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BIOCHEMICAL CHARACTERIZATION OF THE SUBMICROSOMAL MEMBRANE OF THE RAT BRAIN

SELECTIVE SOLUBILIZATION OF THE COMPONENTS OF THE LIGHT SMOOTH-SURFACED MEMBRANE BY LYSOPHOSPHATIDYLCHOLINE

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Summary

The light smooth-surfaced membrane, one of the three membrane fractions derived from the rat brain microsomal fraction, was fractionated into its soluble and insoluble parts by the use of lysophosphatidylcholine and the chemical composition of these was investigated.

Under the condition whereby the maximal amount of the membrane protein was solubilized by lysophosphatidylcholine (0.5% lysophosphatidylcholine at 37°C for 10 min), the insoluble residue, which accounted for approximately 30% of the membrane protein, was ultracentrifugally homogeneous and showed a granular structure under the electron microscope.

The lipid composition of the soluble and insoluble fractions, as well as their protein composition, revealed a preferential and limited solubilization of the constituents of the membrane by lysophosphatidylcholine.

Introduction

In our previous work [1] three membranes were isolated from the microsomal fraction of the rat brain. They were distinct from each other in terms of their buoyant densities, ultrastructures, chemical compositions and enzymatic activities. The galactolipid and ganglioside content of these membranes is one of the most prominent features which differentiated these membranes chemically.

Abbreviations: SDS, sodium dodecyl sulfate; AcNeu, *N*-acetyl neuraminic acid.

Neurobiologically, it may be worthwhile to investigate the molecular topology of these smooth membranes and to establish unequivocally their cellular and subcellular localization in mammalian nervous tissue.

In the present experiment we searched for a condition whereby reproducible and maximal solubilization of protein and endogenous phospholipid of the light smooth-surfaced membrane, which contained both galactolipid and ganglioside, could be accomplished. Under this condition, an ultracentrifugally homogeneous insoluble residue was obtained and then its chemical composition, in comparison with that of the lysophosphatidylcholine-soluble fraction, was analysed.

Materials and Methods

Male Wistar rats weighing approx. 200 g were used. Lysophosphatidylcholine, which contained primarily palmitic and stearic acids, was purchased from Sigma, and was ascertained to give a single spot upon thin layer chromatography.

Crude light smooth-surfaced membranes, separated from the microsomal fraction of the whole brain of rats as described in the previous paper [1], was homogenized in distilled water with a Teflon-glass homogenizer and layered on a linear 25—45% sucrose gradient made over 3 ml of 60% sucrose and centrifuged in a swinging rotor at 67 000 × *g* for 16 h. The density gradient profile was analysed by a LKB-Uvicord III 2089. The main band at the 1.08 M sucrose concentration (Fig. 1), which occupied about 70% of the total absorption at 280 nm and was recognized as a whitish cloudy band in the cellulose tube, was collected by a Pasteur pipette and diluted with 3 vols. of cold water. It was then collected as a pellet after centrifugation at 100 000 × *g* for 90 min. After centrifugal analysis on a sucrose density gradient, this purified membrane fraction, designated as the light smooth-surfaced membrane, was revealed to be pure enough to be used for the lysophosphatidylcholine treatment (Fig. 2a).

Before the analysis the membrane fractions were dialyzed against distilled

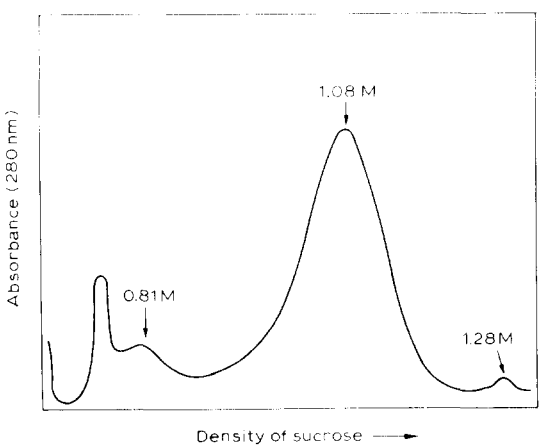

Fig. 1. Linear sucrose density gradient (from 25 to 45%) centrifugal profile of the crude light smooth-surfaced membrane. The figures on the peaks indicate molar concentration of sucrose.

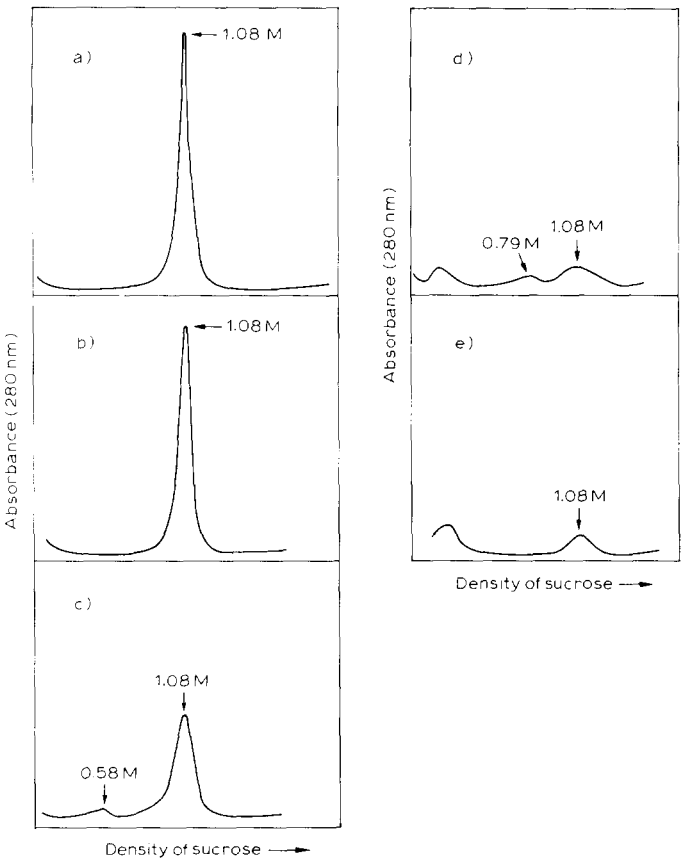

Fig. 2. Linear sucrose density gradient centrifugation profiles of the light smooth-surfaced membrane treated with various concentrations of lysophosphatidylcholine. Thoroughly homogenized light smooth-surfaced membrane, 1 mg as protein in a final volume of 1 ml, was incubated with sucrose at a final concentration of 0.23 M and lysophosphatidylcholine at final concentrations of (a) zero, (b) 0.05%, (c) 0.1%, (d) 0.2% and (e) 0.5%. After incubation at 37°C for 10 min, the reaction mixture was layered on a linear sucrose gradient, 10—45%, and centrifuged at 67 000 X *g* for 16 h and analysed by LKB-Uvicord III at 280 nm.

water for 3 days to remove the contaminating sucrose, and lyophilized. The lipid was extracted with 20 vols. of chloroform/methanol (2 : 1, v/v) and partitioned into the upper and the lower phases as described by Folch et al. [2] and analysed as described in the previous paper [1]. Protein content was determined by the method of Lowry et al. [3].

For SDS polyacrylamide gel electrophoresis, the light smooth-surfaced membrane and the lysophosphatidylcholine-insoluble residue were dissolved in the SDS sample buffer of Laemmli [4] by warming the solution at 95°C for 3—4 min. The lysophosphatidylcholine-soluble fraction was precipitated in 5% trichloroacetic acid, washed several times with ether and dissolved in the SDS sample buffer. Vertical slab gel electrophoresis was carried out on polyacrylamide gel of linear concentration from 7.5 to 15% by the method of Laemmli [4]. Gels were stained with Coomassie Blue.

Pellets of the membrane fractions were fixed for 5 h in 1% OsO_4 in Millonig's

phosphate buffer at pH 7.3 in the cold, dehydrated by ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and observed under a Hitachi HU-11B electron microscope.

Results

Lysophosphatidylcholine treatment of the light smooth-surfaced membrane

a. Concentration of lysophosphatidylcholine. 1 ml of the incubation mixture, which contained 1 mg protein equivalent of the light smooth-surfaced membrane, variable amounts of lysophosphatidylcholine and 0.23 mmol of sucrose was incubated at 37° C for 10 min under shaking. After the incubation, the mixture was diluted with an equal volume of cold distilled water, then layered on 2 ml of 0.5 M sucrose and centrifuged at 100 000 × *g* for 90 min. The pellet and the supernatant thus obtained were analysed as described in Materials and Methods. A constant amount of protein, which was about 28% of the light smooth-surfaced membrane, was recovered in the pellet at lysophosphatidylcholine concentrations above 0.2%. Also, at any lysophosphatidylcholine concentraion higher than 0.2%, the ratio of phospholipid to protein was constant (Fig. 3). These results seem to indicate completion of the solubilizing reaction at lysophosphatidylcholine concentrations above 0.2% and formation of a new, stable and precipitable component. The incubation mixture was also analysed for its ultracentrifugal pattern by linear 10—45% sucrose gradient centrifugation at 67 000 × *g* for 16 h (Fig. 2). Absorbance at 1.08 M sucrose at low concentration of lysophosphatidylcholine (Fig. 2a—c) seemed to be a sum of the absorbance due to turbidity by the membrane vesicle and true ultraviolet absorbance by the membrane protein. This main peak decreased as the lysophosphatidylcholine concentration increased. Minor peaks observed at 0.1%

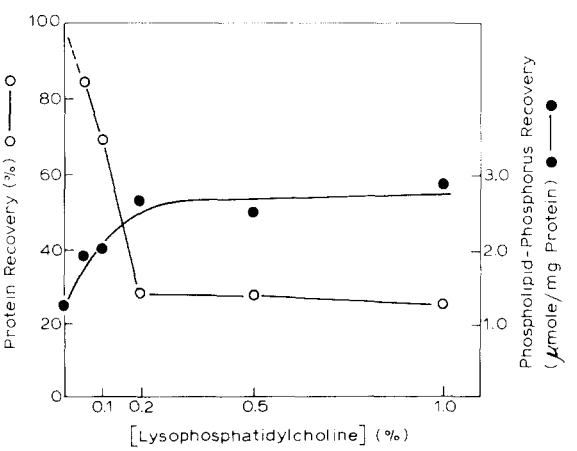

Fig. 3. Effect of lysophosphatidylcholine concentration on the solubilization of the light smooth-surfaced membrane. 1 ml of the reaction mixture consisted of thoroughly homogenized light smooth-surfaced membrane (1 mg as protein), sucrose at 0.23 M and lysophosphatidylcholine at 0.05—1.0%. After the incubation at 37°C for 10 min, the insoluble residue was separated and analyzed as described in the text. ○, Recovery of protein into the lysophosphatidylcholine-insoluble residue (%) left-hand ordinate; ●, recovery of the phospholipid into the lysophosphatidylcholine-insoluble residue (μmol phospholipid phosphorus per mg of protein), right-hand ordinate.

and 0.2% lysophosphatidylcholine (Fig. 2c and d) disappeared at 0.5% lysophosphatidylcholine and only two peaks were observed at the density of 1.08 M sucrose and at between 0.5 M sucrose and the sample solution. From these results, the minimum concentration of lysophosphatidylcholine required for the maximum solubilization of the light smooth-surfaced membrane was determined to be between 0.2% and 0.5% under the present experimental conditions.

b. Incubation time. At lysophosphatidylcholine concentration of 0.5% the recovery of protein and phospholipid phosphorus in the insoluble residue decreased with incubation time and became nearly constant after 10 min of incubation.

Based on the results described above, we prepared the lysophosphatidylcholine-soluble and the lysophosphatidylcholine-insoluble fractions as follows. 1 ml of the reaction mixture containing thoroughly dispersed light smooth-surfaced membrane, 1 mg as protein, sucrose at a final concentration of 0.23 M and freshly dissolved lysophosphatidylcholine at a final concentration of 0.5%, was incubated at 37° C. After 10 min, an equal volume of cold water was added to the reaction mixture and layered over 2 ml of 0.5 M sucrose, and then centrifuged at 100 000 × *g* for 90 min. Supernatant and the pellet designated as the "lysophosphatidylcholine-soluble fraction" and the "lysophosphatidylcholine-insoluble fraction" respectively, or "lysophosphatidylcholine-insoluble residue", were collected separately. The lysophosphatidylcholine-insoluble fraction was washed twice with cold water to remove the possible contamination by lysophosphatidylcholine, employing homogenization followed by centrifugation at 100 000 × *g* for 90 min.

SDS gel electrophoretic patterns of lysophosphatidylcholine-soluble and -insoluble fractions (Fig. 4)

Almost all of the light smooth-surfaced membrane proteins corresponding to bands a, d, f, h, i, j, k and l and most of those corresponding to e and g seemed to be solubilized by lysophosphatidylcholine. In contrast to these proteins, proteins corresponding to bands b and c remained preferentially insoluble. Furthermore, a large quantitative difference was recognizable between some of the corresponding protein bands of the lysophosphatidylcholine-soluble and -insoluble fractions.

Electron micrographs of lysophosphatidylcholine-insoluble fraction (Fig. 5)

The light smooth-surfaced membrane is fairly homogeneous compared to the unpurified light smooth-surfaced membrane [1] and showed vesicular and trilamellar membrane structures (Fig. 5a), while the lysophosphatidylcholine-insoluble fractions displayed granular structures (Fig. 5b).

Lipid composition of lysophosphatidylcholine-soluble and -insoluble fractions (Tables I and II)

Per protein, the lysophosphatidylcholine-soluble fraction contained as much as five times as much endogenous phospholipid (total phospholipid minus lysophosphatidylcholine), three times as much ganglioside, approx. twice as much cholesterol and one-half as much cerebroside as compared to the lysophosphatidylcholine-insoluble fraction. Furthermore, the lysophosphatidylcholine-insol-

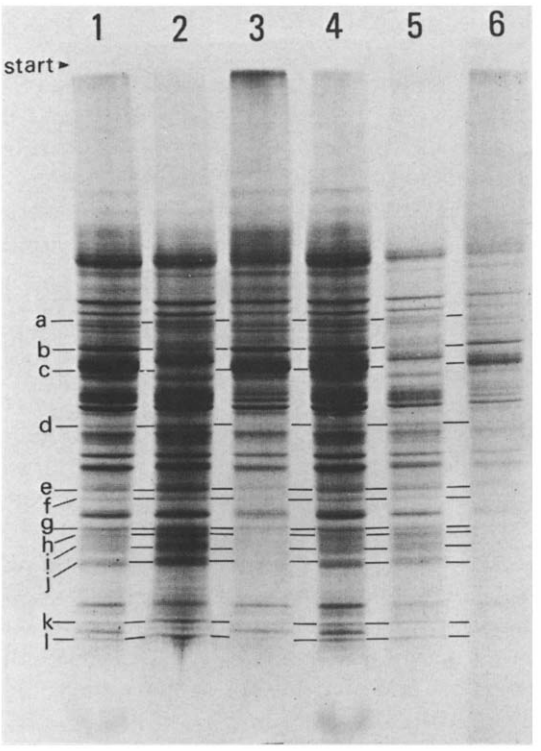

Fig. 4. SDS polyacrylamide gel electrophoresis of the light smooth-surfaced membrane and of lysophosphatidylcholine-soluble and insoluble fractions. 1 and 4, light smooth-surfaced membrane, approx. 50 µg protein; 2, lysophosphatidylcholine-soluble fraction, approx. 50 µg protein; 3, lysophosphatidylcholine-insoluble fraction approx. 50 µg protein; 5, lysophosphatidylcholine-soluble fraction, approx. 35 µg protein; 6, lysophosphatidylcholine-insoluble fraction, approx. 15 µg protein. Protein quantities applied on lanes 5 and 6 were the amounts of protein recovered in the fractions when the light smooth-surfaced membrane equivalent to 50 µg protein was solubilized by lysophosphatidylcholine. Electrophoresis was run in a slab of 7.5—15% linear concentration gradient of acrylamide in the presence of 0.1% SDS at pH 8.8 (0.375 M Tris · HCl buffer). Letter indications, see text.

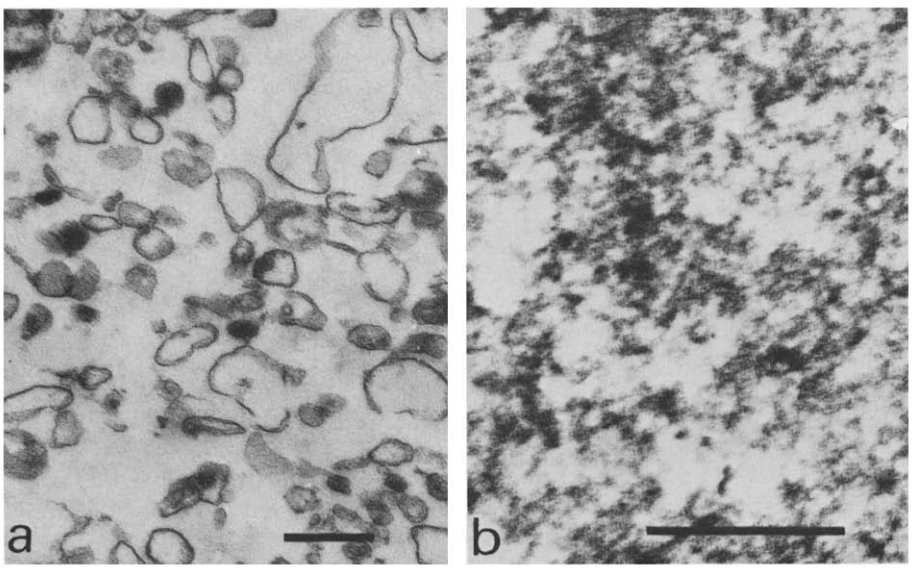

Fig. 5. Electron micrographs of the light smooth-surfaced membrane and lysophosphatidylcholine-insoluble residue. The specimens were fixed with OsO_4 and processed as described in the text. a. Light smooth-surfaced membrane. b. Lysophosphatidylcholine-insoluble residue. Scale marks, 0.5 µm.

TABLE I

PHOSPHOLIPID, CHOLESTEROL, LIPID-AcNeu, AND CEREBROSIDE CONTENTS PER PROTEIN OF SUBFRACTIONS OF LIGHT SMOOTH-SURFACED MEMBRANE ISOLATED FROM RAT MICROSOMAL FRACTION

The light smooth-surfaced membrane, lysophosphatidylcholine-insoluble and -soluble fractions were prepared as described in the text. Data are means of two or three separate experiments.

	Phospholipid (μmol/mg protein)			Cholesterol (μmol/mg protein)	Lipid-AcNeu (nmol/mg protein)	Cerebroside (nmol/mg protein)
	Total	Endogenous	Lysophosphatidylcholine			
Light smooth-surfaced membrane	0.99	0.99	0	0.85	40.8	68.0
Lysophosphatidylcholine-insoluble fraction	1.79	0.29	1.50	0.48	12.7	94.6
Lysophosphatidylcholine-soluble fraction	10.33	1.33	9.00	0.88	40.1	41.0

TABLE II

PERCENTAGE COMPOSITION OF PHOSPHOLIPIDS OF THE LIGHT SMOOTH-SURFACED MEMBRANE AND ITS LYOSPHOSPHATIDYLCHOLINE-INSOLUBLE AND -SOLUBLE FRACTIONS

Values (percentages to the endogenous phospholipid) are expressed as mean ± S.D. (four experiments). PE, phosphatidylethanolamine; PS + PI, phosphatidylserine and phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin.

	PE	PS + PI	PC	SM
Light smooth-surfaced membrane	23.7 ± 3.7	11.8 ± 1.1	53.8 ± 2.9	9.1 ± 1.1
Lysophosphatidylcholine-insoluble fraction	20.9 ± 4.4	15.1 ± 2.4	53.0 ± 5.8	11.1 ± 1.5
Lysophosphatidylcholine-soluble fraction	13.5 ± 2.1	10.1 ± 0.59	62.0 ± 2.0	14.4 ± 2.36

uble fraction contained phosphatidylethanolamine and phosphatidylserine plus phosphatidylinositol at higher percentage and phosphatidylcholine and sphingomyelin at lower percentage than the lysophosphatidylcholine-soluble fraction. Moreover, it is evident that a fairly large amount of lysophosphatidylcholine, which could not be removed by repeated washing with water, was incorporated into the lysophosphatidylcholine-insoluble fraction.

Discussion

Formerly, Wiegandt [5] indicated the heterogeneity of the brain microsome in respect to the distribution of gangliosides. We succeeded in subfractionating the microsomal membrane fraction of the rat brain. Each of the subfractions have characteristic glycolipid distribution and enzyme activity [1]. Their cellular and subcellular origin or origins have not yet been elucidated. The aim of our present experiment was to subfractionate further one of them, the light smooth-surfaced membrane which was designated as the light smooth-surfaced microsomal membrane in our previous paper [1].

Under the conditions which we had used, namely 1 ml of the reaction mixture (which contained 1 mg of the light smooth-surfaced membrane as protein), lysophosphatidylcholine at 0.5% and sucrose at 0.23 M, incubated at 37°C for 10 min, the maximum amounts of protein and phospholipid were solubilized and a precipitable residue having a constant ratio of phospholipid to protein was obtained.

Rather marked biochemical differences between the phosphatidylcholine-insoluble fraction and the soluble fraction indicate preferential solubilization of the components of the light smooth-surfaced membrane. Based on the figures in Tables I and II and the percentage of recovery of the protein in the lysophosphatidylcholine-insoluble residue, it is calculated that more than 90% of the ganglioside, about 50% of the cerebroside and about 90% of the cholesterol in the light smooth-surfaced membrane were solubilized by the detergent. In addition, lysophosphatidylcholine appeared to solubilize membranes rich in choline containing phospholipids more effectively than those rich in other kinds of phospholipids. In contrast to the solubilization of these lipids, lysophosphatidylcholine was incorporated into the insoluble residue. The incorporated lysophosphatidylcholine could not be removed by repeated washing and it amounted to approx. 80% of the phospholipid phosphorus and 60% of the total lipid

content of this insoluble fraction. Consequently, the lipid to protein ratio of the insoluble residue is almost the same as that of the light smooth-surfaced membrane, and this may account for the buoyant density, which was the same in both particles. Incorporation of lysophosphatidylcholine which was used for the isolation of ATPase from lobster muscle microsomes, into the insoluble enzyme complex has also been reported [6].

Our lysophosphatidylcholine-insoluble residue and the Triton X-100 insoluble residue of erythrocyte ghosts [7] closely resembled each other in their fine granular electron-microscopical images, though the lipid content of these residues was rather different.

Characterization of the intermediate fractions, as revealed in Fig. 2, may give further clues towards elucidating the chemical structure and biological significance of the light smooth-surfaced membrane.

Acknowledgement

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