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ACETYLCHOLINESTERASE AND ACETYLCHOLINE PROTEOLIPID RECEPTOR: TWO DIFFERENT COMPONENTS OF ELECTROPLAX MEMBRANES

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SUMMARY

Electroplax membranes from *Electrophorus electricus* were separated by two methods of subcellular fractionation, and in both cases a parallelism between the content of acetylcholinesterase and the binding of cholinergic drugs was observed. Treatment of the membranes with the –SH reagents, *p*-hydroxymercuribenzoate and *p*-chloromercuribenzoate, produced a 16–28 % inhibition in the binding of [^{14}C]acetylcholine. With the –S–S– reagent, 1,4-dithiothreitol, followed by *N*-ethylmaleimide the inhibition was 64–67 %. Membranes depleted of acetylcholinesterase with 1 M NaCl showed no quantitative change in the binding of dimethyl (+)-[^{14}C]tubocurarine and methyl[^{14}C]hexamethonium as compared to the controls. Furthermore the same amount of receptor proteolipid, which binds [^{14}C]acetylcholine, was found after the 1 M NaCl treatment. It is concluded that: (a) acetylcholinesterase and the acetylcholine proteolipid receptor are two different macromolecules present in the electroplax membranes; (b) the receptor proteolipid, previously isolated from the intact tissue, is contained in the electroplax membrane.

INTRODUCTION

The chemical distinction between acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) and the cholinergic receptor at postsynaptic membranes is a subject of long controversy[1,2]. While some investigators have postulated that both the enzyme and the receptor could be part of the same macromolecule[3,4], others have brought indirect autoradiographic[5], physiological and pharmacological evidence[6,7,2] that they are different molecular entities. Recent work has revealed the possibility of a more direct attack on this problem. SILMAN AND KARLIN[8] demonstrated that when electroplax membranes isolated from the *Electrophorus electricus* were submitted to washing with 1 M NaCl, they lost most of their acetylcholinesterase into the supernatant. This made feasible the study of such acetylcholinesterase-depleted membranes, using the binding techniques developed in this laboratory for nerve-ending membranes separated from brain[9,10].

In a previous work several proteolipids (*i.e.* hydrophobic lipoproteins) were

separated from lyophilized electroplaques of Torpedo and Electrophorus using organic solvents and column chromatography[11]. One such proteolipid showed high affinity binding (*i.e.* binding at $1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M concentration) for [^{14}C]acetylcholine, methyl-[^{14}C]hexamethonium and [^{3}H]trimethylphenylammonium-diazonium fluoroborate. This last compound has a cationic head similar to acetylcholine and irreversibly inactivates the electroplax *in vivo*, an action that has been interpreted by some as due to covalent bonding with some amino acid residues of the cholinergic receptor[12]. Because of these binding properties, which are not found in the other proteolipids, it was denominated "receptor" proteolipid.

In the present investigation it will be demonstrated that this receptor proteolipid is present in the isolated electroplax membranes. Furthermore, both from the intact and the acetylcholinesterase-depleted electroplax membranes, the same amount of receptor proteolipid may be extracted. These findings are interpreted as evidence that acetylcholinesterase and this acetylcholine receptor are two different macromolecules present in the electroplax membrane. A preliminary presentation of these findings was made at the recent meeting in Skokloster (Sweden)[13].

METHODS

Tissue fractionation

For each experiment, 10–30 g of electric tissue of *Electrophorus electricus* obtained in frozen condition were used. Two methods of subcellular fractionation were employed:

In Method A, the tissue (20–30 g) was treated by the technique used to separate plasma membranes from liver cells[14]. The homogenization was done in a Virtis blender at 3/4 of the maximum speed in 0.01 M bicarbonate buffer (pH 7.5). The homogenate was treated with collagenase (Sigma, Type 1), 0.1 mg/ml of homogenate, for 1 h at 37° and then filtered through a steel filter of 100-μ mesh. The homogenate (FH in Table I) was then submitted to four successive centrifugations at 3250 rev./min and washings in the same buffer solution. The pellet (Pg) obtained was resuspended in 0.32 M sucrose (pH 7.2) and layered upon a gradient with four steps of sucrose from 1.4 to 0.8 M (Table I). Each gradient was seeded with the equivalent of 4–6 mg protein. Centrifugation of the gradient was carried at 50000 $\times$ *g* for 1.5 h in the SW-25.1 rotor of the Spinco L ultracentrifuge.

In Method B, the technique described by CHANGEUX *et al.*[15] was used. Homogenization was done in 0.2 M sucrose (pH 7.2) in the Virtis blender for 1.5 min at a velocity 3/4 of the maximum. The homogenate was submitted to sonic treatment for 1 min, in an ice jacket, with a sonifier from Branson Inst. Co. in position 5. Then it was centrifuged at 5000 $\times$ *g* for 20 min, and after discarding the pellet, the supernatant was layered on a gradient of two steps of 1.0 and 0.4 M sucrose and centrifuged for 5 h at 25000 rev./min in the SW-25.1 rotor (Table II).

In both methods the layers were separated and sedimented at 10000 $\times$ *g*; the biochemical studies were then done on the pellet.

Electron microscopy

In Method A the steps in the purification of the membranes were monitored with phase microscopy and in its final stage with electron microscopy. The various

fractions of the gradient were fixed in glutaraldehyde, postfixed in osmium tetroxide and processed for electron microscopy. For the various fractions obtained with Method B, only electron microscopy was used.

Assays

Protein was determined by the method of LOWRY *et al.*[16]; acetylcholinesterase by that of ELLMAN *et al.*[17]. Some membranes were treated with the -SH-blocking reagents, *p*-hydroxymercuribenzoate and *p*-chloromercuribenzoate; others were treated with the reducing agent, 1,4-dithiothreitol, followed by the alkylating reagent, *N*-ethylmaleimide. Both in the control and the pretreated membranes, the binding of [^{14}C]acetylcholine was determined by the method of AZCURRA AND DE ROBERTIS[9] (Table III).

The proteolipid protein was extracted from the lyophilized membranes with chloroform–methanol (2:1, by vol.) and purified on a Sephadex LH 20 column by the method of SOTO *et al.*[18]. The elution was done with 40 ml chloroform, 10 ml each of chloroform–methanol mixtures (15:1, 10:1 and 6:1, by vol.), followed by 40 ml of chloroform–methanol (4:1, by vol.). The eluted fractions were monitored for protein with an LKB Uvicord at 278 nm and on each tube protein[16], lipid phosphorus and radioactivity were determined[10]. The radioactive drugs used were: acetyl[*Me*-^{14}C]-choline chloride, specific activity 10.4 mC/mmole (Radiochemical Centre, Amersham); methyl[^{14}C]hexamethonium, specific activity 1.52 mC/mmole (New England Co.); and dimethyl (+)-[^{14}C]tubocurarine, specific activity 7.71 mC/mmole (New England Co.).

RESULTS

Acetylcholinesterase and binding in subcellular fractions

Table I shows the results obtained in two typical fractionation experiments done with Method A. The values of acetylcholinesterase in all the fractions are much lower than those given in the literature[19,15]. This is due to the solubilization of the enzyme during the homogenization and long washing process with the bicarbonate buffer, as could be demonstrated by checking the acetylcholinesterase activity in the supernatants. With Method B, in the total homogenate obtained after the first centrifugation, we found 745 μmoles/mg protein per h of acetylcholinesterase activity which is close to the published figures. With this method the enzyme activity in the membranes separated at 0.4 and 1.0 M sucrose is considerable higher than with Method A (Table II). However, with both methods there is a 4.5–5.5-fold concentration of acetylcholinesterase in the most purified membranes, as compared to the original homogenates.

The binding of methyl[^{14}C]hexamethonium and dimethyl (+)-[^{14}C]tubocurarine was carried out on all the fractions obtained with Method A. In Table I it may be observed that there is a certain parallelism between the binding of these two cholinergic drugs and acetylcholinesterase. The highest binding capacity corresponds to the membranes isolated in Fraction 0.8 M which show a 3-fold increase in binding with respect to the filtered homogenate.

Electronmicroscopy of the subcellular fractions

Figs. 1A and 1B show the type of membranous profiles which may be observed, respectively, in Fractions 0.8 and 1.0 M separated with Method A. The morphology

TABLE I

ACETYLCHOLINESTERASE AND BINDING OF METHYL[^{14}C]HEXAMETHONIUM AND DIMETHYL (+)-[^{14}C]-TUBOCURARINE TO SUBCELLULAR FRACTIONS OF ELECTRIC TISSUE OF ELECTROPHORUS SEPARATED WITH METHOD A

For the binding of methyl[^{14}C]hexamethonium, about 150 μg protein of each fraction were used at a final concentration of $3 \cdot 10^{-6}$ M. For dimethyl (+)-[^{14}C]tubocurarine, 100 μg protein, $5 \cdot 10^{-6}$ M were used. FH, filtered homogenate; Pg, pellet after several washings. For further details on the binding technique see ref. 9.

Fraction	*Protein (mg/g fresh tissue)*		*Acetylcholinesterase (μmoles/h per mg protein)*		[^{14}C]*Hexamethonium (nmoles/mg protein)*	*Dimethyl (+)-[^{14}C]-tubocurarine (nmoles/mg protein)*
	(1)	(2)	(1)	(2)	(1)	(2)
FH	20.4	23.0	167	120	4.7	0.85
Pg	1.1	0.79	315	311	7.8	1.70
Gradient						
0.8 M	0.016	0.013	750	400	15.0	2.60
1.0 M	0.027	0.019	162	152	9.0	1.90
1.2 M	0.110	0.132	90	114	7.4	0.70
1.4 M	0.010	0.132	110	168	5.9	0.73
Pellet	0.800	0.371	75	81	5.2	0.83
Recovery (%)	87.2	84.8				

TABLE II

ACETYLCHOLINESTERASE IN SUBCELLULAR FRACTIONS OF ELECTRIC TISSUE OF ELECTROPHORUS SEPARATED WITH METHOD B

H is the supernatant after the first centrifugation (see METHODS). If H is sedimented at 100 000 $\times$ *g* for 30 min, the acetylcholinesterase of the particulate is 1496 μmoles/h per mg protein.

Fraction	*Protein (mg/g fresh tissue)*	*Acetylcholinesterase (μmoles/h per mg protein)*
H	15.8	745
Gradient		
0.2 M	0.16	350
0.4 M	0.35	4130
1.0 M	1.44	2836
Pellet	1.82	98

of the subfractions of Method B is similar to that illustrated by CHANGEUX *et al.*[15]. With both methods membrane fragments may be observed. Because of the sonic treatment in the fractions obtained with Method B, the membrane fragments are of smaller size (particularly in Fraction 0.4 M) and resemble vesicular elements larger than synaptic vesicles.

Effect of –SH and –S–S– reagents on the binding of [^{14}C]acetylcholine

Table III shows the effect of *p*-hydroxymercuribenzoate and *p*-chloromercuribenzoate on the binding of [^{14}C]acetylcholine to the membranes separated with Method B. This treatment produced a slight inhibition of the binding (*i.e.* 17–28 %).

Similar membranes were successively treated with dithiothreitol to reduce –S–S–, followed by *N*-ethylmaleimide to block the resulting –SH groups[20]. In this case, the inhibition of the [^{14}C]acetylcholine binding was 64–67 % (Table III). In one experiment done with dithiothreitol alone, there was no effect on the binding.

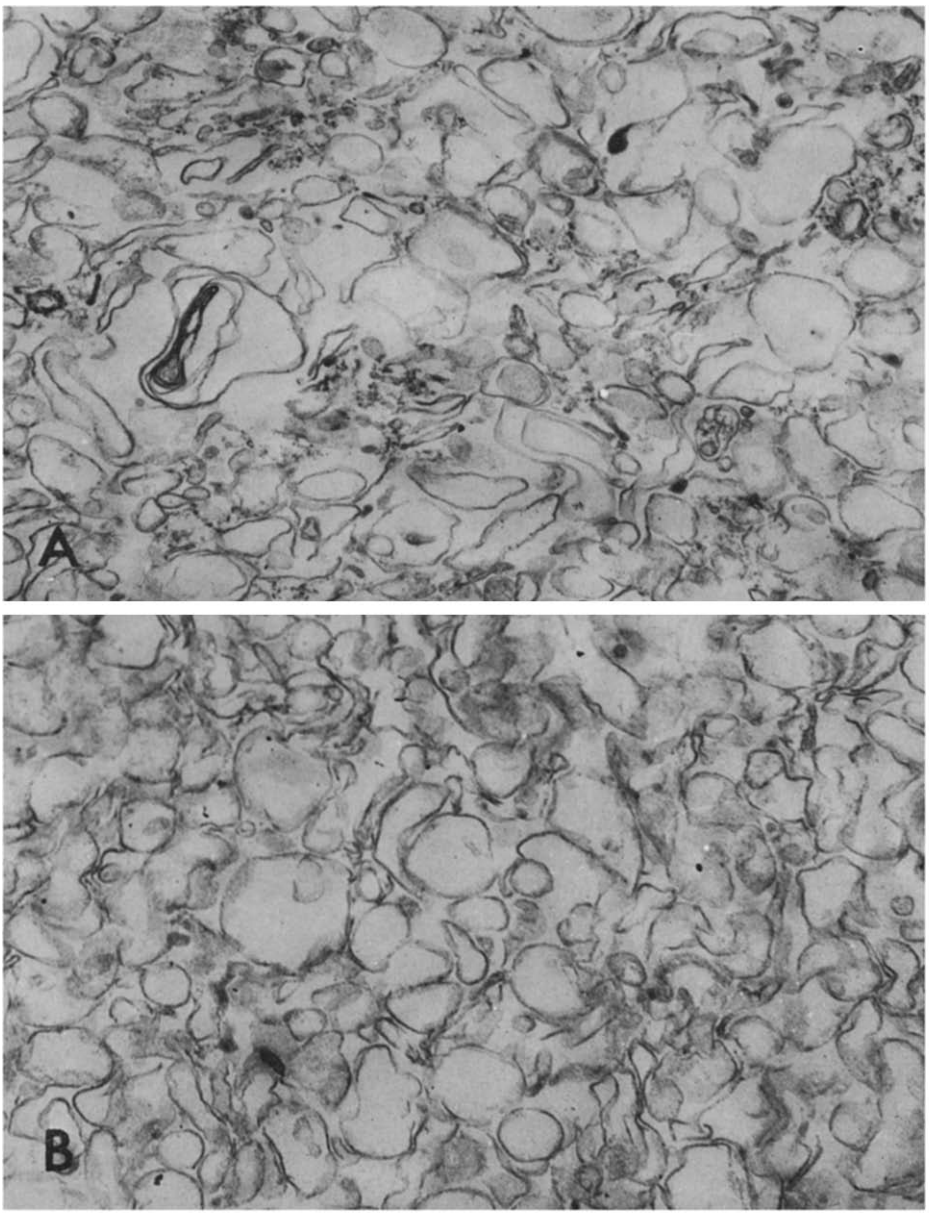

Fig. 1. Electroplax membranes of Electrophorus separated with Method A and observed under the electron microscope. A, Fraction 0.8 M; B, Fraction 1.0 M. Membranous profiles deprived of collagen are seen in both fractions. 40000 ×.

Effect of 1 M NaCl on the acetylcholinesterase and binding of [^{14}C]acetylcholine in electroplax membranes

The results shown in Table IV confirm the findings of SILMAN AND KARLIN[8]; treatment of the isolated electroplax membranes with 1 M NaCl results in an almost complete solubilization of acetylcholinesterase. In these membranes the loss of proteins is minimal. Fig. 2 shows the percentage loss of acetylcholinesterase in three experiments with membranes separated by Methods A and B. The reduction in the bound enzyme by the 1 M NaCl treatment varies between 90 and 97 %. In contrast the binding of the two cholinergic drugs shows practically no change after extraction of the membranes with 1 M NaCl.

TABLE III

ACTION OF DRUGS ON THE BINDING OF [^{14}C]ACETYLCHOLINE TO THE ELECTROPLAX MEMBRANES

The fractions were separated with Method B. For *p*-hydroxymercuribenzoate and *p*-chloromercuribenzoate a 0.1 M glycylglycine buffer (pH 8) was used. After 20 min, the membranes were sedimented and the binding with $1 \cdot 10^{-6}$ M [^{14}C]acetylcholine was done in distilled water (pH 7) with Tris buffer containing $2 \cdot 10^{-5}$ M physostigmine to inactivate acetylcholinesterase. Other membranes, in Tris buffer (pH 8), were treated for 20 min with dithiothreitol, sedimented and then treated again with *N*-ethylmaleimide before the binding.

Fraction	*Protein* (*μg/ml*)	*Drug*	*Drug concn.* (*mM*)	*Disint./min per mg protein*		*Inhibition* (%)
				Control	*Treated*	
0.4 M	600	*p*-Hydroxymercuribenzoate	50	6071	5000	18
1.0 M	1000	*p*-Hydroxymercuribenzoate	50	6576	5580	17
1.0 M	730	*p*-Chloromercuribenzoate	50	6539	4714	28
1.0 M	3000	Dithiothreitol	10	2500	816	67
		N-Ethylmaleimide	10			
1.0 M	3000	Dithiothreitol	10	2500	912	64
		N-Ethylmaleimide	10			

TABLE IV

ACETYLCHOLINESTERASE IN ELECTROPLAX MEMBRANES BEFORE AND AFTER TREATMENT WITH 1 M NaCl

The fractions, separated with Method B, were rehomogenized in NaCl for 2 min, then they were left for 20 min in the cold and sedimented. The enzyme was assayed in the sediment and the supernatant in relation to the total protein content.

Fraction	*Control* (*μmoles/h per mg protein*)	*1 M NaCl* (*μmoles/h per mg protein*)	
		Sediment	*Supernatant*
0.4 M	3700	353	3147
1.0 M	3580	132	3276

Binding of [^{14}C]acetylcholine to the proteolipid from electroplax membranes

Previous experiments on Torpedo and Electrophorus have shown that the binding of [^{14}C]acetylcholine is with a special proteolipid peak which is eluted with chloroform from the Sephadex LH 20 column[11]. This problem was reinvestigated on isolated electroplax obtained with Method B. Fractions 0.4 and 1.0 M were pooled,

lyophilized and extracted with chloroform–methanol (2:1, by vol.)[18]. The lipid extract was incubated with [^{14}C]acetylcholine at a final concentration of $1 \cdot 10^{-6}$ M. From 15 mg of lyophilized tissue, 252 μg of proteolipid protein were found in the extract and 168 μg were recovered from the column. The total amount of lipid phosphorus was 156 μg, and 101 μg were recovered. About 50 % of the radioactivity put on the column

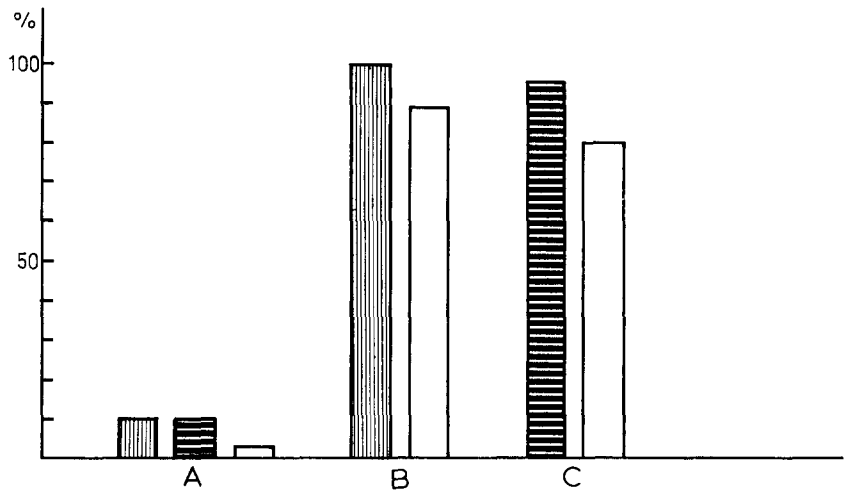

Fig. 2. Acetylcholinesterase and binding of dimethyl (+)-[^{14}C]tubocurarine and methyl[^{14}C] hexamethonium in electroplax membranes after treatment with 1 M NaCl. The results are expressed as percentage of the corresponding control in three different experiments. A, acetylcholine; B, dimethyl (+)-[^{14}C]tubocurarine; C, methyl[^{14}C]hexamethonium.

was recovered in the eluate (Fig. 3A). With free [^{14}C]acetylcholine most of the radioactivity was retained by the column, and only a small amount was eluted at the end of the chromatogram.

A similar experiment was carried out on electroplax membranes treated for 10 min with 1 M NaCl (Fig. 3B). In this case, starting with 32 mg of lyophilized membranes, 488 μg of proteolipid were obtained, and 461 μg were recovered in the elution. The total radioactivity added was 69000 disint./min, and the recovery was 50030 disint./min. As shown in the chromatograms of Figs. 3A and 3B, most of the proteolipids appear in a single peak with a maximum in Tube 13 which coincides with the peak of radioactivity. The maximum of lipid phosphorus is found in Tube 12. There is practically no difference between the control and the acetylcholinesterase-depleted membranes in relation to the content of receptor proteolipid and binding of [^{14}C]acetylcholine. In fact the specific activity in Tube 13 of the control was 0.012 nmole of [^{14}C]acetylcholine per μg of proteolipid protein and in the corresponding tube of the acetylcholinesterase-depleted membranes was 0.010 nmole of [^{14}C]acetylcholine per μg of proteolipid protein.

DISCUSSION

The acetylcholine–receptor interaction at postsynaptic membranes has, in the past, been studied on the assumption that the catalytic site of acetylcholinesterase and the binding site of the acetylcholine receptor have some properties in common.

The enzyme may be easily separated from the electroplax and obtained in a rather purified form[21,22]. It consists of a macromolecule of mol.wt. 260000 made of four subunits with similar molecular weight[22]. Recently CHANGEUX *et al.*[4] suggested that acetylcholinesterase may contain both catalytic and regulatory subunits and that the latter "might be, or contribute to the acetylcholine macromolecular receptor".

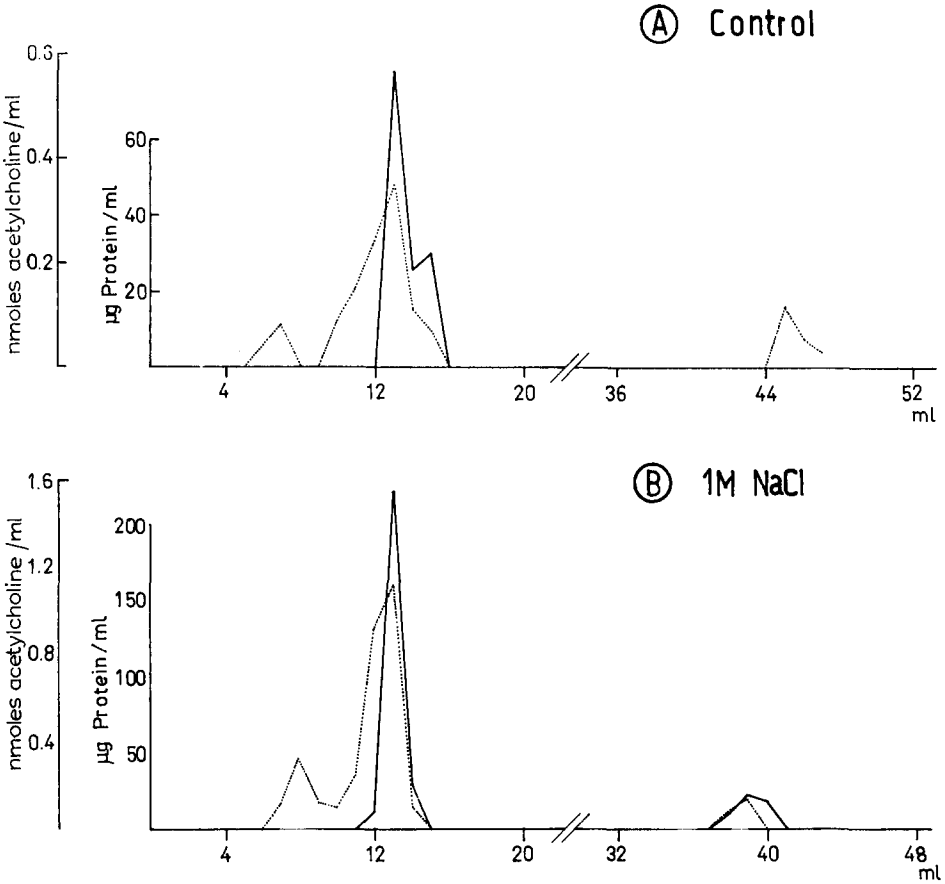

Fig. 3. Chromatograms on Sephadex LH20 of proteolipids extracted from electroplax membranes. A. Control. B. After 1 M NaCl treatment. The fractions were isolated at 1 ml per tube (see the description in the text). ....., protein; —, [^{14}C]acetylcholine.

KARLIN[2] has emphasized that there are large quantitative differences in the apparent dissociation constant of the acetylcholine–receptor complex in the isolated electroplax of Electrophorus (*i.e.* approx. $5 \cdot 10^{-6}$ M) and the K_m of acetylcholinesterase from the same cell (*i.e.* approx. $1 \cdot 10^{-4}$ M). Furthermore KARLIN AND BARTELS[20], using the isolated electroplax preparation, observed that the response to acetylcholine is partially inhibited with *p*-chloromercuribenzoate at concentrations that have no effect on acetylcholinesterase. A more effective inhibition of the receptor was obtained by dithiothreitol followed by *N*-ethylmaleimide[20,23]. Our results on the action of these agents upon the acetylcholine binding to the isolated membranes are in line with the above findings.

The main obstacle in obtaining a clear-cut distinction between acetylcholinesterase and the receptor has been the lack of any direct information on the nature of the receptor. From nerve-ending membranes of the cerebral cortex we isolated a special proteolipid showing affinity for dimethyl (+)-[^{14}C]tubocurarine[24] and atropine sulphate[25] at concentrations of $1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M. Furthermore, it was shown that the binding of cholinergic drugs was confined mainly to subsynaptic membranes, while acetylcholinesterase had a much wider distribution in the limiting membrane of the nerve ending[26]. The isolation of a receptor proteolipid from the electroplax permitted a more direct approach to this problem. LA TORRE *et al.*[11] demonstrated that the cholinergic drugs bind to a special proteolipid of the electroplax which is eluted from the Sephadex LH 20 column in chloroform. (Unpublished results indicate that the apparent dissociation constant for the high affinity binding for acetylcholine is $1 \cdot 10^{-7}$.)

The results presented here demonstrate that, although acetylcholine and the high affinity binding for cholinergic drugs reside in the same membrane fractions of the electroplax, they can be easily differentiated by the following properties:

(a) The binding of [^{14}C]acetylcholine to the electroplax membrane is slightly reduced by –SH-blocking agents, but it is greatly inhibited by dithiothreitol followed by *N*-ethylmaleimide, a treatment which is known to inactivate the acetylcholine receptor in the living electroplax without inhibiting acetylcholinesterase[23].

(b) While acetylcholinesterase may be easily solubilized and extracted from the electroplax membranes by the 1 M NaCl treatment[8], the high affinity binding for cholinergic drugs remains intact in the acetylcholinesterase-depleted membranes.

(c) The same amount of receptor proteolipid having a similar specific radioactivity may be separated both from normal and from acetylcholinesterase-depleted membranes.

These findings indicate that acetylcholinesterase and the proteolipid acetylcholine receptor are two distinct macromolecules. Furthermore, they suggest that their attachment to the membrane is of a different nature. While acetylcholinesterase is easily removed from the surface of the membrane, the proteolipid – a rather hydrophobic protein – is more intimately bound or built into the lipoprotein membrane structure and needs a more drastic treatment to be separated from it. Such a treatment completely inhibits acetylcholinesterase[10].

In a previous paper we separated and purified the receptor proteolipid from the total electroplax[11]; here the demonstration that it is localized in the electroplax membranes adds considerable support to our assumption that this macromolecule represents the acetylcholine receptor in the living electroplax.

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