluted fractions was dissolved in 2% SDS and 1% β-mercaptoethanol for 3 min in a boiling waterbath. The samples were submitted to 9% SDS-polyacrylamide gels containing 6 M urea in tubes and run at 2 mA/gel for 16 h at 20°C. The gels were stained in 0.25% Coomassie brilliant blue. Pyruvate kinase (M_r 36000) and lactate dehydrogenase (M_r 57000) were used as standards after crosslinking with glutaraldehyde so as to give multiple aggregates.

Two-dimensional immunoelectrophoresis was performed between the $(Na^+ + K^+)$-ATPase-enriched fraction from *Torpedo* and rat antibodies directed against the 93 kDa polypeptide, copurifying with nAChR. Immunoelectrophoresis was run in 1% agarose containing 3% poly(ethylene glycol) (M_r 6000, PEG 6000). The presence of PEG 6000 has a positive effect on antigen-antibody reactions by withdrawal of water from the precipitates. In the first dimension SDS-treated and reduced $(Na^+ + K^+)$-ATPase (10 μg) was electrophoresed at 10 V/cm for 20 min. By this procedure only separation by charge is obtained and thus no separation of the α- and β-subunits of the ATPase is necessarily expected, while free SDS is expected to move in advance of the protein material. Electrophoresis was then run perpendicular at 2 V/cm overnight allowing protein to move into the antibody containing gel (15% serum, v/v). The plate was stained with Coomassie brilliant blue. In a comparable study, the SDS-treated $(Na^+ + K^+)$-ATPase antigen was run in the second dimension first through a barrier of Triton X-100 in agarose and then into the antibody-containing gel. Triton X-100 is used to remove excess SDS from protein solutions and non-specific precipitation is eliminated [23].

Results and Discussion

After solubilization with $C_{12}E_8$ in buffer containing dithiothreitol, nicotinic acetylcholine receptor and $(Na^+ + K^+)$-ATPase were eluted from the Sepharose 6-B column with approximately the same volume. Fractions enriched in K^+-phosphatase activity were obtained, but SDS-polyacrylamide gel electrophoresis revealed that they contained other polypeptide bands in addition to the α- and β-subunits of the $(Na^+ + K^+)$-ATPase. In extracts of the crude membrane fraction prepared in the presence of 1 mM dithiothreitol, Hamilton et al. [24] showed that nicotinic acetylcholine receptor mainly existed as a monomer with an M_r of about 270000. This value is close to the apparent molecular weight for $(Na^+ + K^+)$-ATPase from *Electrophorus* (M_r 275000) [16,25,26]. In the absence of dithiothreitol, however, nicotinic acetylcholine receptor eluted mainly as dimers with molecular weight around 500000 [24]. In Fig. 1a it is seen that nicotinic acetylcholine receptor was eluted in fraction 22 with a specific α-[^{125}I]bungarotoxin binding capacity of about 0.3 nmol/mg protein. The aggregation state of $(Na^+ + K^+)$-ATPase was unaffected by dithiothreitol and the enzyme appeared in fractions 25–28 with a specific K^+-phosphatase activity of 0.3 μmol/min per mg (20°C). The nicotinic acetylcholine receptor contamination in these fractions was around 0.1 nmol α-[^{125}I]bungarotoxin binding sites/mg. The $(Na^+ + K^+)$-ATPase-enriched fraction eluted at a volume corresponding to a Stokes radius of 68–73 Å which was identical to that in the same column for pure $(Na^+ + K^+)$-ATPase from the pig kidney [17]. The experiment shows that solubilization of the proteins in the absence of dithiothreitol resulted in a better separation of $(Na^+ + K^+)$-ATPase from nicotinic acetylcholine receptor on the Sepharose 6-B column, but the procedure was not adequate for purification of the proteins.

Since resolution by gel exclusion chromatography was insufficient for immunological studies attempts were made to separate the proteins by differences in charge on DEAE-Sephadex col-

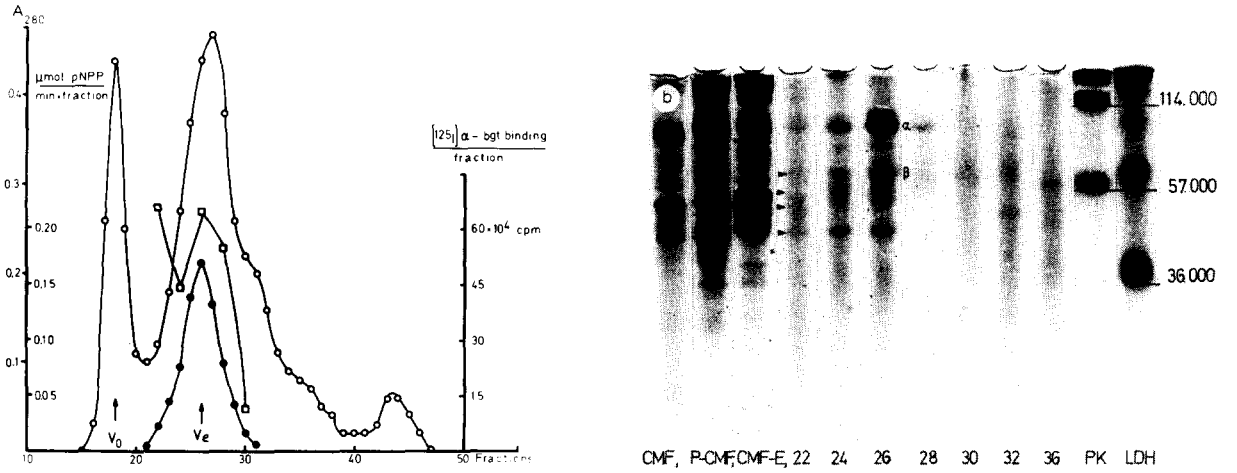

Fig. 1. (a) Elution profile showing separation of crude membrane fraction extract (CMF-E) without dithiothreitol on a Sepharose 6-B column. Fractions of 3 ml were collected. Peak positions (V_e = elution volume, 78 ml) were estimated using Blue dextran as a marker for the void volume (V_o, 50 ml). The column was calibrated with marker proteins with known Stokes radii [17]. ○———○, protein, measured by A_{280}; ●———●, K^+-phosphatase activity; □———□, α-[^{125}I]bungarotoxin binding capacity. (b) 9% SDS-polyacrylamide gel electrophoresis on the above eluted fractions. From left to right: (1) CMF, (2) P-CMF, (3) CMF-E, (4) fraction 22, the four nicotinic acetylcholine receptor polypeptides are marked with arrows, (5) 24, (6) 26, (7) 28, the α- and β-subunits of ($Na^+ + K^+$)-ATPase are pointed out, (8) 30, (9) 32, (10) 36, (11) standard PK (pyruvate kinase, M_r 57000), (12) LDH (lactate dehydrogenase, M_r 36000). Standards were crosslinked with glutaraldehyde to give the monomer, dimer and trimer.

umns. Results are presented in Fig. 2a and in Table I. Most of the solubilized protein was attached to the ion exchange resin, but the first peak contained material which eluted without binding to the column, Fig. 2b, peak 1. After charging the column with 150 mM KCl peaks 3 and 4 were collected containing ($Na^+ + K^+$)-ATPase with specific activities around 0.15–0.2 μmol/min per mg at 20°C and a yield of 10–15 mg protein/100 g *Torpedo marmorata* organ. Both peaks were free

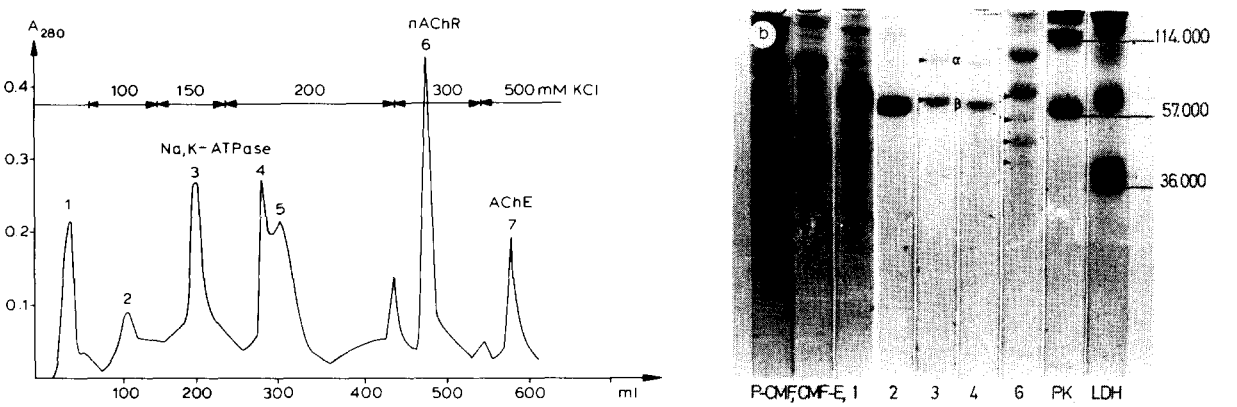

Fig. 2. (a) Elution profile from fractionation of crude membrane fraction extract (CMF-E) with dithiothreitol on a DEAE-Sephadex A-25 column. The column was eluted with 10 mM Tris-buffer (pH 7.5), with increasing concentrations of KCl as pointed out in the figure. Samples from the same peak were pooled and are designated peaks 1–7. AChE, acetylcholinesterase. (b) SDS-polyacrylamide gel electrophoresis on CMF-E and the peaks described in (a). From left to right: (1) P-CMF, (2) CMF-E, (3) peak 1, (4) peak 2, (5) peak 3, the α- and β subunits of the ($Na^+ + K^+$)-ATPase are pointed out, (6) peak 4, (7) peak 6, the four nicotinic acetylcholine receptor (nAChR) polypeptides are marked with arrows. (8) standard PK (pyruvate kinase), (9) standard LDH (lactate dehydrogenase).

TABLE I

FRACTIONATION OF PROTEIN COMPONENTS FROM *TORPEDO* ELECTRIC TISSUE ON DEAE-SEPHADEX COLUMN

– means that the fraction was without activity. CMF-E, crude membrane fraction extract.

Peak	1	2	3	4	4	6	7	Activity in % of total	CMF-E
Protein (mg)	3.1	0.6	3.3	2.7	0.2	2.6	1.1		19.8
% yield	16	3	17	14	1	13	6	70	100
K^+-phosphatase									
μmol/min per mg	0.04	0.07	0.13	0.11	0.07	0.02	–		0.12
% yield	2	1	13	6	–	2	–	24	100
α-[^{125}I]Bungarotoxin									
nmol/mg	–	–	–	–	–	0.92	0.52		0.18
% yield	–	–	–	–	–	68	16	84	100
Acetylcholine esterase									
μmol/min per mg	–	–	–	1.8	–	21	103		27
% yield	–	–	–	–	–	10	20	30	100

from α-[^{125}I]bungarotoxin binding capacity and acetylcholinesterase activity. Table I shows that only 24% of the K^+-phosphatase activity loaded on the column was recovered in the fractions. This suggests that part of the enzyme is inactivated during chromatography, as discussed later. In SDS-polyacrylamide gel electrophoresis both peaks showed two bands with apparent molecular weights of 90 000–100 000 and 50 000–60 000. These values are close to those obtained by Peterson and Hokin [26] for the α- and β-subunits of (Na^+ + K^+)-ATPase from *Electrophorus*. In peak 3 and 4 the α/β ratio based on Coomassie blue staining of the gels was much lower than expected [17,26]. Assuming that the material consists of (Na^+ + K^+)-ATPase subunits, the low ratio can be explained by loss of material from the α-subunit band or by contamination of the β-subunit band.

Our activity compares favourably with that of a Lubrol extract from *Torpedo marmorata* electric organ with a (Na^+ + K^+)-ATPase activity of 0.25 μmol P_i/min per mg at 37°C [18]. The enzyme activity is also very sensitive to temperature but is more stable at 20°C than at 37°C [17]. The (Na^+ + K^+)-ATPase of peak 3 (fig. 2a) was completely inhibited by 1 mM ouabain and the I_{50}-value was $3 \cdot 10^{-5}$ M, which is in accordance with values given in [18]. The (Na^+ + K^+)-ATPase activity in our preparation was low as compared to values of 10–20 μmol/min per mg at 37°C, reported for an 80% pure *Electrophorus* electric organ enzyme [16,25]. This may be explained by a dissociation of the α- and β-subunits. The somewhat higher specific K^+-phosphatase activity obtained by gel exclusion chromatography compared to ion exchange chromatography may depend on different degrees of delipidation [19,27].

In fractions 3 and 4 the (Na^+ + K^+)-ATPase activity was unstable. Both the K^+-phosphatase and the ATPase activities disappeared when the gel matrix-bound protein was freed from detergent by extensive washing of the column [17,19]. At an elevated detergent concentration of 0.05% (0.09 mM) and in the presence of glycerol [27] the enzyme was stabilized. This modification increased the specific activity at least ten times. It is, however, not clear whether this stabilization completely restored the enzymatic properties of the (Na^+ + K^+)-ATPase.

Peak 6 in Fig. 2a is a nicotinic acetylcholine receptor-enriched fraction with a specific α-bungarotoxin binding capacity of 1 nmol α-[^{125}I]bungarotoxin/mg protein. Pure *Torpedo* nicotinic acetylcholine receptor has a specific α-bungarotoxin binding of 8–9 nmol/mg [11]. In addition to polypeptides with molecular weights corresponding to the polypeptides of the nicotinic acetylcholine receptor complex (Fig. 2b, peak 6)

obtained after purification by Triton X-100 solubilization and affinity chromatography a polypeptide band with molecular weight corresponding to the α-subunit of $(Na^+ + K^+)$-ATPase [17,26] is seen. Only traces of K^+-phosphatase activity could be found in this peak (Table I). If this polypeptide band originates from $(Na^+ + K^+)$-ATPase the enzyme activity has been inactivated presumably because it has been dissociated from the β-subunit.

It cannot be completely excluded that the 93 kDa polypeptide chain might consist of an aggregate of the smaller polypeptides [11] though the precaution had been taken to include EDTA throughout the purification to avoid polypeptide cross-linking. Repeated SDS-treatment under reducing conditions and 10% SDS-polyacrylamide gel electrophoresis could, however, not dissociate the 93 kDa polypeptide band into smaller units [12].

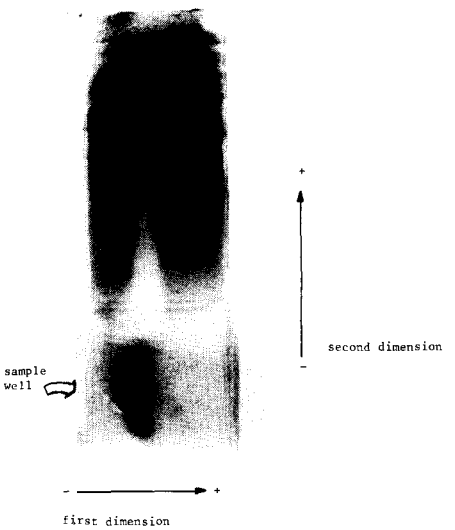

Fig. 3. Cross-reactivity between antibodies against the 93 kDa polypeptide, copurifying with nicotinic acetylcholine receptor and the $(Na^+ + K^+)$-ATPase fraction (peak 3) from *Torpedo*. Two-dimensional immunoelectrophoresis was run in the first dimension with $(Na^+ + K^+)$-ATPase (10 μg) (SDS-treated under reducing conditions) at 10 V/cm for 20 min. The bottom part of the slide shows staining of the protein after this run. Electrophoresis was then run perpendicular at 2 V/cm overnight into the antibody containing gel (15% serum, v/v). The plate was stained with Coomassie blue. The heavy background staining is due to the high concentration of sera in the gel, which in the presence of 3% PEG 6000 is difficult to wash out without influencing the precipitate.

In Fig. 3 two-dimensional immunoelectrophoresis demonstrated cross-reactivity between the obtained $(Na^+ + K^+)$-ATPase fraction (peak 3, Fig. 2) and antibodies against the 93 kDa polypeptide. Experiments performed with a Triton X-100 barrier inserted between the first dimension gel and the antibody containing gel in the second dimension resulted in the same precipitation peak as seen in Fig. 3. Consequently the observed precipitate is not due to a non-specific reaction between SDS and serum proteins [23]. The 93 kDa polypeptide therefore has immunological determinants in common with the $(Na^+ + K^+)$-ATPase, suggesting that this is the α-subunit.

An important issue raised by the present experiments is why it is difficult to separate the nicotinic acetylcholine receptor complex and the α-subunit of the $(Na^+ + K^+)$-ATPase by the present and previous techniques [6,11–14]. It is not possible now to answer the question whether the close proximity of the $(Na^+ + K^+)$-ATPase and the nicotinic acetylcholine receptor complex reflects a structure-function relationship in the membrane, which is essential for cholinergic neurotransmission, or if it is due to an unspecific aggregation of proteins in the detergent solution.

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