Biochimica et Biophysica Acta, 595 (1980) 244—256

BBA 78576

SPONTANEOUS AND DETERGENT-INDUCED VESICULATION OF THYMOCYTE PLASMA MEMBRANES

ERNST FERBER, BRIGITTE SCHMIDT and HANS U. WELTZIEN
Max-Planck-Institut für Immunbiologie, Freiburg (F.R.G.)
(Received June 7th, 1979)

Key words: Membrane vesiculation; Plasma membrane; Detergent; Thymocyte

Summary

An analog of lysophosphatidylcholine (1-dodecyl-propanediol-3-phosphocholine) which does not impair membrane-bound enzymes was used for the induction of shedding of membrane vesicles from intact calf thymocytes. Without liberation of intracellular enzymes such as lactate dehydrogenase (EC 1.1.1.27) the shedded membranes contained 15—25% of the total activity of the plasma membrane enzymes alkaline phosphatase (EC 3.1.3.1), nucleotide pyrophosphatase (EC 3.1.4.1) and γ-glutamyl transferase (EC 2.3.2.2). Membrane-free supernatants only exhibited trace activities of these enzymes. Without further purification, the specific enzyme activities in shedded membranes were of the same order of magnitude as in purified plasma membranes prepared after nitrogen cavitation of thymocytes.

Small amounts of membrane vesicles which showed a different composition could be removed without detergent. These membranes exhibited a 3-fold lower specific activity of the γ-glutamyl transferase while that of the alkaline phosphatase and nucleotide pyrophosphatase was similar as in detergent induced membrane vesicles. Distinct differences also were found in the protein pattern. The content of total cholesterol and phospholipid in vesicles shed spontaneously or after detergent treatment was nearly identical, however, significant differences were found in the fatty acid composition of the main phospholipids. The content of polyunsaturated fatty acids (linoleic and arachidonic acid) increased in the order: spontaneously shedded membranes, detergent induced vesicles, conventional purified plasma membranes. These results are discussed in terms of the heterogeneous composition of areas of the thymocyte plasma membrane.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; ET-12-H, 1-dodecyl-propanediol-3-phosphocholine.

Introduction

There is accumulating evidence that under physiological conditions certain components are spontaneously released from the outer cell membrane (for review see [1]). Thus macromolecules such as surface immunoglobulins [2] or receptors [3] could be obtained from the medium of cultured lymphocytes. However, there has also been some indication that the macromolecules are not released as defined soluble entities but together with fragments of the membrane [2]. Spontaneous release of complete membrane vesicles was observed from normal and tumor cells [4—6].

In many of these studies it was difficult to exclude that the material was released from dying or dead cells, in particular, in cases where biological fluids were used as a source of the shedded membrane vesicles. The extent of vesiculation can be increased by chemical agents such as formaldehyde [7] or detergents [8]. However, there is evidence that this type of vesiculation often is a response of the cell to injury [9]. Therefore, the aim of this study was to analyze in detail the function and structure of membrane fragments obtained from thymocytes treated with a detergent which is known not to impair sensitive membrane structures [10]. Emphasis was placed on the strict control of sublytic conditions. A comparison of shedded membranes with normal purified plasma membranes should reveal whether all characteristics of plasma membranes such as marker enzymes and lipid composition are identical or differ in the shedded vesicles, which would indicate a heterogeneous composition of the outer cell membrane.

Materials and Methods

Chemicals

1-Dodecyl-propanediol-3-phosphocholine (ET-12-H) was synthesized as described previously [11,12]. Hepes was obtained from Serva, Heidelberg, F.R.G. All other chemicals were analytical grade reagents.

Preparation of membrane vesicles from intact thymocytes

Calf thymus cells were prepared as described previously [13]. Suspensions of thymocytes were prepared at a concentration of $1.5 \cdot 10^8$ cells/ml in phosphate-buffered saline and incubated for 10 min, 37°C with and without detergents. The standard preparation was carried out using 100 ml cell suspension ($1.5 \cdot 10^{10}$ cells). After incubation, the cell suspension was centrifuged at $250 \times g_{av}$, 5 min, 20°C, and two thirds (66 ml) of the supernatant was saved. The remaining supernatant and cells were discarded. In order to remove any contaminating intact and broken cells this supernatant was centrifuged at $9000 \times g_{av}$, 2 min (10 000 rev./min, rotor JA-20, Beckman). Finally, the cell-free supernatant (55 ml) was centrifuged at $228\,000 \times g_{av}$, 60 min (50 000 rev./min, rotor 50.2 Ti, Beckman). The membrane-free supernatant (48 ml) was collected and the membraneous pellet was resuspended in 1.0 ml of 10 mM Hepes buffer, pH 7.2.

Preparation of thymocyte plasma membranes

Plasma membranes from calf thymus cells were prepared by the nitrogen

cavitation method as described previously [14] with the modifications introduced later [15].

Enzyme assays

Alkaline phosphatase (EC 3.1.3.1): The activity of the alkaline phosphatase was followed with *p*-nitrophenylphosphate as substrate. The reaction mixture contained in a total volume of 1.0 ml, 100 μmol diethanolamine, pH 9.5, 1.0 μmol $MgCl_2$, 5 mg Triton X-100, 2.5 μmol *p*-nitrophenylphosphate and 3—10 μg membrane protein. After 10 min incubation at 37°C the reaction was stopped by addition of 0.2 ml 5 M NaOH. Liberated nitrophenol in supernatants was measured at 405 nm and calculated using a molar extinction coefficient of 17 840 $l \cdot mol^{-1} \cdot cm^{-1}$. We determined a Michaelis constant for the alkaline phosphatase in plasma membranes from calf thymus to be K_m = 75 μM.

Nucleotide pyrophosphatase (EC 3.1.4.1): The determination of the activity of the nucleotide pyrophosphatase was carried out under identical conditions as described for the alkaline phosphatase with the exception that *p*-nitrophenyl-5'-thymidylate (3.0 mM) was used as substrate. We determined a Michaelis constant for the nucleotide pyrophosphatase in plasma membranes from calf thymocytes to be K_m = 1.2 mM.

γ-Glutamyl transferase (EC 2.3.2.2): The activity of γ-glutamyl transferase was determined according to Novogrodsky et al. [16] using 1-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine (Boehringer, Mannheim, F.R.G.) as substrates. Trichloroacetic acid (10%) was used instead of acetic acid to stop the reaction.

(Mg^{2+} + Na^+ + K^+)-ATPase (EC 3.6.1.3): ATPase activity was assayed using a modification of the technique of Crane and Lipmann [17] using γ-^{32}P-ATP and adsorption of nucleotides on activated charcoal. The reaction mixture contained in a total volume of 1.0 ml: 100 μmol imidazole buffer pH 7.4, 5 μmol $MgCl_2$, 30 μmol KCl, 150 μmol NaCl, 600 nmol ATP containing 0.4 μCi γ-^{32}P-ATP and 10—40 μg of membrane protein. After an incubation time of 10—30 min at 37°C, 2 mg of bovine serum albumin was added to fascilitate membrane protein precipitation, and the reaction was stopped by addition of 4 ml 5% (w/v) icecold trichloroacetic acid. The extract was centrifuged, and 3 ml of the supernatant were adsorbed on approximately 2 g of trichloroacetic acid-washed activated charcoal (Norit A, Serva, Heidelberg). After centrifugation 1 ml extract was mixed with 10 ml Aquasol (New England Nuclear) and counted in a Packard Tricarb scintillation counter.

Acyl-CoA:1-acylglycero-3-phosphocholine O-acyltransferase (EC 2.3.1.23): The activity of the acyltransferase was determined by reaction of arachidonyl-CoA with ^{14}C-labelled lysophosphatidylcholine (1-[1'-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine) as described earlier [18].

Succinate dehydrogenase (EC 1.3.99.1): The activity of the succinate dehydrogenase was determined using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride as electron acceptor according to Pennington [19].

β-N-Acetylglucosaminidase (EC 3.2.1.30): The acetylglucosaminidase was determined using a modification of the techniques of Sellinger et al. [20] and Kornfeld and Siemers [21]. The reaction mixture contained in a total volume

of 1.0 ml, 100 μmol sodium citrate, pH 4.6, 5.0 μmol *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and 5—20 μg membrane protein. After 30 min incubation at 37°C the reaction was stopped by addition of 0.2 ml 1 M NaOH. Liberated nitrophenol in supernatants was measured at 405 nm and calculated using a molar extinction coefficient of 17 840 $l \cdot mol^{-1} \cdot cm^{-1}$.

Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Wróblewski and LaDue [22].

Lipid extraction and analysis

Lipids were extracted from membrane suspensions by a modification of the method of Ways and Hanahan [23]. 1.0 ml of membrane suspension containing 0.5—1.5 mg protein was extracted with 5 ml methanol and 5 ml chloroform. 1.0 mg butylated hydroxytoluene was added as antioxydant. The precipitate was re-extracted with 2 ml methanol and 5 ml chloroform. The combined extracts were washed with 3 ml of 0.1 M KCl followed by washing with 5 ml methanol + 3 ml 0.1 M KCl and the chloroform layer was taken to dryness under nitrogen.

The separation of lipids was carried out by thin-layer chromatography on 0.75-mm silica plates (20 × 16 cm, Kieselgel H, Merck, Darmstadt) containing 0.07% (w/v) Tylose 10 000 P (Farbwerke Hoechst) which were developed according to Wood and Harlow [24] in chloroform/methanol/acetic acid/isotonic saline (50 : 25 : 8 : 4, v/v). The lipids were visualized with ultraviolet light after staining with rhodamin 6 G (0.01% in water).

The fatty acid composition of phospholipids was determined by gas-liquid chromatography as described previously [25]. Total cholesterol in purified lipid extracts was determined enzymatically using cholesterol oxidase according to the method of Röschlau et al. [26] with a Biochemica Test Combination Kit (Boehringer, Mannheim). For this, aliquots of the dried lipid extract were dissolved in 20 μl acetone.

The phospholipid content was measured by phosphorus determination in purified lipid extracts according to the method of Lowry et al. [27] and on silica after thin-layer chromatography according to the method of Gerlach and Deuticke [28].

Protein was determined by the ninhydrin method according to Moore and Stein [29].

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out using commercially available gradient gels (Pharmacia, Frankfurt, F.R.G.) ranging from 4 to 30% polyacrylamide. 30 μg of membrane protein were applied on each gel. Solubilization, buffer composition, electrophoretic conditions, gel calibration and the staining procedure was identical with the methods described by Fairbanks et al. [30] with the exception that for complete solubilization the material was heated for 3 min at 100°C.

Results

Membrane-bound enzymes

Holdsworth and Coleman [31] using intact pig lymph-node cells reported

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES IN PELLETS AND SUPERNATANTS OF EXTRACTS PREPARED FROM INTACT CALF THYMOCYTES

Total protein and activities refer to $1.5 \cdot 10^{10}$ cells. Specific activities are expressed as nmol · mg^{-1} · min^{-1}, total activities as percentage of the sum of total activities of supernatants and pellets (alkaline phosphatase and γ-glutamyl transferase) and for lactate dehydrogenase as percentage of the homogenate. In homogenate the specific activity of lactate dehydrogenase was determined to be 270.2 nmol · mg^{-1} · min^{-1} and the total activity 123.01 μmol · min^{-1}.

	Protein		Alkaline phosphatase		γ-Glutamyl transferase		Lactate dehydrogenase	
	Total (mg)	%	Specific activity	% total activity	Specific activity	% total activity	Specific activity	% total activity
Glycocholate (214 nmol/ml)								
Supernatant	29.4	96.5	0.78	1.6	0.07	17.9	127.0	3.0
Pellet	1.07	3.5	1312.0	98.4	8.5	82.1	—	—
Glycocholate (536 nmol/ml)								
Supernatant	29.0	95.9	0.86	1.2	0.10	14.6	134.0	3.1
Pellet	1.24	4.1	1614.0	98.8	14.2	85.4	—	—
Glycocholate (1072 nmol/ml)								
Supernatant	43.4	94.5	0.64	1.7	0.11	8.9	241.0	8.5
Pellet	2.54	5.5	643.0	98.3	18.5	91.1	—	—
ET-12-H (20 nmol/ml)								
Supernatant	25.9	95.6	0.91	1.6	0.10	10.5	167.0	3.5
Pellet	1.18	4.4	1212.0	98.4	19.1	89.5	—	—
ET-12-H (40 nmol/ml)								
Supernatant	29.0	94.8	0.95	1.4	0.10	5.4	184.0	4.3
Pellet	1.60	5.2	1200.0	98.6	32.5	94.6	—	—

that sublytic concentrations of glycocholate and taurocholate can solubilize up to 20% of the total activities of plasma membrane-bound alkaline phosphatase, nucleotide pyrophosphatase and 5'-nucleotidase. The first series of experiments were carried out to adapt this method to calf thymus cells and to extend it to other detergents. Appreciably amounts of these plasma membrane marker enzymes can also be removed from calf thymocytes. However, as shown in Table I, the activities were not found in the soluble supernatant but upt to 90% in the membraneous pellet. Thus, obviously these membrane fragments are removed by a shedding process.

From previous studies [10] it was known that short chain analogs of lysophosphatidylcholine had certain advantages for the solubilization of membrane-bound enzymes. One of these compounds (ET-12-H) which exhibited the highest potential for the solubilization without affecting sensitive membrane-bound enzymes was compared with glycocholate. Under sublytic conditions ET-12-H also removed parts of the membrane which contain enzyme activities of the plasma membrane (alkaline phosphatase, nucleotide pyrophosphatase and γ-glutamyl transferase) (Table I). Compared with glycocholate the efficiency of this compound is higher since similar effects were achieved with 10-fold lower concentrations. Moreover, as can be seen from the liberation of lactate dehydrogenase, the sublytic range of ET-12-H is greater than that of glycocholate. Therefore, in the following experiments, only ET-12-H was used. Using

TABLE II

ENZYME ACTIVITIES IN MEMBRANES PREPARED FROM INTACT CALF THYMOCYTES

Total protein and activities refer to $1.5 \cdot 10^{10}$ cells. Specific activities are expressed as nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}. The enrichment factors are given in brackets. Total activities are given as percentage of the total activity of the homogenate. Homogenates were prepared from cell suspensions ($1.0 \cdot 10^8$ cells/ml) by nitrogen cavitation.

	Control			Detergent ET-12-H (40 nmol/ml)	
	Homogenate	Supernatant	Membranes	Supernatant	Membranes
Protein					
Total mg	362.9	29.5	1.32	28.9	2.53
%	100.0	8.13	0.36	7.80	0.70
Alkaline phosphatase					
Specific activity	40.61 (1.0)	1.04 (0.03)	1094.9 (27.0)	1.18 (0.03)	1449.7 (35.7)
Total activity %	100.0	0.21	9.81	0.23	24.89
Nucleotide pyrophosphatase					
Specific activity	17.78 (1.0)	0.42 (0.02)	312.3 (17.6)	0.43 (0.02)	390.3 (22.0)
Total activity %	100.0	0.19	6.39	0.19	15.30
γ-Glutamyl transferase					
Specific activity	1.6 (1.0)	0.25 (0.16)	9.23 (5.80)	0.22 (0.14)	32.5 (20.31)
Total activity %	100.0	1.27	2.10	1.07	14.16
Mg^{2+}-ATPase					
Specific activity	3.14 (1.0)		12.60 (3.71)		17.21 (5.48)
Total activity %	100.0		1.46		3.82
Lactate dehydrogenase					
Specific activity	340.7 (1.0)	161.9 (0.48)	53.82 (0.16)	149.3 (0.44)	36.68 (0.11)
Total activity %	100.0	3.86	0.06	3.42	0.08

this compound it was possible to remove up to 25% of the total activity of the alkaline phosphatase as membrane-bound enzyme without any increased liberation of lactate dehydrogenase (Table II). The data of Table II also reveal that appreciable proportions of the plasma membrane can be removed by simply incubating and washing the cells without detergent. Thus, about 10% of the membrane containing alkaline phosphatase is removed in these controls. The most interesting feature, however, comes from the fact that the enzyme composition is different in control membanes and in those obtained after ET-12-H treatment. In contrast to the alkaline phosphatase and nucleotide pyrophosphatase membrane vesicles with high activities of the γ-glutamyl transferase can only be obtained by the use of ET-12-H. While control membranes contain only 2% of the total activity of the γ-glutamyl transferase ET-12-H-membranes contain 14% of the total activity. In contrast to this the alkaline phosphatase activity increases only from 9.8% (control) to 24.9% (ET-12-H-membranes). The enrichment factors (homogenate = 1.0) are increased correspondingly: a 4-fold increase in γ-glutamyl transferase while that of the alkaline phosphatase is increased only 1.3-fold. Fig. 1 shows the changes in the enrichment of these plasma membrane enzymes with increasing concentrations of ET-12-H. Lysosomal (β-*N*-acetyl-glucosaminidase) as well as mitochondrial enzymes (succinate dehydrogenase) were found in shedded membranes only in trace activities

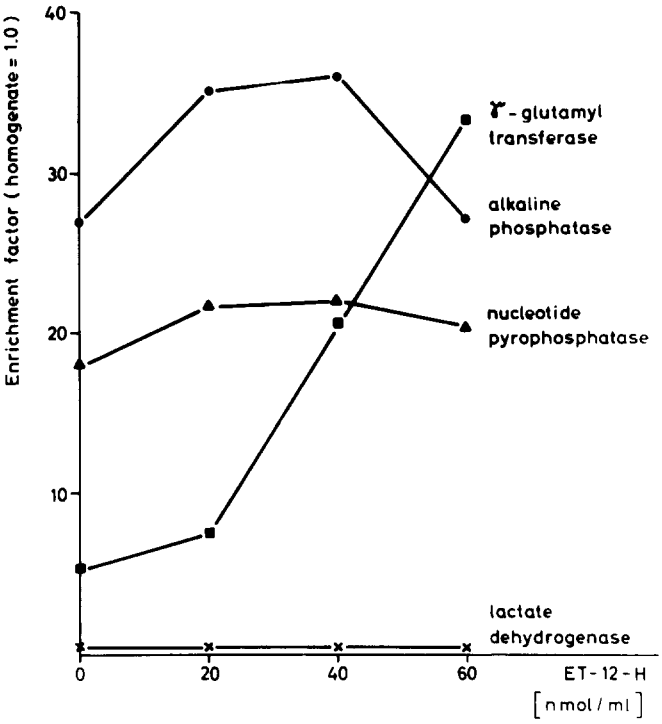

Fig. 1. Shedding of membrane vesicles. Relative specific activities in membranes shed at increasing concentrations of ET-12-H.

which did not exceed 0.3% of the total activity of the homogenate. The only plasma membrane-bound enzyme which is not preferentially removed during shedding is the acyl-CoA:lysolecithin acyltransferase (not shown in Table II).

Lipid composition

Table III shows that cholesterol and phospholipid are not solubilized during

TABLE III

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF MEMBRANE-FREE SUPERNATANTS AND OF SHEDDED MEMBRANES

Specific contents are expressed as nmol/mg protein. Total amounts are given as nmol and correspond to fractions prepared from $1.5 \cdot 10^{10}$ cells.

	Cholesterol			Phospholipid			Molar ratio
	Specific content	Total amount	%	Specific content	Total amount	%	
Control							
Homogenate	20.6	7475	100	80.8	29 332	100	0.255
Supernatant		<14			<7		
Membranes	215.0	188.8	2.5	430.0	377.5	1.3	0.500
Detergent ET-12-H (40 nmol/ml)							
Supernatant		<9			1759		
Membranes	320.1	464.8	6.2	574.1	833.4	2.8	0.557

TABLE IV

PHOSPHOLIPID DISTRIBUTION OF MEMBRANE-FREE SUPERNATANTS AND OF SHEDDED MEMBRANES

Total amounts refer to $1.5 \cdot 10^{10}$ cells.

	Control			Detergent ET-12-H (40 nmol/ml)		
	Supernatant (nmol)	Membranes (nmol)	(%)	Supernatant (nmol)	Membranes (nmol)	(%)
Lysophosphatidyl choline	—	3.9	1.0	1740	42.5	5.0
Sphingomyelin	—	42.9	11.0	—	102.0	12.0
Phosphatidyl choline	<12	206.7	53.0	<20	416.5	49.1
Phosphatidyl serine + phosphatidyl inositol	—	62.4	16.1	—	110.5	12.9
Phosphatidyl ethanolamine	—	74.1	18.9	—	178.5	21.0

the shedding procedure neither without (control) nor with the addition of detergent. Both lipids are recovered completely in the membranes. The high phospholipid content in membrane-free supernatants of ET-12-H treated cells is due exclusively to this lysophosphatidylcholine analog (Table IV). Compared to control membranes ET-12-H-membranes exhibit only a slightly increased cholesterol/phospholipid ratio but significantly higher specific contents (per mg protein) of both cholesterol and phospholipid. In Table IV, the percentage distribution of the main phospholipids is depicted. With the exception of ET-12-H-membranes which contain a few percent of the lysophosphatidylcholine analog, the distribution of the main phospholipids is very similar to that found in membranes shedded without detergent (control membranes).

Significant differences were observed in the fatty acid composition. Total lipids, as well as the major phospholipids phosphatidylcholine and phosphatidylethanolamine of shedded membranes contain lower amounts of highly unsaturated fatty acids (arachidonic and linoleic acid) than purified plasma membranes prepared by the nitrogen cavitation method (Table V). Moreover, control membranes also differ in their fatty acid composition from membranes induced with ET-12-H. As can be seen from the ratio of polyenoic to saturated fatty acids there is a progressive tendency to increasing amounts of polyunsaturated acids in the order: control membranes, ET-12-H membranes, conventional plasma membranes.

Comparison of shedded membranes and purified plasma membranes

A comparison of the main characteristics of shedded membranes (induced with ET-12-H) and purified plasma membranes prepared by the nitrogen cavitation method [14] revealed that the composition is very similar (Table VI). Purified plasma membranes contain higher amounts of both total phospholipids and cholesterol. Therefore, the molar ratio of cholesterol to phospholipid is nearly identical. No significant differences were found in the phospholipid distribution. Moreover, the plasma membrane marker enzymes determined exhibit activities of the same order of magnitude. However, two enzymes show

TABLE V

FATTY ACID COMPOSITION OF SHEDDED MEMBRANES AND OF PURIFIED PLASMA MEMBRANES

Shedded membranes induced without addition (control) and with addition of ET-12-H. Purified plasma membranes (PM) were prepared by the nitrogen cavitation method. Values are expressed as mol%.

	16:0	16:1	18:0	18:1	18:2	20:1 + 18:3	20:4	22:5	22:6	Polyenoic/ saturated
Total lipids										
Control	44.9	1.7	18.9	25.4	7.2	—	1.7	—	—	0.139
ET-12-H (20 nmol/ml)	40.3	1.8	18.3	30.8	6.8	—	2.0	—	—	0.150
ET-12-H (40 nmol/ml)	38.2	2.3	18.6	29.2	6.3	1.1	3.2	1.0	—	0.204
PM	29.4	2.6	18.3	32.3	9.3	0.9	5.2	1.7	0.2	0.363
Phosphatidyl choline										
Control	52.6	3.0	11.6	26.3	5.6	0.9	—	—	—	0.101
ET-12-H (20 nmol/ml)	56.9	3.5	7.5	27.5	3.6	0.9	—	—	—	0.070
ET-12-H (40 nmol/ml)	54.4	3.6	9.1	26.1	5.8	0.9	—	—	—	0.106
PM	41.1	2.6	9.5	33.2	11.1	0.9	1.6	—	—	0.269
Phosphatidyl ethanolamine										
Control	31.7	—	19.4	30.1	16.4	—	2.6	—		0.370
ET-12-H (20 nmol/ml)	23.7	—	22.4	33.2	13.9	1.1	5.7	—	—	0.451
ET-12-H (40 nmol/ml)	24.7	—	21.9	31.5	11.4	—	10.5	—	—	0.470
PM	13.0	3.6	21.7	33.3	11.8	1.3	15.2	—	—	0.779

TABLE VI

COMPARISON OF THE COMPOSITION OF SHEDDED MEMBRANES (ET-12-H) WITH PURIFIED PLASMA MEMBRANES (NITROGEN CAVITATION)

Values are means ± standard deviation from 3 preparations with 3 determinations each.

	Shedded membranes	Purified plasma membranes
Total lipids (nmol/mg protein)		
Cholesterol	320.1 ± 18.2	580.2 ± 25.3
Phospholipid	574.1 ± 28.6	980.2 ± 42.3
Molar ratio	0.557 ± 0.02	0.592 ± 0.03
Phospholipid distribution (%)		
Lysophosphatidyl choline	5.0	—
Sphingomyelin	12.0	12.1
Phosphatidyl choline	49.1	49.2
Phosphatidyl serine + phosphatidyl inositol	12.9	15.0
Phosphatidyl ethanolamine	21.0	23.7
Enzymes (nmol · mg^{-1} · min^{-1})		
Alkaline phosphatase	1250.1 ± 78.1	1150.2 ± 62.1
Nucleotide pyrophosphatase	420.1 ± 22.1	510.3 ± 28.3
γ-Glutamyl transferase	30.2 ± 2.0	31.5 ± 1.5
Mg^{2+}-ATPase	18.0 ± 1.0	42.1 ± 2.0
Acyl-CoA:lysolecithin acyltransferase	6.0 ± 0.5	30.2 ± 1.9

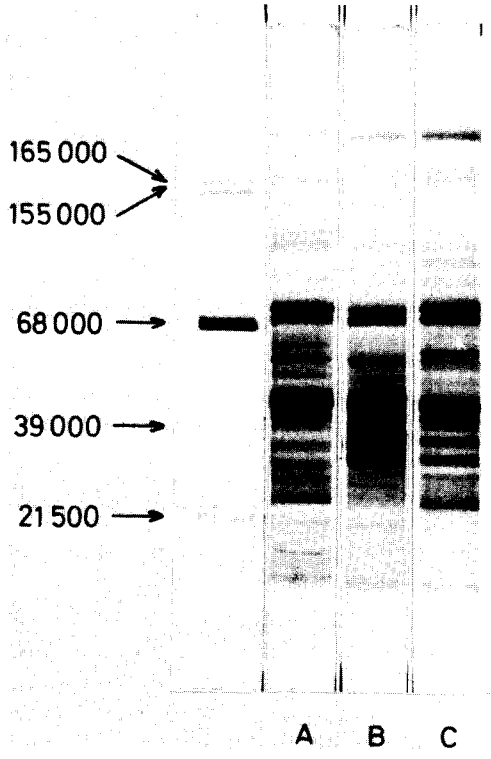

Fig. 2. SDS-polyacrylamide gel electrophoresis of shedded vesicles and purified plasma membranes. 30 μg of protein were applied on each gel. Gradient gels containing 4—30% polyacrylamide were used. A, shedded membranes, induced with ET-12-H (40 nmol/ml); B, shedded membranes, without detergent (control membranes); C, purified plasma membranes (nitrogen cavitation). The reference proteins used were: Trypsin inhibitor from soybean (M_r 21 500), RNA-polymerase from *Escherichia coli* α-subunit (M_r 39 000), bovine serum albumin (M_r 68 000), RNA-polymerase β-subunit (M_r 155 000) and RNA-polymerase β′-subunit (M_r 165 000). These reference proteins were purchased from Boehringer, Mannheim, F.R.G. Ribosomal contaminations were removed from shedded membranes by centrifugation on 35% (w/w) sucrose in 10 mM Hepes buffer for 2 h at 177 000 × g_{av}.

a different behaviour: the Mg^{2+}-ATPase and, in particular, the acyl-CoA : lysophosphatidylcholine acyltransferase exhibit lower activities in shedded membranes. Another important difference concerns the fatty acid composition as described above (Table V).

SDS-polyacrylamide gel electrophoresis

Fig. 2 shows the SDS-gel electrophoresis of spontaneously and ET-12-H-induced vesicles. As can be seen most bands occur in both types of shedded vesicles and compare well with purified plasma membranes. However, distinct differences can be detected e.g. two bands (35 000 and 33 000 dalton) are more prominent in control membranes.

Discussion

The preparation of pure plasma membranes after disruption of cells by shearing or cavitation generally suffers from the fact, that contamination with non-characterized intracellular membranes (e.g. nuclear membranes) cannot be excluded. Therefore, for many purposes it is an advantage to prepare plasma membrane vesicles without cell lysis by a shedding process *.

Using the lysophosphatidylcholine analog ET-12-H we have established a method which yields membrane vesicles from calf thymocytes which exhibit all characteristic features of pure plasma membranes prepared after nitrogen cavitation. Without further purification, the specific activities of the plasma membrane marker enzymes γ-glutamyl transferase, alkaline phosphatase and nucleotide pyrophosphatase as well as the cholesterol and phospholipid content of shedded vesicles compare favorably with those from purified plasma membranes.

Our results differ in many respects from the numerous reports concerning the release of membrane components by shedding. Scott [7] described the formaldehyde-induced plasma membrane vesiculation and Holdsworth and Coleman [31] used glycocholate for the release of plasma membrane marker enzymes. Vesicles spontaneously shed from tumor cells were analyzed by Blitterswijk et al. [4]. For purposes of plasma membrane preparation these materials have never been sufficiently characterized. Moreover, some of these agents employed for vesiculation are known to cause cell injury as judged by morphological and enzymatic criteria [7,9].

In cases of spontaneously shed vesicles, in particular when they are obtained from wash fluids or ascites fluid [4,5,33], it is very difficult if not impossible to decide whether the membraneous material was not derived from dying or dead cells. This is documented by characterization data, when given at all, showing a poor purification, e.g. only a 3-fold enrichment of 5'-nucleotidase [5] over the homogenate. We compared the release of plasma membrane marker enzymes by glycocholate and ET-12-H. In both cases in the order of 20% of the total activities were released from the cells without lysis. In contrast to the report of Holdsworth and Coleman [31] these activities were almost

* The term shedding is used here exclusively for the release of membrane vesicles and not for the liberation of defined components such as enzymes or receptors.

exclusively associated with sedimentable material. The advantages of ET-12-H over glycocholate in this system are that 10-fold lower concentrations are required and that the sublytic concentration range is wider. These low detergent concentrations do not impair biochemical analysis, in particular, phospholipid and cholesterol determinations and SDS-gel electrophoresis.

Although the method allows the preparation of pure plasma membranes it should be emphasized that shedded vesicles do not represent a statistical average of the entire plasma membrane. This is documented by the fact that the composition of spontaneously released vesicles (control vesicles) and ET-12-H induced vesicles differ. Of particular interest are the relatively high specific activities of γ-glutamyl transferase in ET-12-H-induced vesicles. This enzyme was enriched up to 7-fold over spontaneously shedded membranes with increasing ET-12-H concentrations. The overall phospholipid composition of control and ET-12-H-induced vesicles was similar to conventionally purified plasma membranes. However, we found significant differences in the composition of their fatty acids. The lowest amounts of unsaturated fatty acids we found in spontaneously shed vesicles while increasing amounts were present in detergent-prepared vesicles and normal purified plasma membranes. The most likely explanation for this observation is that the plasma membrane consists of areas of different structure and function [15,32] which are anchored at different strengths within the membrane. Obviously, those parts of the membrane which are more rigid are preferentially shed. This is in accordance with the findings of Blitterswijk et al. [4] who found that vesicles, spontaneously shed from tumor cells, are more rigid than the entire plasma membrane. In this case, however, this difference was not achieved by different fatty acids but was due to a higher cholesterol content in shedded membranes. Thus, the controlled vesiculation with ET-12-H is not only an alternative method for the preparation of plasma membranes, but may also be a useful tool for the analysis of the plasma membrane substructure.

Acknowledgements

The skillfull technical assistance of K. Rinno, I. Aehnelt and K. Hansen is greatfully acknowledged. This study has been financially supported by the Deutsche Forschungsgemeinschaft (Fe 130/4).

References

1 Doljanski, F. and Kapeller, M. (1976) J. Theor. Biol. 62, 253–270
2 Uhr, J.W. and Vitetta, E.S. (1973) Fed. Proc. 32, 35–40
3 Owen, F.L. and Fanger, M.W. (1975) J. Immunol. 115, 765–770
4 Van Blittersijk, W.J., Emmelot, P., Hilkmann, H.A.M., Oomenmeulemans, E.P.M. and Inbar, M. (1977) Biochim. Biophys. Acta 467, 309–320
5 Raz, A., Goldman, R., Yuli, I. and Inbar, M. (1978) Cancer Immunol. Immunother. 4, 53–59
6 De Broe, M.E., Wieme, R.J., Logghe, G.N. and Roels, F. (1977) Biochim. Biophys. Acta 81, 237–245
7 Scott, R.E. (1976) Science 194, 743–745
8 Billington, D. and Coleman, R. (1978) Biochim. Biophys. Acta 509, 33–47
9 Hoerl, B.J. and Scott, R.E. (1978) Virchows Arch. B 27, 335–345
10 Weltzien, H.U., Richter, G. and Ferber, E. (1979) J. Biol. Chem. 254, 3652–3657
11 Arnold, D., Weltzien, H.U. and Westphal, O. (1967) Liebigs Ann. Chem. 709, 234–239
12 Weltzien, H.U., Arnold, B. and Westphal, O. (1973) Liebigs Ann. Chem. 1973, 1439–1444

13 Resch, K. and Ferber, E. (1972) Eur. J. Biochem. 27, 153—161
14 Ferber, E., Resch, K., Wallach, D.F.H. and Imm, W. (1972) Biochim. Biophys. Acta 266, 494—504
15 Brunner, G., Heidrich, H-G., Golecki, J.R., Bauer, H.C., Suter, D., Plückhahn, P. and Ferber, E. (1977) Biochim. Biophys. Acta 471, 195—212
16 Novogrodsky, A., Tate, S.S. and Meister, A. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2414—2418
17 Crane, R.K. and Lipmann, F. (1953) J. Biol. Chem. 201, 235—243
18 Ferber, E. and Resch, K. (1973) Biochim. Biophys. Acta 296, 335—349
19 Pennington, R.J. (1961) Biochem. J. 80, 649—654
20 Sellinger, O.Z., Beaufay, H., Jacques, P., Doyen, A. and De Duve, C. (1960) Biochem. J. 74, 450—456
21 Kornfeld, R. and Siemers, C. (1974) J. Biol. Chem. 249, 1295—1301
22 Wróblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. Med. 90, 210—213
23 Ways, P., Reed, C.F. and Hanahan, D.J. (1963) J. Clin. Invest. 42, 1248—1260
24 Wood, R. and Harlow, R.D. (1969) Arch. Biochem. Biophys. 131, 495—501
25 Ferber, E., de Pasquale, G.G. and Resch, K. (1975) Biochim. Biophys. Acta 398, 364—376
26 Röschlau, P., Bernt, E. and Gruber, W. (1974) Z. Klin. Chem. Klin. Biochem. 12, 403—407
27 Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. and Farr, A.L. (1954) J. Biol. Chem. 207, 1—17
28 Gerlach, E. and Deuticke, B. (1963) Biochem. Z. 337, 477—479
29 Moore, S. and Stein, W.H. (1948) J. Biol. Chem. 176, 367—388
30 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
31 Holdsworth, G. and Coleman, R. (1976) Biochem. J. 158, 493—495
32 Resch, K., Loracher, A., Mähler, B., Stoeck, M. and Rode, H.N. (1978) Biochim. Biophys. Acta 511, 176—193
33 Novotny, A., Grohsman, J., Abdelnoor, A., Rote, N., Yang, C. and Waltersdorff, R. (1974) Eur. J. Immunol. 4, 73—78