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ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM FRIEND ERYTHROLEUKAEMIC CELLS

A STUDY WITH SPHINGOMYELINASE C

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Plasma membranes have been prepared from Friend erythroleukaemic cells using a Dounce homogenization technique followed by differential and sucrose gradient centrifugations. (1) A plasma membrane fraction was obtained which showed a 20- to 30-fold enrichment in 5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase and in ^{32}P-labeled (poly)phosphoinositides. About 1% of the total protein, 6–7% of phospholipid, 8–9% of cholesterol and 12–15% of each of the above markers were recovered in the plasma membrane fraction with an average yield of 15–20%. The plasma membrane was characterized by a high cholesterol to phospholipid molar ratio (0.626), a 2-fold enrichment in sphingomyelin and in phosphatidylserine as compared to the whole cell and by the complete absence of diphosphatidylglycerol. (2) When compared to the phospholipid composition of the mature mouse erythrocyte membrane, the plasma membrane of the Friend cell only differs by a higher phosphatidylcholine and a lower phosphatidylethanolamine content, whereas the levels of sphingomyelin and phosphatidylinositol plus phosphatidylserine are similar. (3) Friend cells were treated with sphingomyelinase C (*S. aureus*) under non-lytic conditions and subsequently submitted to subcellular fractionation. The results showed that the plasma membrane accounted for 38.5% of the total phospholipid, 64.1% of the total cholesterol and about 4.4% of the total protein content of Friend cells. (4) Sphingomyelin appeared to be asymmetrically distributed in the plasma membrane of Friend cells, with about 85% of this phospholipid being present in the outer monolayer.

Introduction

Murine erythroleukaemic cells derived from mouse spleens infected with the Friend virus complex (Friend cells) have been widely used as a model to study differentiation in vitro [1–3]. Following exposure to a variety of chemically unrelated compounds [2], Friend cells undergo a number of alterations, among which early and late plasma membrane changes have been distinguished [2]. The involvement of phospholipids in the differentiation-related processes has been emphasized. For instance, dimethylsulfoxide and other inducers, which affect the phospholipid transition phase temperature of unilamellar liposomes [4], were found to alter the fluorescence polarization of diphenylhexatriene-labeled Friend cells [5,6]. Mouse myeloid leukaemia M1 cell cultures supplemented with choline analogs were recently found to undergo differentiation, suggesting the involvement of phospholipid metabolism in their mechanism of differentiation [7].

The phospholipid turnover in Friend cells, in relation to cell differentiation, has been studied by several groups [8–10]. In all these studies, however, the biochemical events following induction of Friend cells have been investigated on whole cells only and no attempt has been made so far to

isolate the plasma membrane of these cells as a pure, fully characterized subcellular membrane preparation. The availability of a pure plasma membrane fraction would enable one to identify more precisely, among the changes observed in induced cells, those which are susceptible to occur specifically at the plasma membrane level. Moreover, one could then readily compare the chemical composition of the erythroblast plasma membrane with that of the corresponding mature erythrocyte, whereas such a comparison is not possible when analytical data based on whole cells are used.

Recently, we reported that the phosphorylation of (poly)phosphoinositides in intact Friend cells incubated with ^{32}P for a short time was a convenient marker for the plasma membrane [11]. In this paper, we present an extensive analysis of the subcellular fractionation of Friend cells. Characterization of the subcellular fractions was achieved by the use of marker enzymes and by phospholipid and cholesterol analysis. In addition, the treatment of Friend cells with sphingomyelinase C under non-lytic conditions, followed by isolation and analysis of the plasma membrane, enabled us to estimate the percentage of the cell phospholipids which is present in the plasma membrane. These data were used in turn to estimate the percentage of total cell cholesterol and protein which belong to the plasma membrane. Finally, an asymmetric distribution of sphingomyelin in the plasma membrane of Friend cells is reported.

Materials and Methods

Cells. Friend cells (clone 745) were cultivated as described in Ref. 10. Cells were harvested after 3 days, centrifuged at 480 × *g* for 5 min and washed three times at 37°C with buffer I (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 5 mM Tricine/NaOH, pH 7.5). The cell pellet was resuspended in buffer I and the cell number determined by counting a suitably diluted aliquot in a hematocytometer. This cell suspension was either submitted to sphingomyelinase C treatment before cell fractionation or directly processed for cell fractionation.

Sphingomyelinase C treatment. Sphingomyelinase C (*S. aureus*) was purified as described by Zwaal et al. [12]. About $600 \cdot 10^6$ cells (corresponding to about 65 mg total protein) in 10 ml buffer I were incubated at 37°C in the presence of 30 IU sphingomyelinase C. As a control, an equal number of cells were incubated under the same conditions but without the enzyme. After 15 min, the enzymatic reaction was terminated by adding 10 ml cold 10 mM EDTA in buffer I. Aliquots of 2.5 ml (corresponding to $75 \cdot 10^6$ cells) were further diluted with 4.5 ml 5 mM EDTA in buffer I and used for the determination of cell lysis and for the analysis of the cell phospholipid and cholesterol composition. The remaining cells were then spun down at 2200 × *g* for 5 min and submitted to cell fractionation.

Determination of cell lysis. The release of lactate dehydrogenase (EC 1.1.1.27) was used as an index of sphingomyelinase C-induced lysis of cells. The diluted cells were centrifuged for 5 min at 2200 × *g*. The amount of lactate dehydrogenase in the supernatant was compared to that found in this cell suspension before centrifugation; the latter value was taken as 100%. The reaction mixture contained 42 mM potassium phosphate buffer (pH 7.6), 0.9 mM sodium pyruvate, 0.2 mM NADH, 0.1% Triton X-100 and the sample (50 µl cell suspension and up to 500 µl supernatant) in a total volume of 3 ml. The linear rate of NADH oxidation was followed at 340 nm during 2 min.

Cell fractionation. The procedure used for the fractionation of Friend cells is described in Fig. 1.

Enzyme assays. Marker enzyme assays for subcellular fractions were carried out using established procedures, referred to in parentheses: 5′-nucleotidase (EC 3.1.3.5) [13], the released inorganic phosphate being measured as in Ref. 14; alkaline phosphodiesterase I (EC 3.1.4.1) [15]; alkaline phosphatase (EC 3.1.3.1) [13]; NADH-diaphorase (EC 1.6.99.3) [16]; antimycin-insensitive NADH-cytochrome *c*-reductase (EC 1.6.2.4) [13]; succinate dehydrogenase (EC 1.3.99.1) [17]; β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) (18); β-D-glucuronidase (EC 3.2.1.31) [13]. ^{32}P-labeling of intact Friend cells through (poly)phosphoinositide kinases was as described [11].

Phospholipid and cholesterol determinations. Lipid extracts from whole cell pellets were obtained using the procedure of Folch et al. [19] including one washing of the extract with the

Folch theoretical upper phase. Isolated membrane fractions were extracted by the method of Renkonen et al. [20]. Total lipid phosphorus was determined according to Ref. 21 after digestion of aliquots of lipid extracts with 70% $HClO_4$ at 190–200°c for 1 h. Phospholipids were separated by two-dimensional thin-layer chromatography and the phospholipid composition determined as described in Ref. 22. Cholesterol was determined colorimetrically [23] after saponification of aliquots of lipid extracts [24].

Protein determination. Protein was measured by the method of Lowry et al. [25] after coprecipitation with sodium deoxycholate (0.5 mg/ml) in the presence of 10% trichloroacetic acid [26].

Results and Discussion

A short description of the characterization of subcellular fractions of Friend cells by marker enzymes and in particular by the application of (poly)phosphoinositide phosphorylation has been presented recently [11]. A full account of the data is given here in Table I. Briefly, the fractionation procedure described in Fig. 1 yielded four major different membrane fractions after the sucrose gradient step. Fractions E and D represented mainly mitochondrial and microsomal membranes, respectively. However, some cross-contamination of microsomes by mitochondrial material occurred, as evidenced by the 2-fold enrichment of succinate dehydrogenase in fraction D. On the basis of the 20- to 30-fold enrichment in plasma membrane markers and of a similar enrichment in ^{32}P-labeled (poly)phosphoinositides, fraction C was considered to be representative of plasma membrane material. Although fraction B was also enriched in plasma membrane, it accounted only for half of the protein and of the plasma membrane markers present in fraction C (Table I). In addition, fraction B exhibited a significantly lower enrichment in ^{32}P-labeling of (poly)phosphoinositides and a higher enrichment in lysosomal material than did fraction C. Finally, fraction A represented a minor component (< 0.1% of the total homogenate protein) to which no clear identity could be assigned despite a high enrichment in lysosomal markers. We therefore consid-

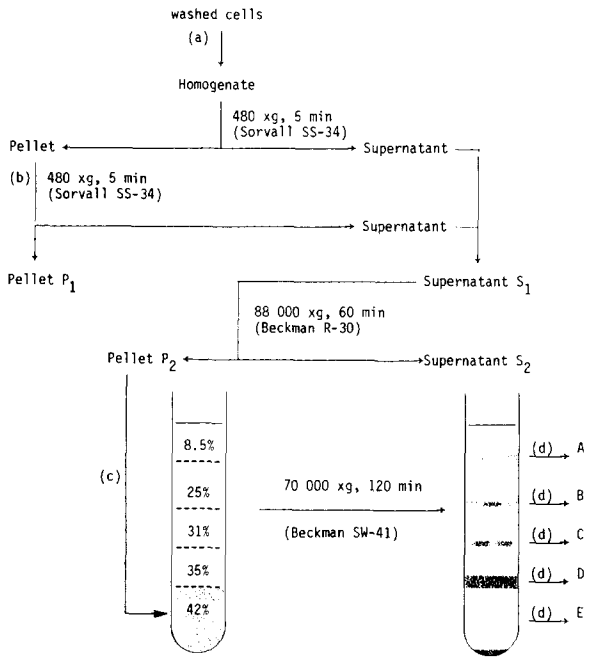

Fig. 1. The whole fractionation procedure was carried out at 0–4°C. (a) Control or sphingomyelinase C-treated Friend cells were pelleted as described in Materials and Methods. The cell pellet was resuspended in 7 ml buffer II (10 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.5) and allowed to swell for 10 min. Cells were then disrupted in the same medium with 10 strokes of the tight-fitting pestle of a glass Dounce homogenizer. Enough 2 M sucrose in buffer II was added to reach 0.25 M sucrose; this suspension was taken as the starting homogenate. (b) The first $480 \times g$ pellet was resuspended in 10 ml buffer III (0.25 M sucrose and 10 mM Tris-HCl, pH 7.5) and homogenized as above. (c) The 'crude membrane' pellet P_2 was resuspended in 4 ml buffer III with a loose-fitting glass Dounce pestle. The sucrose concentration was slowly brought to 42% (w/v) by dropwise addition of 60% (w/v) sucrose in 10 mM Tris-HCl (pH 7.5) under gentle vortexing. Aliquots of 3 ml of this dense suspension were injected below a discontinuous gradient made of 4 layers (2 ml each) of (w/w) 35%, 31%, 25% and 8.5% sucrose in 10 mM Tris-HCl (pH 7.5). (d) The four interfacial bands (A at 8.5%/25%, B at 25%/31%, C at 31%/35%, D at 35%/42%) and the pellet E were collected, diluted with about 20 ml 50 mM sucrose in 10 mM Tris-HCl (pH 7.5) and centrifuged at $88000 \times g$ for 45 min in a Beckman R-30 rotor. These washed fractions were then resuspended in a suitable amount of 25 mM sucrose/10 mM Tris-HCl (pH 7.5) and assayed for protein, enzyme activity and phospholipid and cholesterol content as described in Materials and Methods. When sphingomyelinase-treated Friend cells were fractionated, all solutions contained 1 mM EDTA except the gradient solutions which were made 0.1 mM in EDTA. This ensured a complete inhibition of any sphingomyelinase present during the whole procedure.

TABLE I

DISTRIBUTION OF SUBCELLULAR MARKER ENZYMES AND ^{32}P-LABELED DI- AND TRIPHOSPHOINOSITIDES IN THE FRACTIONATION OF FRIEND CELLS

For each constituent, relative specific activities (expressed as recovered activity/mg protein or ^{32}P cpm/mg protein in each fraction, divided by activity/mg protein or ^{32}P cpm/mg protein in the homogenate) are given. The amount of each constituent in each fraction, expressed as percentage of the amount found in the homogenate, is given in parentheses. The relative specific activity in the homogenate is 1.0. Recovery is the sum of recovered activities in all fractions expressed as percentage of the activity present in the homogenate. Markers for plasma membrane were constituents 1, 2, 3, 9 and 10; for endoplasmic reticulum (microsomal fraction) 4 and 5; for mitochondria 6, for lysosomes 7 and 8. The specific activities of the marker enzymes in the starting homogenate, expressed as nmol·(mg protein)$^{-1}$·h^{-1}, were; 5′-nucleotidase, 106; alkaline phosphodiesterase I, 18; alkaline phosphatase, 702; NADH-diaphorase, 1314; NADH-cytochrome *c*-reductase, 564; succinate dehydrogenase, 173; β-D-glucuronidase, 20 and β-*N*-acetyl-D-glucosaminidase, 735.

Constituent	Fraction												Recovery (%)
	P_1		S_2		A		B		C		D		E		
1 5′-Nucleotidase	0.46	(*12.6*)	0.47	(*19.4*)	15.98	(*0.4*)	31.72	(*7.6*)	28.85	(*15.3*)	7.97	(*13.0*)	1.70	(*13.9*)	*82.2*
2 Alkaline phosphodiesterase I	0.58	(*15.8*)	0.64	(*26.6*)	16.72	(*0.5*)	22.17	(*5.4*)	19.67	(*10.0*)	6.34	(*10.4*)	1.49	(*12.1*)	*80.8*
3 Alkaline phosphatase	0.38	(*10.3*)	0.46	(*19.2*)	2.14	(*0.1*)	29.15	(*7.0*)	29.11	(*14.9*)	7.82	(*12.8*)	1.62	(*13.2*)	*77.5*
4 NADH-diaphorase	0.56	(*16.3*)	0.44	(*19.5*)	1.36	(*0.1*)	0	(*0*)	0.21	(*0.2*)	7.23	(*71.2*)	1.64	(*3.7*)	*110.9*
5 NADH-cytochrome *c*-reductase	0.98	(*28.3*)	0.06	(*2.6*)	0.37	(*0.01*)	0.43	(*0.2*)	0.88	(*0.8*)	7.12	(*70.0*)	2.03	(*4.6*)	*106.5*
6 Succinate dehydrogenase	1.17	(*31.7*)	0.79	(*32.9*)	0	(*0*)	0	(*0*)	0	(*0*)	1.97	(*3.2*)	4.57	(*37.4*)	*105.2*
7 β-D-Glucuronidase	0.59	(*17.2*)	0.37	(*16.4*)	11.90	(*0.5*)	5.63	(*2.2*)	4.71	(*4.5*)	2.72	(*26.7*)	1.15	(*2.6*)	*70.1*
8 β-*N*-acetyl-D-glucosaminidase	0.44	(*12.9*)	0.24	(*10.6*)	11.54	(*0.5*)	7.82	(*3.0*)	6.75	(*6.1*)	3.87	(*38.0*)	1.68	(*3.8*)	*74.9*
9 Phosphatidylinositol 4-[^{32}P]phosphate	1.00	(*33.4*)	0.48	(*22.5*)	8.74	(*1.1*)	11.63	(*3.6*)	18.75	(*12.3*)	4.48	(*12.1*)	1.13	(*5.7*)	*90.7*
10 Phosphatidylinositol 4,5-[^{32}P]bisphosphate	1.15	(*38.1*)	0.52	(*24.3*)	7.03	(*0.9*)	14.03	(*4.4*)	37.66	(*24.6*)	8.67	(*23.5*)	1.47	(*7.4*)	*123.2*
Recovered protein		*36.7*		*51.2*		*0.1*		*0.4*		*0.9*		*5.1*		*5.9*	

ered that reasonably pure plasma membranes were present in fraction C.

Table II shows the percentages of the homogenate content in protein, phospholipid, cholesterol, 5'-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase and ^{32}P-labeled (poly)phosphoinositides which can be recovered in the plasma membrane fraction C. Although these values are not very high (see, however, Ref. 27) and lead to moderate yields only (see below), there was always enough material present for reliable phospholipid and cholesterol determinations to be made. Moreover, high yields can generally be obtained only at the expense of purity. Since purity was the main requirement of our experimental approach using sphingomyelinase C (see below), moderate yields were normally expected.

The phospholipid and cholesterol composition of gradient fractions B, C, D and E is depicted in Table III and compared to that of whole Friend cells. Our data on whole cells agree well with those obtained on Friend cells by Harel et al. [28] and on Rauscher cells by Montfoort and Boere [29]. On the other hand, the lipid phosphorus and cholesterol to protein ratios as well as the cholesterol to phospholipid molar ratio observed in our Friend cells differ somewhat from the results of Zwingelstein et al. [8,30], whereas the strong discrepancy between the abovementioned ratios (Refs. 28, 29; this study) and those reported by Rittman et al. [9] cannot be easily explained.

TABLE II

RECOVERIES OF CELLULAR COMPONENTS IN THE PLASMA MEMBRANE

Recoveries are expressed as % of the amount of component present in the homogenate and are given as mean value ± S.D. The number of determinations is given in parentheses.

Component	Recovery
Protein	0.92 ± 0.20 (7)
Phospholipid	6.66 ± 1.40 (4)
Cholesterol	8.63 ± 0.72 (2)
5'-Nucleotidase	14.2 ± 3.0 (3)
Alkaline phosphodiesterase I	12.0 ± 2.6 (3)
Alkaline phosphatase	13.9 ± 2.1 (5)
Phosphatidylinositol 4-[^{32}P]phosphate	5–12.3 (range)
Phosphatidylinositol 4,5-[^{32}P]bis phosphate	10–24.6 (range)

The relative phospholipid composition of Friend cells was characterized by slightly higher amounts of phosphatidylcholine, phosphatidylinositol and sphingomyelin and by lower amounts of phosphatidylethanolamine and diphosphatidylglycerol, than in earlier reports [8,9,28,30]. No striking differences were apparent, with the exception, however, of the rather high content of diphosphatidylglycerol observed by Rittman et al. [9] and Zwingelstein et al. [8,30]. Table III shows that mitochondrial and microsomal fractions E and D were the only subcellular membranes to be significantly enriched in diphosphatidylglycerol, whereas this phospholipid was absent in the plasma membranes. On the other hand, plasma membranes were highly enriched in sphingomyelin and phosphatidylserine and showed a somewhat decreased phosphatidylcholine and phosphatidylinositol content as compared to whole cells. Plasma membranes isolated from Friend cells are thus similar in this respect to plasma membranes isolated from many other types of cells [31]. It was shown recently that in addition to phospholipid exchange between Friend cells and culture medium, a net incorporation of phospholipids (and particularly of lysophosphatidylcholine) from the serum into the cells significantly contributed to the final phospholipid composition of Friend cells [10]. We always observed the presence of a small amount of lysophosphatidylcholine in whole Friend cells; this phospholipid was mostly found (and consistently enriched) in isolated plasma membranes (Table III). This result supports the view that at least part of the cellular content of lysophosphatidylcholine is due to a net uptake from the lipoproteins present in the culture medium [10].

When the phospholipid composition of plasma membranes isolated from Friend cells is compared to that of mature mouse erythrocytes [10,29], it appears that these two membranes only differ in that the former has a higher content of phosphatidylcholine and a lower content of phosphatidylethanolamine (55.2% and 16.5%, respectively), than the latter (46–47% and 23–25%, respectively). On the other hand, the plasma membrane of Friend cells contains comparable amounts of sphingomyelin and of phosphatidylinositol plus phosphatidylserine. This is further illustrated by

TABLE III

PHOSPHOLIPID AND CHOLESTEROL COMPOSITION OF WHOLE FRIEND CELLS AND OF ISOLATED MEMBRANE FRACTIONS B, C, D AND E

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol; LPC, lysophosphatidylcholine; n.d., not detected. The number of determinations is given in parentheses.

Component		Whole Friend cell	Membrane fraction			
			B	C	D	E
Total lipid phosphorus (μmol/mg protein)		0.127 ± 0.009 (4)	1.181 ± 0.003 (2)	0.907 ± 0.026 (4)	0.431 ± 0.006 (2)	0.209 ± 0.105 (2)
Total cholesterol (μmol/mg protein		0.039 ± 0.003 (4)	0.665 ± 0.000 (2)	0.568 ± 0.034 (2)	0.098 ± 0.028 (2)	0.063 ± 0.004 (2)
Phospholipid composition (mol%)						
	PC	59.9 ± 2.1 (3)	54.8 ± 0.1 (2)	55.2 ± 4.3 (4)	64.6 ± 0.6 (2)	63.3 ± 4.2 (2)
	PE	18.5 ± 1.6 (3)	13.3 ± 2.8 (2)	16.5 ± 2.3 (4)	18.1 ± 1.3 (2)	16.1 ± 5.7 (2)
	Sph	5.6 ± 0.7 (3)	10.3 ± 1.3 (2)	10.2 ± 0.9 (4)	4.0 ± 1.6 (2)	3.8 ± 0.7 (2)
	PI	8.3 ± 0.4 (3)	6.1 ± 0.9 (2)	5.9 ± 0.7 (4)	6.6 ± 0.5 (2)	9.2 ± 0.1 (2)
	PS	4.0 ± 0.3 (3)	13.6 ± 2.5 (2)	10.6 ± 0.9 (4)	2.5 ± 1.1 (2)	1.1 ± 0.3 (2)
	DPG	3.0 ± 0.2 (3)	n.d.	n.d.	4.4 ± 1.3 (2)	6.4 ± 0.8 (2)
	LPC	0.7 ± 0.2 (2)	1.9 ± 2.4 (2)	1.4 ± 0.7 (4)	n.d.	n.d.
Molar ratio cholesterol : phospholipid		0.307	0.563	0.626	0.227	0.301
Molar ratio Sph : PC		0.093	0.188	0.185	0.062	0.060
Molar ratio PS : PC		0.067	0.248	0.192	0.039	0.017

the comparison of the sphingomyelin/phosphatidylcholine, phosphatidylserine/phosphatidylcholine and cholesterol/phospholipid ratios in Friend cells (0.185, 0.192 and 0.626, respectively, see Table III) with those in mature mouse erythrocytes (0.262, 0.242 and 0.77, respectively; see Table II in Ref. 29). Any alteration of the plasma membrane composition during differentiation of Friend cells could thus imply a modification of the relative amounts of the two major phospholipids, leaving constant the relative amounts of the others, whatever the involved mechanisms would be [32,33].

Treatment of Friend cells with sphingomyelinase C under non-lytic conditions, followed by isolation and analysis of the plasma membrane, allowed us to determine the percentage of the total cell phospholipids which is present in the plasma membrane. The rationale of this approach (see also the legend to Table IV) has been discussed by Chap et al. [34] and successfully applied to pig [34] and human [35] platelets. Table IV shows that the extent of sphingomyelin degradation, expressed as percent of the total phospholipids, was higher in isolated plasma membranes (8.2%) than in intact cells (3.15%). The ratio of the latter to the former was remarkably constant, the average value being 38.5% ± 1.1 for three separate experiments. This value is lower than that found in pig and human platelets (63% and 57%, respectively; see Refs. 34 and 35) but is merely a reflection of the amount of intracellular membranes in Friend cells [36]. Moreover, the contribution of the microvillae to the surface area of the plasma membrane of Friend cells is probably much lower than that of the open canalicular system [37] in the case of platelets.

From the percentage of sphingomyelin degradation determined in the extract from whole cells as well as in that from isolated plasma membranes, we calculated that the plasma membrane contained an average of 70.8% of the cellular content of this phospholipid. This high value is reflected by the analytical data of Table III and is in full agreement with the general trend of this phospholipid to be essentially located at the plasma membrane level [31].

The comparison between sphingomyelin hydrolysis (expressed as % of the total phospholipids) in the plasma membrane of treated cells and the relative amount of this phospholipid in plasma membranes isolated from control cells gives the percentage of the plasma membrane sphingomyelin which can be degraded by sphingomyelinase C without lysis of the cells (see Ref. 34). In two experiments, we found an average value of 84.8% ± 2.0. Consequently, up to 85% of the sphingomyelin in the plasma membrane of Friend cells is present in the outer leaflet of this membrane. It must be stressed that although this degree of hydrolysis was obtained after 15 min of incubation with sphingomyelinase C, an extension of the incubation time up to 120 min failed to increase the extent of sphingomyelin hydrolysis (data not shown). This suggests that the non-available sphingomyelin pool, presumably located at the inner leaflet, does not experience any detectable transbilayer movement within the time scale (120 min) of this experiment. We can conclude from

TABLE IV

NON-LYTIC DEGRADATION OF SPHINGOMYELIN BY SPHINGOMYELINASE C IN FRIEND CELLS AND IN ISOLATED PLASMA MEMBRANES

Cells were treated as described in Materials and Methods. After 15 min incubation, sphingomyelin hydrolysis reached a maximal value which could not be increased by further incubation. The extent of cell lysis was negligible ($\leq 0.8\%$ for three experiments). The percentage of total cell phospholipid present in the plasma membrane is derived as follows: Suppose that A and B are the total amounts of phospholipid present in the plasma membrane and in the intracellular membranes, respectively, and that H is the amount of phospholipid degraded by sphingomyelinase C under non-lytic conditions. Expressed on a total phospholipid basis, the extents of hydrolysis in whole cells and in isolated plasma membranes are, respectively, $H/(A+B)$ and H/A. The ratio of these values is $A/(A+B)$ and represents the percentage of total cell phospholipid originating from the plasma membrane. Here, $H/(A+B) = 3.15\%$ and $H/A = 8.20\%$. Thus, $3.15/8.20 = 0.384$ or 38.4%.

	Friend cells	Plasma membranes
% sphingomyelin degraded	56.0 ± 2.2	79.3 ± 6.5
Hydrolysis (% of total phospholipid)	3.15 ± 0.48	8.20 ± 1.47
% total cell phospholipid present in plasma membrane	38.45 ± 1.06	

these results that sphingomyelin is asymmetrically distributed over the two layers of the plasma membranes of Friend cells. This distribution is almost identical to that found in mature human [12,22] and mouse (Roelofsen, B. and Van Linde, M., unpublished results) erythrocytes and very similar to that found in platelets [34,35]. This implies that, as far as sphingomyelin is concerned, the definitive asymmetry which is observed in mature erythrocytes appears to preexist already at an early stage of the differentiation pathway (the erythroblast). This is most remarkable since several studies on totally unrelated cells such as BHK-21 cells or MDBK cells have revealed a reversed distribution for sphingomyelin [38,39]. Since it has been suggested that membrane lipid asymmetry of blood cells might be involved in the regulation of haemostasis [40–42], it is tempting to consider that such a property could be shared by all those cells which have to enter into the blood circulation.

It is also of interest to estimate the amount of total cell protein which can be accounted for by the plasma membrane. Two independent methods can be applied using the present data. Firstly, the amount (on a protein basis) of a given organelle relative to the whole cell is given by the ratio of the specific activities of any marker for this organelle in the homogenate and in the isolated membrane fraction [43]. From the data of Table I for 5′-nucleotidase, alkaline phosphodiesterase I and alkaline phosphatase, an average value of $4.0 \pm 0.9\%$ is found. Secondly, the yield of plasma membrane (on a protein basis) can be obtained by dividing the yield based on phospholipid by the phospholipid/protein ratio. Using the data of Tables III and IV, one can calculate a 19.1% yield. The amount of plasma membrane protein relative to cell protein, expressed by the ratio of protein recovery (see Table II) to protein yield, would be 4.8%, which is in good agreement with the value derived from the specific activities. Similar values can be calculated, using the specific activity ratio method, for human leukaemic lymphocytes [27], Burkitt's lymphoma cells [44] or for mouse ascitic leukaemia cells [45]. On the other hand, cells of an entirely different morphology such as platelets [35] would contain up to 18–19% of the total cell protein in their plasma membrane, which is in line with their high plasma membrane/cell phospholipid ratio [34,35].

Table III shows also that in isolated plasma membranes, the enrichment factor for cholesterol (14.5) is 2-fold higher than that for phospholipid (7.2). We could expect the amount of cell cholesterol present in plasma membrane to be similarly increased when compared to phospholipid. Assuming an average value of 4.4% for the cell protein originating from the plasma membrane, and taking into account the data of Table III, we calculate that $64.1 \pm 6.0\%$ of the total cellular cholesterol could be located in the plasma membrane. This high value shows that in Friend cells, cholesterol is primarily located in the plasma membrane, in agreement with the localization generally observed for this component in most cells [46].

Finally, it should be mentioned that most of these data are derived from a central measurement, namely the ratio of plasma membrane to total cell phospholipid as determined after sphingomyelinase C treatment of whole cells. Since this ratio was found to be remarkably constant despite some variations in the extent of hydrolysis from one experiment to another, we can consider the data derived from it as reasonable estimations of their actual value.

The general conclusion which can be drawn from the present results is that the simple technique of non-lytic treatment of whole cells with sphingomyelinase C, combined with an adequate subcellular fractionation procedure, can provide a number of useful data about the plasma membrane of a eukaryotic system such as the Friend cell. The next step will be to investigate whether (and if so, how) the percentual ratios determined in undifferentiated Friend cells for phospholipid, protein and cholesterol, as well as the (asymmetric) distribution of phospholipids in the plasma membrane, will change during the chemically-induced differentiation of these cells. Such studies are now in progress in this laboratory.

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