RECONSTITUTION OF ACETYLCHOLINESTERASE ACTIVITY FROM ELECTROPLAX MEMBRANE FRAGMENTS INTO PHOSPHATIDYLCHOLINE VESICLES

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Received 21 March 1978

1. Introduction

The enzyme acetylcholinesterase (EC 3.1.1.7) is known to be associated with the cell membrane of the electroplax [1]. Together with the nicotinic acetylcholine receptor, it is one of the key proteins involved in the process of synaptic transmission occurring at the electric organs of certain marine fishes.

Nevertheless, neither its location within the electroplax membrane nor its relationship with the lipids of the membrane matrix have yet been determined. Both matters are relevant to a better understanding of the structure and function of electric organ synapses.

The solubilization of acetylcholinesterase activity from electroplax membrane fragments and its reconstitution into the walls of phosphatidylcholine vesicles is reported here. The newly assembled structures proved to be a useful model for further examination of the enzyme interactions (if any) with membrane or exogenously added lipid and/or other membrane molecules.

2. Materials and methods

Male *Discopyge tschudi* (Heckel, 1846) specimens, an electric fish of the Torpedinidae family [2], were obtained at the Marine Biology Station at Mar del Plata. The electric organs were dissected within 1 h of capture and kept frozen at −60°C until further use. Membrane fragments were prepared from 10 g thawed tissue by the method in [3]. Lipid phosphorus, protein and acetylcholinesterase activities were measured by the methods in [4], [5] and [6], respectively. In every case the activity was measured using the same buffer employed to prepare the phospholipid vesicles in order to prevent osmotic changes.

Egg yolk phosphatidylcholine(s) was prepared in the laboratory as in [7].

The detergent-dialysis procedure in [8] was used for the reconstitution experiments. A membrane suspension in 20 mM Tris–HCl, 80 mM KCl buffer, pH 7.3 (0.83 mg protein/g fresh tissue, 0.23 mg phospholipid/g fresh tissue) was incubated with either 0.5% Triton X-100 or 1 M NaCl and gently agitated in the cold room with a magnetic stirrer for 30 min. It was then centrifuged at 100 000 × *g* for 60 min. The supernatant fraction obtained from the Triton-treated membranes was added to a glass vial containing a certain amount (specified in the text) of N_2-vacuum dried phosphatidylcholine. The whole mixture was mechanically shaken in a Vortex until all the lipid had come off the glass walls, sonicated in a bath type sonicator for 30 min as in [7] and dialyzed against 500 vol. detergent-free Tris–KCl buffer under N_2, at 4°C, for 48 h with 8 dialysis changes. After this, samples of the dialysis bag contents were taken for measurement of enzyme activity and electron microscopy. The latter were negatively stained with 2% phosphotungstic acid and examined under a Siemens Elmiskop I electron microscope at 80 kV. The remaining suspension was centrifuged at 100 000 × *g* for 60 min. Protein, lipid phosphorus and enzyme activity were determined in the pellet and supernatant fractions.

Table 1
The incubation of *D. tschudi* electroplax membrane fragments with 1 M NaCl or 0.5% Triton X-100

	Before incubation	After incubation	Pellet	Supernatant
NaCl (1 M)	8.45	8.98	5.98	2.32(28)
Triton X-100 (0.5%)	9.68	12.00	3.23	9 (75)

Enzyme specific activities are expressed as μmol acetylthiocholine/mg protein/min. Percentages of extraction are indicated between parentheses

3. Results and discussion

Table 1 shows a typical enzymatic activity extraction experiment, 1 of 5. It can be seen that the enzyme is not easily released by the high ionic strength treatment, whereas Triton proved to be more effective. The same results were obtained by homogenizing whole electric organ tissue in buffered 1 M NaCl or 0.5% Triton X-100. This result suggests a stronger association of the enzyme with the membrane fragments than that reported for *E. electricus* membranes [9]. It is worth recalling here that the butyrylcholinesterase activity of *D. tschudi* electroplax membrane fragments was almost nil, as in other members of the Torpedinidae family [10].

The Triton-extracted material neither sediments at 100 000 X *g* after 60 min, nor shows vesicle formation under the electron microscope. This is even true after 48 h dialysis against Triton-free Tris–KCl buffer.

When such an extract is added to dried phosphatidylcholine and dialyzed against Triton-free Tris–KCl buffer, there are vesicles measuring 200–500 nm (external diameter), with a large central cavity and possessing 2 or 3 lamellae (data not shown). This material forms a pellet after being spun down at 100 000 X *g* and contains about 73% orginal enzyme activity (see table 2). The latter is associated with the pellet fraction somewhat strongly, since it remains after one wash with buffer.

The presence of phospholipid vesicles might help detergent removal from the preparation and lead to aggregation of membrane proteins including acetylcholinesterase. This can be tested by performing sucrose density gradient centrifugation of the reconstituted vesicles (fig.1). It can be demonstrated that the enzyme activity comigrates along with the phospholipid at the beginning of the gradient, as it is the

Table 2

	Supernatant	Pellet
Protein (mg)	6.78	0.90
Phospholipid (mg)	2.47	11.32
Enzyem spec.act.	3.92	10.80

A 0.5% Triton X-100 supernatant (see table 1), spec.act. 12, 2 mg phospholipid, 8.2 mg protein, was added to 15 mg egg yolk phosphatidylcholine and the detergent-dialysis procedure in [8] was used. Rest of experimental details in section 2. Exogenously added lipid was 88% total lipid in this and about the same in other reconstitution experiments reported throughout this paper. Lipid and protein recovery after dialysis was about 65%

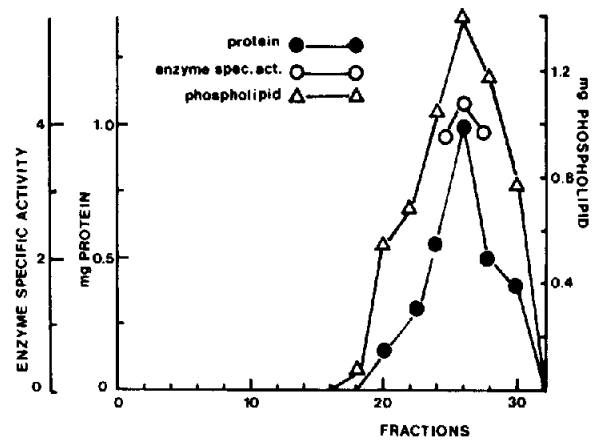

Fig.1. A membrane preparation (3.50 mg protein, 0.5 mg lipid) was supplemented with 5 mg egg phosphatidylcholine and reconstitution was carried out as in section 2. The contents of the dialysis bag was then placed on top of a discontinuous sucrose gradient in Tris–KCl buffer (0.5, 1 and 1.7 steps, all molar concentrations) and centrifuged for 4 h at 25 000 rev./min using a SW25 rotor in a Spinco ultracentrifuge. Fractions of 2 ml were collected and assayed for protein, lipid phosphorus and acetylcholinesterase activity. The bottom of the gradient is on the left hand side.

case for pure phospholipid vesicles. This result suggests a true association between the enzyme and the phospholipids.

The observed activities can be explained by either of two hypotheses:

1. There are enzyme molecules incorporated into the wall of the vesicles.

2. Such molecules are trapped inside.

Although it is likely that during the reconstitution process both events take place, I prefer to assume that the former hypothesis is a plausible one, since further treatment of the vesicles with 1% Triton X-100 produces no increase in the rate of hydrolysis of acetylthiocholine. This detergent concentration does not affect the activities either of the 0.5% Triton-solubilized enzyme or that of a commercial preparation (Sigma) obtained from *E. electricus*. Control experiments also revealed that phosphatidylcholine liposomes do not alter enzyme activities.

The kinetic characteristics of the vesicle-associated enzyme are not dissimilar from those of the original membrane-bound variety: it displays Michaelis-Menten kinetics, the K_m for acetylthiocholine is in the order of 5×10^{-5} mol. l^{-1} and it is inhibited by 1 μM eserine.

If the vesicles are incubated in 1 M NaCl, there is no increase in enzyme activity. This is the opposite to what occurs with *E. electricus* membrane fragments [9] (see table 3). Since phospholipid vesicles do not sediment in high ionic strength media [7], it was not possible to perform centrifugation experiments to demonstrate an eventual enzyme detachment from the membrane induced by salt.

The above results were further substantiated by an experiment such as that shown in table 3. A membrane suspension (about 5 mg total protein and 1.5 mg phospholipid) was treated with 1 M NaCl and its acetylcholinesterase activity measured before and after incubation. The suspension was then centrifuged

Table 3

		1st. centrifugation		1 M NaCl wash	
Before 1 M NaCl	After 1 M NaCl	Pellet	Supernatant	Pellet	Supernatant
10.56	11.00	9.67	1.97	–	–
11.66	11.20	10.00	2.15	9.57	0.22

0.5% Triton X-100 treatment of 1st. centrifugation pellet

	Pellet	Supernatant
Enzyme spec.act.	2.30	6.87
Protein (mg)	2.00	2.80
Phospholipid (mg)	0.60	0.80

Supernatant addition to 7 mg egg yolk phosphatidylcholine plus dialysis

	Pellet	Supernatant
Enzyme spec.act.	9.57	1.16
Protein (mg)	0.24	0.89
Phospholipid (mg)	0.35	0.26

A pellet fraction such as that depicted in table 1, previously exposed to 1 M NaCl, was treated with 0.5% Triton X-100. The supernatant was added to phosphatidylcholine and reconstitution was achieved as described in section 3

at 100 000 × *g* for 60 min and the resulting pellet was redissolved in fresh 1 M NaCl and recentrifuged in the same way. It is noteworthy that even after a high-ionic-strength wash of the pellet, more than 95% original activity remains associated with the new pellet (table 3).

The pellet derived from the first centrifugation was incubated with 0.5% Triton X-100 (see section 2) and the mixture was centrifuged as described, for 60 min. It can be seen from table 3 that 75% activity is extracted by detergent. The supernatant fraction is then added to dried phosphatidylcholine, and the reconstitution experiment carried out as stated. After this procedure 82% original activity precipitates along with the lipid (table 3).

There are some reports showing a possible role of membrane lipids in the functioning of acetylcholinesterase. By using human red cells [11] a drop in enzymic activity with deoxycholate concentrations above 60 mM and a further recovery after the addition of membrane lipids was demonstrated. These molecules could be involved in the catalytic activity of the erythrocyte enzyme [11].

When the acetylcholinesterase molecules are reassembled into the phospholipid vesicles, they also maintain a close association with phospholipids (tables 2 and 3).

On the basis of the afore-mentioned results, I should like to suggest that, at least in *D. tschudi* electroplax membrane fragments, the acetylcholinesterase molecules are closely related to a membrane component, presumably phospholipid in nature.

Whether whilst being reconstituted the enzyme is assembled as a separate entity without interacting with other molecules, or whether it requires other membrane component(s) derived from the native membrane framework, is a matter of further experimental enquiry.

Acknowledgements

I should like to thank Professor M. B. Cousseau from the Marine Biology Station at Mar del Plata for kindly providing the electric fish. This work was supported by a grant from the CONICET (Argentina). E.L.M.O. is Career Investigator from the CONICET.

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