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PLASMA MEMBRANE AMINOPHOSPHOLIPID DISTRIBUTION IN TRANSFORMED MURINE FIBROBLASTS

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Summary

The possibility that the asymmetric distribution of aminophospholipids may be an intrinsic property of mammalian plasma membranes was examined in LM cells, a transformed murine fibroblast cell line. The cells were grown in suspension culture in a chemically defined medium without lipid, protein, or serum and then treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS). A maximum of 4% of LM cell plasma membrane phosphatidylethanolamine and 5% of the phosphatidylserine was labelled with TNBS. Furthermore, long chain and unsaturated fatty acids were preferentially esterified to the non-derivatized phosphatidylethanolamine (inner monolayer) as compared to phosphatidylethanolamine derivatized with TNBS (outer monolayer). Isethionyl acetimidate, an alternative non-penetrating reagent, confirmed the results obtained with TNBS and provided supportive evidence for the highly asymmetric distribution of phosphatidylethanolamine; 6% of the phosphatidylethanolamine was labelled with isethionyl acetimidate. When the penetrating reagent methyl acetimidate was used, more than 80% of the phosphatidylethanolamine was derivatized. Although the growing of the LM cells in 10% calf serum significantly increased plasma membrane phosphatidylcholine while decreasing phosphatidylethanolamine, calf serum had no significant effect on phosphatidylethanolamine or phosphatidylserine asymmetry.

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TNP-phosphatidylethanolamine, trinitrophenylphosphatidylethanolamine; TNP-phosphatidylserine, trinitrophenylphosphatidylserine.

Introduction

The asymmetric distribution of phospholipid species between the inner and outer monolayer of biological membranes has been implicated as having a role in platelet aggregation, sickle cell anemia, cell transformation, and coupling of the β-receptor with adenylate cyclase [1—5]. The vertical asymmetry in phospholipid distribution may also account for differences in fluidity between bilayer halves of tumour cell plasma membranes [6]. However, the origin and regulation of membrane lipid asymmetry are not understood at this time. It has been suggested that the sequential methylation of phosphatidylethanolamine in the erythrocyte membrane may be a significant mechanism for generation of membrane lipid asymmetry and for regulation of membrane fluidity [4,5]. Such a methylation system, however, could not account for the asymmetric distribution of lipids in neuronal and LM cell plasma membranes. Methylation of phosphatidylethanolamine to form phosphatidylcholine does not occur in either LM cells or the central nervous system, yet neuronal membrane phospholipids and LM cell phospholipids are asymmetrically distributed [7—9].

An alternative theory for the origin of the asymmetric distribution of lipids may be that the phospholipids in the plasma membrane are largely derived from exchange of serum lipid components with the surface membrane. Previous investigations imply that the asymmetric distribution of phospholipid may be an intrinsic property of eucaryotic cells. However, all investigations on the asymmetric distribution of phospholipids in plasma membranes of eucaryotic cells thus far reported have been conducted on cells exposed to serum, e.g., erythrocytes [10,11], hepatocytes [12] and fibroblasts [3]. Thus it may be possible that phospholipid asymmetry may not be an intrinsic property of eucaryotes, since the asymmetric distribution of phospholipid may be affected or induced by exchange processes. Herein we present data on the asymmetric distribution of plasma membrane aminophospholipids in a tumorigenic, transformed, murine fibroblast grown in the absence of serum, lipids and proteins.

Materials and Methods

Cell culture. LM cells, a choline-requiring strain of mouse fibroblasts (ATCC CCL 1.2), were grown in suspension culture in a serum-free chemically defined medium as described by Schroeder et al. [13].

Labelling reactions. TNBS, isethionyl acetimidate and methyl acetimidate, which bind covalently with primary amines of phospholipids and proteins, were utilized to chemically label phospholipid amino groups [10,14,15]. Whole LM cells or isolated subcellular organelles were treated with TNBS according to a previously published procedure [10] modified as follows: Reagent A (TNBS reagent) contained 4 mM TNBS, 30 mM NaCl, 120 mM $NaHCO_3$, 11 mM glucose, and 1% delipidized bovine serum albumin. The bovine serum albumin (fraction V, Pentex, Miles Research Labs., Elkhart, IN) was delipidized by the procedure of Wilcox et al. [16]. The reagent pH was adjusted to 8.5 with NaOH. Reagent B (Reaction termination reagent) was 0.15 M Tris-HCl, pH 7.0 (Sigma Chem. Co., St. Louis, MO). The treatment procedure was as follows: (a) 40×10^6 cells (exponential phase) were washed twice with phosphate

buffered saline [13] and incubated with 10 ml of TNBS reagent, (b) unless otherwise specified the treatment was carried out with 4 mM TNBS reagent at 4°C for 2 h, (c) the cells were maximally labelled by shaking at 140 rev./min on a New Brunswick Gyrotary Shaker (model G-10, New Brunswick, NJ), (d) the reaction was terminated by the addition of 40 ml of 0.15 M Tris-HCl, pH 7.0, followed by centrifugation, (e) the cells were washed again with 20 ml of the Tris-HCl buffer and then once with 10 ml of phosphate buffered saline. All washes were carried out with Tris-HCl and phosphate buffered saline solutions at 4°C. The isethionyl acetimidate or methyl acetimidate reactions with LM cells were conducted with the same buffer as the TNBS reaction except that the pH was 8.0. The incubation was for 2 h.

Membrane fractionation. Plasma membrane, microsome, and mitochondrial fractions were isolated from cells treated with TNBS, isethionyl, acetimidate, or methyl acetimidate as described previously [13]. The membrane fractionation was not altered by TNBS treatment since a similar degree of plasma membrane purification was obtained. There was an 8-fold increase in $(Na^+ + K^+)$-ATPase activity in each case. Plasma membranes, microsomes, and mitochondria were also isolated from non-treated cells [13] and subjected to TNBS treatment as described above. In addition, a lipid extract [17] of isolated membrane fractions was also treated for 2 h with 4 mM TNBS, 15 mM TNBS, 10 mM isethionyl acetimidate, or 10 mM methyl acetimidate in 1 ml chloroform/methanol (1 : 1) containing 0.1 ml 5% sodium bicarbonate [10] while either mixing on a Vortex Genie Mixer or sonicating for 2 h on a Branson Sonogen water bath sonifier (Cole-Parmer Co., Chicago, IL).

Analytical procedures. Aminophospholipid labelling by TNBS and lipid composition of isolated LM cell membranes was determined as follows: Lipids were extracted by the method of Bligh and Dyer [17]. Phospholipids were separated by two dimensional thin-layer chromatography (silica gel G plates, 250 μM thick, Analtech Inc., Newark, NJ). The plates were developed in the first direction with chloroform/methanol/water (65 : 25 : 4), dried under vacuum for 1 h, and then developed in the second direction with *n*-butanol/glacial acetic acid/water (6 : 2 : 2). After air drying for 4 h the plates were either (a) packaged with Kodak X-Omat L film (Eastman Kodak Co., Rochester, NY) for visualizing phospholipids prelabelled with $^{32}PO_4$ [13]; the phospholipid spots were then scraped into scintillation vials containing 3A70 (Research Products, Elk Grove Village, IL), and the radioactivity was determined with a Beckman LS 3155T liquid scintillation counter (Beckman Inc., Palo Alto, CA); or (b) the phospholipid spots were visualized lightly with iodine vapor, scraped, and then eluted with chloroform/methanol/1.5% ammonium hydroxide (6 : 5 : 1) with a scintered glass funnel. The phospholipids were transmethylated and the fatty acid composition determined by gas-liquid chromatography as described elsewhere [13]. TNP-[^{32}P]phosphatidylethanolamine and TNP-[^{32}P]phosphatidylserine were identified by comparison with standards synthesized from [^{32}P]phosphatidylethanolamine or [^{32}P]phosphatidylserine after reaction with TNBS. The percentage aminophospholipid labelling was determined from the ratio (cpm TNP-[^{32}P]phosphatidylethanolamine) to (cpm TNP-[^{32}P]phosphatidylethanolamine + [^{32}P]phosphatidylethanolamine) on each thin-layer chromatography plate. The percent TNP-phosphatidylserine was calculated similarly.

Ouabain sensitive ($Na^+ + K^+$)-ATPase activity was determined as previously described [13]. Protein was measured by the method of Lowry et al. [18].

Results

Effects of TNBS labelling on LM cell viability

There are several indications that TNBS labelling of the whole cells did not result in large alterations in cell membrane function or cell viability, thereby affecting aminophospholipid labelling. First, TNBS treatment did not affect the ($Na^+ + K^+$)-ATPase activity of the plasma membrane to any great extent, since there was only approximately a 20% decrease in the ($Na^+ + K^+$)-ATPase activity of cells treated with 4 mM TNBS as compared to non-treated cells (data not shown). These results are consistent with those of others who treated sarcoplasmic reticulum with an amino-reactive reagent and found little effect on ATPase activity [19]. Second, trypan blue exclusion indicated that exposure of the cells to a pH of 8.5 for 2 h at 4°C had little effect on cell viability since less than 1% of the cells did not exclude the dye. After 2 h treatment with 4 mM TNBS, pH 8.5, at either 4°C or 23°C, the cells remained 90% viable. Nonviable cells quickly lysed either during the treatment or the wash procedure, and since membranes of broken cells did not co-sediment with intact cells in our low speed centrifugation, these membrane fragments did not affect our results.

Validation of labelling procedure

The reactivity of TNBS with whole LM cells, isolated membrane fractions, and lipid extracts was tested to determine: (1) if the reagent was active and present in excess during our assay, (2) if penetration of reagent into the cell interior occurred, and (3) if all of the aminophospholipids were available for reaction with TNBS. Lipids extracted from a quantity of LM plasma membranes equivalent to that used in the whole cell TNBS treatment were dissolved in 1 ml of chloroform/methanol (1 : 1), containing 0.1 ml of 5% $NaHCO_3$ [10]. TNBS at 4 mM and at 15 mM labelled greater than 96% of the aminophospholipids at both 4°C and 23°C. These results indicated that the reagents were active and that the aminophospholipids reacted essentially to completion, provided that they were accessible to TNBS.

The effects of labelling temperature and of TNBS concentration on whole LM cells were investigated. After treatment of whole cells with TNBS the plasma membranes, microsomes, and mitochondria were purified as described previously [13]. Reagent penetration can be monitored by isolating the internal membranes of the cell and checking for labelled aminophospholipids. As shown in Table I, at both 4°C and 23°C, 15 mM TNBS labelled between 80—92% of both phosphatidylethanolamine and phosphatidylserine in all membrane fractions. Other investigators have also found that TNBS above 4 mM induced permeation of the label into the cell [20]. These cells were probably leaky to TNBS since at concentrations of 15 mM TNBS, Trypan Blue was not excluded well. Significant amounts of microsomal and mitochondrial phosphatidylethanolamine were also derivatized with 4 mM TNBS at 23°C (31.7% and 5.5%, respectively), indicating that TNBS penetrated the plasma membrane of intact cells at this temperature. In contrast, under optimal condi-

TABLE I

DETERMINATIONS OF TNBS PENETRATION INTO LM FIBROBLASTS

LM cells were treated with TNBS (pH 8.5 for 2 h) as described in Methods. Determinations with ± values indicated the mean of three experiments ± S.E.

Treatment temperature (°C)	TNBS (mM)	% Phosphatidylethanolamine labelled		
		Plasma membrane	Microsomes	Mitochondria
23	1.5	19.5	7.5	1.5
23	4.0	26.9	31.7	5.5
23	15.0	89.5 *	87.0 *	92.0 *
4	1.5	4.1 ± 0.6	1.1 ± 0.2	0.6 ± 0.2
4	4.0	4.3 ± 0.3	1.7 ± 0.2	1.9 ± 0.4
4	15.0	80.5 *	84.0 *	91.0 *

* Under these conditions greater than 90% of the phosphatidylserine was also labelled.

tions for labelling only one side of the plasma membrane (4°C and 1.5—4 mM TNBS) little leakage of TNBS into the cells was evident since only 0.6—1.9% of microsomal or mitochondrial phosphatidylethanolamine was labelled.

Isolated microsomes and mitochondria were also treated at 4°C and 23°C (Table II). In all cases more than 50% of the phosphatidylethanolamine and phosphatidylserine were labelled. The data for microsomes labelled at 4°C can also be interpreted as being consistent with the absence of an asymmetric distribution of phosphatidylethanolamine in microsomes (assuming no penetration of the TNBS into the interior matrix of the microsomes). Reaction of isolated plasma membranes with 4 mM TNBS at pH 8.5 for 2 h at 23°C resulted in more than 80% labelling of phosphatidylethanolamine (data not shown). In addition, 40—50% of plasma membrane phosphatidylethanolamine was labelled within 3 min under conditions in which the isolated membrane fraction was first frozen at −20°C and then labelled at 4°C. These results indicate that plasma membrane, microsomal and mitochondrial phosphatidylethanolamine and phosphatidylserine can be derivatized with TNBS provided these aminophospholipids are available for reaction.

TABLE II

ISOLATION OF INTRACELLULAR MEMBRANE FRACTIONS FOLLOWED BY TREATMENT WITH TNBS

Microsomes and mitochondria were isolated from LM cells grown in suspension as described in Methods. The purified microsomes and mitochondria were then treated with 4 mM TNBS, pH 8.5 at the temperature indicated for 80—120 min.

Membrane Fraction	Treatment temperature 4°C		Treatment temperature 23°C	
	% Phosphatidylethanolamine labelled	% Phosphatidylserine labelled	% Phosphatidylethanolamine labelled	% Phosphatidylserine labelled
Microsomes	53.1 ± 1.5 (n = 5)	50.0	66.3 ± 2.8 (n = 3)	70.0
Mitochondria	62.8 ± 2.8 (n = 3)	58.5 ± 5.5 (n = 2)	78.5 ± 0.5 (n = 2)	74.0 ± 0.1 (n = 2)

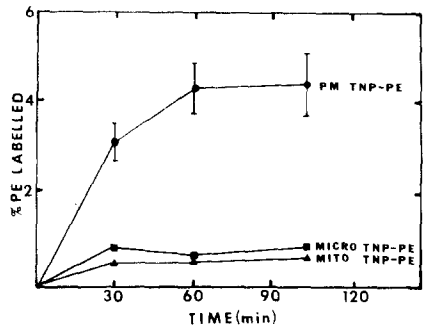

Fig. 1. TNBS labelling of aminophospholipids in membranes of choline-supplemented LM cells. About 40×10^6 cells were incubated in 10 ml of 1.5 mM TNBS, pH 8.5 at 4°C. The cells were then homogenized and the membrane fractions were isolated as described in Methods. Percent labelling was determined as described in Methods and calculated from [TNP-phosphatidylethanolamine]/[phosphatidylethanolamine + TNP-phosphatidylethanolamine]. Values with error bars indicate the mean of three determinations ± S.E. The values for microsomes and mitochondria deviated less than ±0.2 from the mean. PE refers to phosphatidylethanolamine.

TNBS labelling of whole cells

LM cells were grown in suspension culture and washed to remove all medium constituents. The cells were then treated with 1.5 mM TNBS at pH 8.5 and 4°C to label amino groups in the outer monolayer of the plasma membrane. As shown in Fig. 1, the plasma membrane phosphatidylethanolamine isolated from these cells was labelled to a maximum of 4%. Similar results were obtained with 4 mM TNBS, therefore 4 mM TNBS was henceforth utilized to ensure maximal labelling. In addition, as can be seen in Fig. 1, small amounts of microsomal and mitochondrial phosphatidylethanolamine were labelled indicating little leakage of TNBS into the cell. If there were leakage of TNBS into the cell, the percent microsomal and mitochondrial phosphatidylethanolamine labelled should have increased continuously with time. The plateau of microsomal and mitochondrial phosphatidylethanolamine labelling could be due to (a) the rapid labelling of phosphatidylethanolamine in a small number of damaged cells and/or (b) the cells leaky to TNBS were probably also leaky to other ions thus causing these damaged cells to lyse. The resulting highly labelled membrane fragments were then separated from the intact cells by the low speed centrifugation ($150 \times g \times 5$ min). In conclusion, our results of 4% labelled phosphatidylethanolamine in the purified plasma membrane fraction indicate a maximum of 4% phosphatidylethanolamine in the outer monolayer.

The 4% labelling of phosphatidylethanolamine in the eucaryotic LM cell agrees well with the most recent report of 5% labelled phosphatidylethanolamine with TNBS in the human erythrocyte [21]. To ascertain that the labelling pattern we observed with TNBS was not peculiar to this reagent we conducted the labelling treatment with two other covalent labelling reagents. In Table III are presented the results of labelling experiments utilizing an alternative non-penetrating reagent, isethionyl acetimidate [15]. Only 6% of plasma membrane phosphatidylethanolamine was acetimidated following treatment of LM cells with isethionyl acetimidate at 4°C, a value comparable to that obtained after treatment with TNBS. Temperature also appeared to be important with isethionyl acetimidate treatment, since the labelling of phosphatidyl-

TABLE III

WHOLE CELL LABELLING OF PHOSPHATIDYLETHANOLAMINE WITH IMIDATING REAGENTS

Imidating reagent	Treatment temperature (°C)	% Phosphatidylethanolamine labelled		
		Plasma membrane	Microsomes	Mitochondria
Isethionyl acetimidate *	4	6.1 ± 0.4	6.4 ± 0.1	10.3
	23	78.4	54.0	48.3
Methyl acetimidate **	4	80.6 ± 5.8	83.3 ± 3.9	68.9
	23	89.4	—	91.2

* LM cells were grown in the presence of choline as described in Methods. The cells were suspended in 30 ml of the same buffer (pH 8.5) used for the TNBS labelling reagent except that TNBS was omitted. The cells were treated by adding 0.25 ml 0f 0.8 M isethionyl acetimidate, pH 7.0, at 30, 60 and 90 min. At 120 min. the reaction was terminated with 0.15 M TRIS-HCl and the cells were washed with phosphate buffered saline as described in Methods. Membranes were isolated and phosphatidylethanolamine labelling was determined as described in Methods. Determinations with ± values represent the mean of 3 experiments ±S.E.

** All procedures same as above except that methyl acetimidate was used instead of isethonyl acetimidate.

ethanolamine at 23°C was 50% or more in all three membrane fractions. Methyl acetimidate, a penetrating reagent [15] significantly labelled the phosphatidylethanolamine in all three membrane fractions at both 4°C and 23°C. These results indicate that if both sides of the bilayer membrane were exposed to TNBS, isethionyl acetimidate or methyl acetimidate about 80—90% of the plasma membrane phosphatidylethanolamine would probably be derivatized by these reagents. The remaining 10—20% phosphatidylethanolamine may be tightly bound to membrane proteins and thus inaccessible to these labelling reagents.

TABLE IV

DISTRIBUTION AND COMPOSITION OF THE PHOSPHATIDYLETHANOLAMINE ACYL GROUPS BETWEEN THE INSIDE AND OUTSIDE MONOLAYERS OF THE LM CELL PLASMA MEMBRANE

Whole LM cells were treated with 4 mM TNBS, pH 8.5 and 4°C for 2 h. The phosphatidylethanolamine and trinitrophenylated phosphatidylethanolamine were separated by thin-layer chromatography and the fatty acid composition was analyzed by gas-liquid chromatography as described in Methods. L/S refers to the ratio of long chain fatty acids (18 carbons or more) to short chain fatty acids (14—17 carbons). U/S refers to the ratio of unsaturated fatty acids. Values represent the average of two determinations with individual values deviating less than 10% from the average.

Fatty acid	Fatty acid composition (% by wt.)	
	Trinitrophenylphosphatidylethanolamine	Phosphatidylethanolamine
14 : 0	2.3	0.6
15 : 0	0.1	0.1
16 : 0	41.1	9.9
16 : 1	2.8	6.5
18 : 0	34.1	9.0
18 : 1	3.1	47.9
18 : 2	1.2	1.7
C > 18	11.4	21.0
L/S	1.0	3.9
U/S	0.1	2.4

TABLE V

PHOSPHOLIPID COMPOSITION OF SUBCELLULAR MEMBRANE FRACTIONS OF LM CELLS CULTURED IN THE PRESENCE AND ABSENCE OF CALF SERUM

Values were determined by thin-layer chromatography on lipid extracts of membrane fractions from cells homogeneously labelled with $^{32}PO_4$. Each value represents the mean ± S.E. Numbers in parenthesis represent n, the number of experiments.

Supplement	Phospholipid composition (%)			
	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Phosphatidyl-serine
Choline				
Plasma membrane	36.60	33.86	3.81	4.30
Microsomes	31.65	35.10	2.93	3.97
Mitochondria	44.47	30.43	6.63	2.49
Choline + serum				
Plasma membrane	51.25 ± 2.16 (4)	21.69 ± 0.53 (4)	7.41 ± 0.53 (4)	4.96 ± 0.57 (4)
Microsomes	49.93 ± 2.09 (4)	22.74 ± 0.98 (4)	7.78 ± 1.03 (4)	5.69 ± 0.31 (4)
Mitochondria	43.52 ± 0.91 (4)	29.45 ± 0.44 (4)	6.68 ± 0.49 (4)	3.19 ± 0.31 (4)
Calf serum *	95.1	0.8	—	—

* Lipids were extracted from 10 ml of calf serum and the phospholipids were resolved by thin-layer chromatography as described in Methods. The spots were scraped, eluted, and the quantity was determined by phosphate assay.

Phosphatidylethanolamine acyl group asymmetry

The above studies indicated an asymmetric distribution of aminophospholipids across the LM surface membrane. The possibility that phospholipids from the outer monolayer have a different acyl group composition than phospholipids from the inner monolayer was examined. Table IV shows the result of fatty acid analysis of phosphatidylethanolamine and of TNP-phosphatidylethanolamine obtained from the plasma membranes of cells grown in the presence of choline and then treated with TNBS. The data indicated that the unlabelled phosphatidylethanolamine (most of which we consider to be in the inner monolayer of the plasma membrane bilayer) had a higher ratio of unsaturated to saturated fatty acids (U/S ratio) and a higher ratio of long chain (18 carbons or more) to short chain fatty acids (14—17 carbons).

In order to determine if TNBS may react preferentially with phosphatidylethanolamine acylated with more saturated fatty acids, plasma membrane and microsomal membrane fractions were isolated from LM cells and treated with 4 mM TNBS at 4°C. Aliquots were taken at 3, 5, 7, 10, 20 and 40 min. In both membrane fractions the fatty acid composition of the TNP-phosphatidylethanolamine compared to that of phosphatidylethanolamine was the same at all time points. Similar investigations were performed with purified dipalmitoylethanolamine and bacterial phosphatidylethanolamine and both were equivalently labelled with TNBS. This indicated that TNBS did not selectively label phosphatidylethanolamine with saturated fatty acyl groups.

Phospholipid composition of LM cells grown with serum

Table V shows that, when LM cells were grown for six days in medium con-

Sphingomyelin	Phosphatidyl-glycerol	Lysophos-phatidyl-choline	Cardiolipin	Other
2.68	9.80	5.15	—	3.80
6.19	6.52	7.77	—	5.87
1.45	2.07	2.57	4.61	5.28
2.99 ± 0.40 (4)	2.38 ± 0.19 (4)	4.64 ± 0.91 (4)	—	5.45 ± 1.80 (4)
2.89 ± 0.56 (4)	1.89 ± 0.22 (4)	4.32 ± 0.29 (4)	—	6.86 ± 1.32 (4)
1.54 ± 0.28 (4)	2.38 ± 0.38 (4)	1.58 ± 0.26 (4)	6.67 ± 0.40 (4)	4.95 ± 0.53 (4)
2.5	0.8	—	0.8	—

taining 10% calf serum, the phosphatidylcholine content was increased 1.4- and 1.6-fold in plasma membrane and microsomal membrane fractions respectively while the phosphatidylcholine content of the mitochondrial fraction was not affected significantly. The increases in phosphatidylcholine were compensated for by corresponding decreases in phosphatidylethanolamine. The changes in phosphatidylcholine and phosphatidylethanolamine are reasonable in view of the lipid content of serum (Table V) and the known ease of exchange of lipids between plasma membranes and the media bathing them [22]. It is interesting to note that the phosphatidylinositol content of the plasma membrane and microsomes from serum supplemented cells was almost twice that of choline

TABLE VI

TNBS LABELLING OF PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE IN WHOLE LM CELLS

Cells were treated with 4 mM TNBS at 4°C and pH 8.5 for 120 min as described in Methods.

Culture media	Phospholipid	% Aminophospholipid labelled		
		Plasma membrane	Microsomes	Mitochondria
without calf serum *	Phosphatidylethanolamine	4.3 ± 0.3	1.7 ± 0.2	1.9 ± 0.4
	Phosphatidylserine	4.6 ± 2.0	11.8 ± 1.3	4.9
with 10% calf serum **	Phosphatidylethanolamine	3.5 ± 0.3	3.1 ± 0.5	2.1 ± 0.4
	Phosphatidylserine	2.1 ± 0.6	2.3 ± 0.6	1.1 ± 0.7

* Values indicate the mean of three experiments ±S.E.

** Values indicate the mean of four experiments ±S.E.

supplemented cells. The reason for this is not known.

Since LM cells did show an asymmetric distribution of phosphatidylethanolamine when grown in medium without serum, the possibility that serum could have an influence on the asymmetric distribution of phosphatidylethanolamine or phosphatidylserine was investigated. Cells propagated for six days in medium containing 10% calf serum had 4.3% ± 0.9 (n = 4) of the plasma membrane phosphatidylethanolamine trinitrophenylated (Table VI). Phosphatidylserine also appeared to be distributed asymmetrically in the plasma membrane. About 5% of the phosphatidylserine was trinitrophenylated when cells propagated without calf serum were treated with 4 mM TNBS at 4°C. If the cells were grown in medium containing 10% calf serum, the percent of labelled phosphatidylserine decreased to approx. 2%.

Discussion

The present studies indicate that the asymmetric distribution of aminophospholipids in LM cell plasma membranes is an intrinsic property of the cell. The advantage of using LM cells in our studies lies in the fact that they will grow in the absence of serum or hormones. Our simple chemically-defined medium supports LM cell growth indefinitely and permits easy manipulation of individual factors that may regulate or affect membrane phospholipid asymmetry. Phospholipids of serum can exchange with those of the plasma membrane. We have shown that, although supplementing the medium with 10% calf serum increased phosphatidylcholine and decreased phosphatidylethanolamine by an approximately equivalent amount, serum had no large effect on the asymmetric distribution of phosphatidylethanolamine and phosphatidylserine. This indicates that the asymmetric distribution of aminophospholipids is an intrinsic property of at least some mammalian cells.

The results presented here also indicated that a greater percent of unsaturated phosphatidylethanolamine acyl groups were in the inside monolayer phosphatidylethanolamine. An asymmetric distribution of acyl chains has recently also been shown in vesicular stomatitis virus and *Acholeplasma laidlawii* [23,24]. Based on the knowledge of the acyl group composition of the major phospholipids and their asymmetric distribution in plasma membranes Emmelot and Van Hoeven [25] hypothesized that the inside monolayer would contain about twice as many double bonds as the outer monolayer. Unsaturation and polar head group composition appear to be important determinants of lipid fluidity. It has been shown that the asymmetric distribution of dimyristoylphosphatidylethanolamine between the two monolayers of small single-walled vesicles is the result of the combined properties of the acyl chain and polar head group composition [26]. Therefore, since phosphatidylethanolamine is located primarily in the inner monolayer, lipid fluidity in the two monolayers of the plasma membrane bilayer may be asymmetrically distributed. Recent investigations utilizing a fluorescence quenching technique indicate that the inner monolayer of LM plasma membranes is more rigid than the outer monolayer [6]. However, this is contrary to what one would expect based only on fatty acyl group asymmetric distribution. The polar head group as well as acyl composition of the phospholipid may therefore both be important factors in

determining the fluidity gradient. Recent model membrane investigations also indicate that the two bilayer halves are only weakly coupled and that their fluidity may be independently regulated [27].

It might be expected that the TNBS reagent used herein should label all of the phosphatidylethanolamine at penetrating conditions. However, 100% labelling of phosphatidylethanolamine in the plasma membrane did not occur. Even under penetrating treatment conditions (4 or 15 mM TNBS, 23°C, 2 h, Table I) 10—20% of the membrane-bound phosphatidylethanolamine was unavailable for reaction. Other investigators have also made similar reports [20]. This may reflect the presence of some phosphatidylethanolamine that is tightly bound to integral membrane protein such as is found with the phospholipid annulus surrounding the calcium transport protein [28]. In support of this, it has also been found in plasma membrane vesicles of suspension-grown LM cells that approximately 10% of the lipid is inaccessible to the fluorescent probe β-parinaric acid [29]. In conclusion, the trinitrophenylated phosphatidylethanolamine may represent molecules present in easily accessible domains. Nevertheless, these values still demonstrate a large asymmetric distribution of phosphatidylethanolamine in the neoplastic LM cell.

Our data also represent a first demonstration of an asymmetric-distribution of phosphatidylethanolamine and phosphatidylserine in plasma membranes isolated from a neoplastic cell line. Previous investigators of aminophospholipid asymmetry in transformed cells did not isolate the plasma membranes but used whole cell lipid extracts instead [3]. In agreement with the asymmetric distribution of aminophospholipids in LM cell plasma membranes described herein, other investigators have found that phagosome membrane derivatives of LM cells also had an asymmetric distribution of phosphatidylethanolamine [30].

In summary, the data presented here indicate that serum does not affect the distribution of aminophospholipids across the LM cell plasma membrane. In addition the results indicate that the plasma membrane of transformed cells is also asymmetric with regard to aminophospholipid acyl group distribution.

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