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LIPID REQUIREMENTS OF FUNCTIONAL MEMBRANE STRUCTURES AS INDICATED BY THE REVERSIBLE INACTIVATION OF (Na^+–K^+)-ATPase

J. JÄRNEFELT
Department of Medical Chemistry, University of Helsinki, Helsinki (Finland)
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SUMMARY

1. Extraction of the (Na^+–K^+)-ATPase of the electric organ of *Electrophorus electricus* with hexane removes almost all of the cholesterol from the particles. ATPase activity and phospholipid content are left essentially intact.

2. Subsequent removal of the major portion (70–80 %) of the phospholipids by hexane–ethanol extraction reduces the ATPase activity to about 10–15 % of the original.

3. Recombination experiments show that cholesterol and phospholipids are taken up by the extracted proteins. Compared to the amounts originally present, the amounts of cholesterol bound are small, about 20 %, whereas the relative phospholipid concentration is returned to levels existing prior to hexane–ethanol extraction.

4. In recombination experiments cholesterol has a considerably higher ability to reactivate the ATPase than phospholipids, especially if the relative amounts of bound lipids are considered.

INTRODUCTION

Several general models for the arrangement of proteins and lipids in membranes have been proposed, whereas the detailed and specific arrangement of the components is not very well understood. The behaviour of membrane-bound enzyme activities, under conditions where the interactions between proteins and lipids are altered, may provide a sensitive indicator of structural features in membranes[1–4].

In the present study the highly active (Na^+–K^+)-ATPase obtained from the electric organ of the electric eel (*Electrophorus electricus*), was subjected to stepwise extraction with organic solvents. Enzymic activity and lipids were removed in a specific order. Recombinations of the extracted protein residue with lipids indicates that cholesterol has the ability to reactivate, both in the case where primarily cholesterol has been removed and in the case where also the major portion of phospholipids were removed. Reactivation with phospholipid was less conspicuous.

METHODS

The (Na^+–K^+)-ATPase was prepared from the electric organ of *E. electricus* according to the method of Albers[5], and stored as a 0.25 M sucrose suspension at

—70°. For the experiments the preparation was diluted 10-fold with water, centrifuged, suspended in water and lyophilized.

The solvent extraction took place at —20°. The extraction with hexane (1 ml/mg dry enzyme) for 30 min was either followed by two re-extractions with hexane or by one extraction with 5 % ethanol–hexane and one wash with hexane. The two preparations had thus undergone three extractions each. The extracted preparations were dried under vacuum at —20°.

The hexane and ethanol–hexane extracts were used for preparation of the lipids used. The combined extracts were evaporated to dryness, dissolved in chloroform–methanol–water (5:4:1, by vol.) and converted to their sodium salts by passage through a column prepared from Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). The resin was neutralized with acetic acid to pH 7.5 and transferred into chloroform–methanol–water (5:4:1, by vol.). The lipid was applied to the column in this solvent and eluted with 15 column volumes of the same solvent. The eluate was evaporated, dissolved in chloroform and fractionated on a silicic acid column. The column was first eluted with chloroform (about 20 vol.) and then with chloroform–methanol (2:1, by vol.) (about 100 vol.). The eluates were evaporated and the residues taken up in hexane. The insoluble materials were discarded. Analysis indicated, that the first eluate contained cholesterol and no phospholipids, whereas the opposite held for the second eluate. No significant amounts of neuraminic acid were found in either fraction. Accordingly, the fractions were named the C and the PL fraction, respectively. The total unfractioned lipid extract was used in some experiments, and named TL.

For recombination experiments the lipid in question was dissolved in 0.1 ml hexane and a suitable aliquot of the extracted enzyme was incubated with it for 30 min at —20°. After dilution with 2 ml hexane and centrifugation the residue was dried under vacuum at —20° and suspended in 1 ml of 0.02 M Tris–HCl buffer, pH 7.4. The cholesterol used in some experiments was a commercial preparation from Nutritional Biochemicals Corp., Chicago, Ill.

ATPase was assayed by measurement of P_i liberation, according to a modification of the procedure described by Kylin and Gee[6]. The substrate was 5 mM ATP as the Tris salt, and the incubation mixture contained 5 mM Mg^{2+}, and 100 mM Na^+ and 10 mM K^+, where appropriate. The total volume was 0.5 ml, incubation time 3.5 min, and the temperature 37°. The amount of enzyme protein added to each assay was 10–15 μg. The incubation was terminated by addition of 0.2 ml of 20 % trichloroacetic acid, after which 0.2 ml of 2 M sodium acetate was added with vigorous mixing followed by centrifugation. A suitable aliquot of the supernatant was diluted with water to 1.5 ml, and 0.6 ml each of ammonium molybdate (0.44 % in 0.6 M H_2SO_4) and reductant (0.05 % $SnCl_2$ and 0.2 % hydrazine sulfate in 0.6 M H_2SO_4) were added with mixing in between. The color was measured after 20 min at 680 nm.

In absence of Na^+ and K^+, the activity was very low, maximally about 10 % of the Na^+–K^+-stimulated activity. Furthermore, this activity did not change upon manipulations used in this study. Consequently it has been omitted from results.

Phospholipids were measured by the method of Bartlett[7], as modified by Kankare and Suovaniemi[8] and cholesterol according to Pearson *et al.*[9]. The method of Lowry *et al.*[10] was used for protein determination.

TABLE I

EXTRACTION OF $(Na^+–K^+)$-ATPase WITH HEXANE AND ETHANOL–HEXANE. RECOMBINATION WITH CHOLESTEROL AND PHOSPHOLIPIDS

Expt.	*Tube No.*	*Extraction*	*Lipid added*	*Activity (μmoles/min per mg)*	*Relative composition*			*Uptake of added lipid (μg)*
					Protein	*PL*	*Chol.*	
A	1	—	—	9.1	10	5.7	2.0	—
	2		—	6.8	10	4.2	0.12	—
	3	Hexane	Chol. 100 μg	10.7	10	3.6	0.20	6 (Chol.)
	4		Chol. 200 μg	8.9	10	3.5	0.21	10 (Chol.)
	5		Chol. 400 μg	10.5	10	3.0	0.39	26 (Chol.)
	6		C 250 μg	8.8	10	3.1	0.28	16 (Chol.)
B	1	Hexane	—	6.3	10	—	0.10	—
	2		—	1.5	10	—	0.10	—
	3	Ethanol–hexane	Chol. 100 μg	2.8	10	—	0.20	13 (Chol.)
	4		Chol. 200 μg	3.2	10	—	0.31	28 (Chol.)
	5		Chol. 500 μg	3.4	10	—	0.41	46 (Chol.)
	6		C 200 μg	3.2	10	—	0.22	19 (Chol.)
C	1	Hexane	—	5.6	10	3.6	0.10	—
	2		—	0.97	10	1.5	0.10	—
	3	Ethanol–hexane	PL 1.3 mg	1.6	10	2.4	0.09	133 (PL)
	4		PL 2.6 mg	1.3	10	3.1	0.09	285 (PL)
	5		PL 5.2 mg	1.7	10	3.3	0.07	359 (PL)
	6		Chol. 400 μg	2.1	10	1.4	0.51	57 (Chol.)
	7		C 250 μg	2.4	10	1.6	0.30	31 (Chol.)
D	1	Hexane	—	5.7	10	4.7	—	—
	2		—	0.80	10	1.7	—	—
	3	Ethanol–hexane	C 200 μg	2.7	10	2.3	—	—
	4		PL 0.5 mg	0.33	10	2.2	—	129 (PL)
	5		C 200 μg + PL 0.5 mg	1.8	10	2.6	—	134 (PL)
	6		TL 2.5 mg	2.0	10	2.6	—	170 (PL)

Abbreviations: Chol., cholesterol; C, cholesterol fraction; PL, phospholipid fraction; TL, total lipid extract, unfractionated.

RESULTS

Hexane extraction (Table I) removes only a small proportion of the phospholipids (about 25 %), while most of the cholesterol is lost (about 95 %). Simultaneously the ATPase activity is reduced by some 20–30 %.

If the hexane-extracted particles are incubated in a solution of commercial cholesterol or the "C" fraction in hexane at low temperature (—20°), the preparation takes up a certain amount of cholesterol. At the same time the ATPase activity is also increased. The "uptake" of cholesterol is concentration-dependent. About 5–7 % of the cholesterol present in the reincubation mixture is taken up. The situation obtained is to be expected, if the association between particles and cholesterol is freely reversible. No signs of saturation are seen at the concentrations used, these being limited by the poor solubility of cholesterol in cold hexane. The ATPase activities are restored to the pre-extraction level.

Ethanol–hexane extraction caused a further, and drastic (about 80 %) drop in activity. Simultaneously, a large proportion, some 60 % of the remaining phospholipids are lost, as Exp. C, 6 and 7 show. No further cholesterol is removed by this treatment (Expts. B 2 and C 2). In recombination experiments restoration of activity was observed with cholesterol, or a cholesterol-containing fraction of the extracted lipids. The ethanol–hexane-extracted particles take up somewhat more cholesterol than the hexane-extracted particles (10–14 %). The activity was restored to a level approx. 40–50 % of the activity after hexane extraction, or about 30 % of the original. In absolute numbers, the reactivation by cholesterol of the ethanol–hexane-extracted particles is only about half of that seen in the hexane-extracted ones. It can not be excluded that a certain unspecific denaturation of the enzyme may have occurred during the ethanol–hexane extraction, since the total activity obtained after reactivation was also considerably lower than the original.

Recombination experiments with the phospholipid fraction led to a marked uptake of added phospholipids, restoring the relative composition before ethanol–hexane extraction. Despite this massive uptake, the ATPase activity was increased less than with cholesterol (Expt. C, 1–5), and was in some cases even less than in the control (Expt. D, 2 and 4). Again, assuming free reversibility of the phospholipid–protein association, uptake would be likely to occur, even if the solvent had remained the same. About 10 % of the added phospholipid was taken up, in a concentration dependent manner, but apparently, the particles were almost saturated with phospholipid at the highest level used.

Expt. D shows an attempt to reactivate the ATPase by using cholesterol and phospholipid simultaneously. As a matter of fact the simultaneous application of cholesterol and phospholipids resulted in somewhat lower activities than with cholesterol alone.

DISCUSSION

No major loss of (Na^+–K^+)-ATPase activity occurs when the lyophilized enzyme is subjected to extraction with hexane in absence of water. Simultaneously almost all the cholesterol in the particles is removed. Extraction with a more hydrophilic solvent, *i.e.* 5 % ethanol in hexane drastically reduces the activity, and removes a sizable proportion of phospholipids.

In recombination experiments, some rebinding of cholesterol occurs, as well as partial reactivation of the ATPase. Phospholipids are taken up to a high extent, but enzymic reactivation remains on a level below that seen with cholesterol. A somewhat paradoxical situation arises. Removal of the cholesterol does not remove activity, whereas stripping the particle of its phospholipids kills the activity. Recombination with phospholipids is less effective in restoring activity than cholesterol in particles from which the major portion of both lipid classes has been removed.

Some studies by other workers have revealed results of interest in relation to the above mentioned. Roelofsen *et al.*[2] extracted red cell ghosts, and found that removal of the loosely bound lipids did not reduce the ATPase activities, whereas extraction with ethanol–ether caused considerable loss of activity. Their conclusion was that cholesterol did not play any major role in providing the critical organization of the lipoprotein enzyme. However, they did not report any reconstruction attempts. In the hands of Zamudio *et al.*[3] anhydrous pentane extraction of erythrocyte membranes did not reduce the activity of either (Na^+–K^+)-ATPase or NAD dehydrogenase, whereas extraction with more polar solvents inactivated the enzymes. The recent observation by Noguchi and Freed[11] that extraction of frozen brain tissue with chloroform–methanol at low temperature abolishes the (Na^+–K^+)-ATPase and permits recombination experiments, is very similar to the results reported above. Reactivation by cholesterol, but not by phospholipids, was also found by Noguchi and Freed[11].

Thus it seems inevitable that the conclusions reached by a number of workers[2,4,12,13], that phospholipids play a central and possibly very specific role in the activity of the (Na^+–K^+)-ATPase, have to be modified somewhat. Cholesterol seems to be a component that is required for full activity at least under certain conditions. Noguchi and Freed[11] did not report the lipid contents of their residual particles, and a direct comparison is therefore not possible. A striking feature in our experiments is, in good accordance with results of Roelofsen *et al.*[2] and Zamudio *et al.*[3], the fact that almost complete removal of cholesterol does not remove activity. This extraction step therefore seems to leave the original lipoprotein structure, or those parts of it which are critical for enzyme activity fairly intact. Subsequent removal of the major part of the phospholipids causes this structure to break down, and as a consequence, the activity is lost. Cholesterol then seems to have a capability partially to reorganize the remaining structure, possibly using the proteins and the phospholipids left behind as a framework. The removal of phospholipids does not seem to be reversible in the same degree. Phospholipids can be added back and are taken up by the particles, but are less effective than cholesterol in reactivating the enzyme. Thus it appears that the original lipoprotein structure, on which enzymic activity is dependent, can be reversibly altered by extraction of lipids under carefully controlled conditions.

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