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TRANSLOCATION AND TURNOVER OF PHOSPHOLIPID ANALOGS IN PLASMA MEMBRANE-DERIVED VESICLES FROM CELL CULTURES

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Summary

The time-dependent accumulation of phosphatidyldimethylethanolamine in formaldehyde-induced vesicles obtained from a somatic cell hybrid line was investigated. From a number of considerations including a two-fold enrichment of cholesterol and sphingomyelin it was concluded that these vesicles were derived from the cell plasma membrane.

A progressive depletion of phosphatidylcholine, the major vesicle phospholipid, was observed in cells supplemented for various time periods with dimethylethanolamine. This depletion was accompanied by a concomitant increase in the amount of lipid analog.

The time-dependent alteration of the phospholipid polar head group in intact cells was almost identical to that observed in isolated plasma membrane vesicles, suggesting a rapid equilibration of the de novo synthesized phospholipid with the cell surface compartment. From the initial velocity rate, the time required for the phosphatidylcholine pool to double was about 12 h.

Agarose-linked phospholipase A_2 was used to measure the relative composition of choline- and dimethylethanolamine-phosphoglycerides in the outer surface of vesicles prepared from cells with different degrees of polar head group substitution. The gradual appearance of lysodimethylethanolamine lipid analog in vesicles treated with phospholipase A_2 suggested an asymmetric distribution of the phospholipid between the interior and the exterior part of the vesicle. This asymmetry was maximal up to about 4 h following the addition of dimethylethanolamine to the culture medium and was of a transient nature as the lipid analog accumulated on both sides of the plasma membrane. Based on these measurements a fast followed by a slow translocation component could be distinguished with apparent doubling times of 7 and 43 h for the lipid analog, respectively. As the analog becomes the predominant cellular phospholipid a significant increase in the vesicle lipid fluidity was measured.

Introduction

In recent years methods have been devised for altering the phospholipid composition of mammalian cells by introducing into cell culture systems fatty acids of various chain length and degree of unsaturation [1—4], as well as base analogs which are incorporated into polar head groups [5—7]. These probes may shed light on the intimate relationship between the dynamic characteristics of the membrane lipids and the mobility of membrane proteins, both concertedly contributing to the wide spectra of cell membrane functions [3,8—11].

Unlike fatty acids which can often be integrated into preassembled membrane phospholipids by direct exchange mechanisms [12], incorporation of nitrogen base analogs seems to proceed entirely via the de novo synthesis [13,14] and therefore requires several enzymes associated with more than one subcellular compartments. Two aspects of this model which require further clarification are the time course during which these phospholipid analogs are conveyed to the plasma membrane and the mechanism by which they appear at the outer leaflet of the plasma membrane.

In the search for a method for plasma membrane sampling to match the rapid rates at which polar head groups are substituted as a result of base analog supplementation to living cells, we have adopted the formaldehyde vesicle shedding technique recently described by Scott [15]. Using this approach we have obtained estimates for the apparent rates at which dimethylethanolamine and choline-containing phospholipids are conveyed and subsequently distribute between the inner and the outer leaflet of the cell membrane.

Materials and Methods

Cell culture. Somatic hybrid cells, NG108-15, of neuroblastoma N18TG-2 x glioma C6BU-1 parent lines were developed in the laboratory of Dr. M. Nirenberg (NIH, Bethesda). Cells were routinely grown as monolayer cultures in Dulbecco-Vogt modified Eagle's medium (Cat. No. H-21, Grand Island Biological Co., Grand Island, NY) supplemented with 4% fetal calf serum (Rehatuin, Reheis, Chicago, IL), 4.8 mg/l thymidine and 12 mg/l hypoxanthine, at 37° C in a humidified atmosphere consisting of 4% CO_2/96% air. Cell passage was done at weekly intervals by dislodging the cell monolayer with a pasteur pipette in Puck's D_1 solution. All metabolic studies were performed on cells between passage No. 15—25.

Isolation of formaldehyde-induced plasma membrane vesicles. The procedure for the isolation of plasma membrane vesicles was largely based on the method described by Scott [15]. Monolayer cultures grown in 10-cm diameter dishes were rinsed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, pH 7.4, and subsequently incubated at 37° C in an atmosphere of 100% air with 5 ml of 155 mM formaldehyde, 2 mM dithiothreitol and 1 mM Ca^{2+}, 0.5 mM Mg^{2+} in phosphate-buffered saline. Appearance of vesicles was routinely monitored by phase contrast microscopy. After 1.5—2 h the formaldehyde solution containing less than 15% of the total vesicle yield was discarded and the cultures were washed with 2.5 ml of vesicle-releasing buffer consisting of cold Hank's, adjusted to pH 8.6

with 0.18 g/l degassed sodium bicarbonate. Following a brief trituration the great majority of vesicles were released into solution. The latter suspension was further used for vesicle harvesting from 2—3 additional dishes and finally transferred to a glass conical tube. The cell monolayer was subsequently rinsed with 2.5 ml of the releasing buffer and the combined supernatants centrifuged at $100 \times g$ for 3 min, to remove possibly dislodged cells. The supernatant containing the purified vesicles was either used directly for biochemical analyses or further centrifuged at $10\,000 \times g$ for 1 h at 10°C. The resulting pellet was suspended in the experimental buffer described above and stored at 4°C or subjected immediately to lipid extraction.

Lipid analyses. Lipids were extracted and purified by a two-directional thin-layer chromatography procedure on precoated silica gel plates (Merck, Darmstadt) as described in detail elsewhere [16]. Phosphorus was determined according to Bartlett [17]. Whenever the phospholipid content in vesicles was below chemical determination, the concentration was calculated from the corresponding ^{32}P-labeled cellular phospholipids after extended incubation with $^{32}P_i$ [18]. Cholesterol was determined either on the total lipid extract prior to chromatography or after extraction from the silica gel by using the enzymatic kit obtained from Calbiochem. The amounts of the purified cholesterol observed were slightly higher (10—15%) than those which were obtained from the total lipid extract prior to chromatography.

Metabolic studies. The experimental conditions for introducing polar head group alterations employing either 5 mM dimethylethanolamine or the reversal using 5 mM choline have been described in detail elsewhere [13]. Unless otherwise stated all experiments were done in triplicate cultures and were repeated at least twice.

Enzymatic assay of phospholipase A_2. Agarose-linked phospholipase A_2 from bee venom (Sigma, St. Louis, MO) was centrifuged at $3000 \times g$ for 15 min and the pelleted beads washed twice with 0.8% NaCl, 0.04% KCl, 0.02% $MgSO_4$ in 20 mM Tris-HCl buffer (pH 8.5), and finally suspended in the above buffer at a concentration of 5 units/ml. The $10\,000 \times g$ vesicle pellet was suspended in the same buffer, and the reaction was started by adding 0.5 unit of the enzyme and 1 mM $CaCl_2$/0.2 μmol vesicle phosphorus. The incubation was carried out at 37°C with gentle shaking to prevent settling of the beads. At designated times the reaction was stopped by adding 5 mM EDTA and 12 volumes of chloroform/methanol (2/1) and three volumes of water. After mixing and phase separation, the ensuing chloroform layer was subjected to thin-layer chromatography and ^{32}P-labeled phospholipids exposed to autoradiography (Kodak, XRP-54 film) and radioactivity and inorganic phosphorus determined as described elsewhere [16].

Miscellaneous analytical methods. Protein was determined according to Lowry et al. [19] and DNA according to Burton [20] on lipid-extracted material as described previously [13]. Fluorescence polarization of vesicles was determined on an instrument designed by Dr. M. Shinitzky. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene ($1 \cdot 10^{-6}$ M) was incubated for 20 min at 37°C with the vesicles (0.05—0.1 μmol phosphorus) in phosphate-buffered saline (pH 7.4) and the temperature profile was measured as described by Shinitzky and Inbar [21].

Results

Characterization of formaldehyde-induced plasma membrane vesicles

A typical phase-contrast micrograph of the somatic hybrid cell line fol-

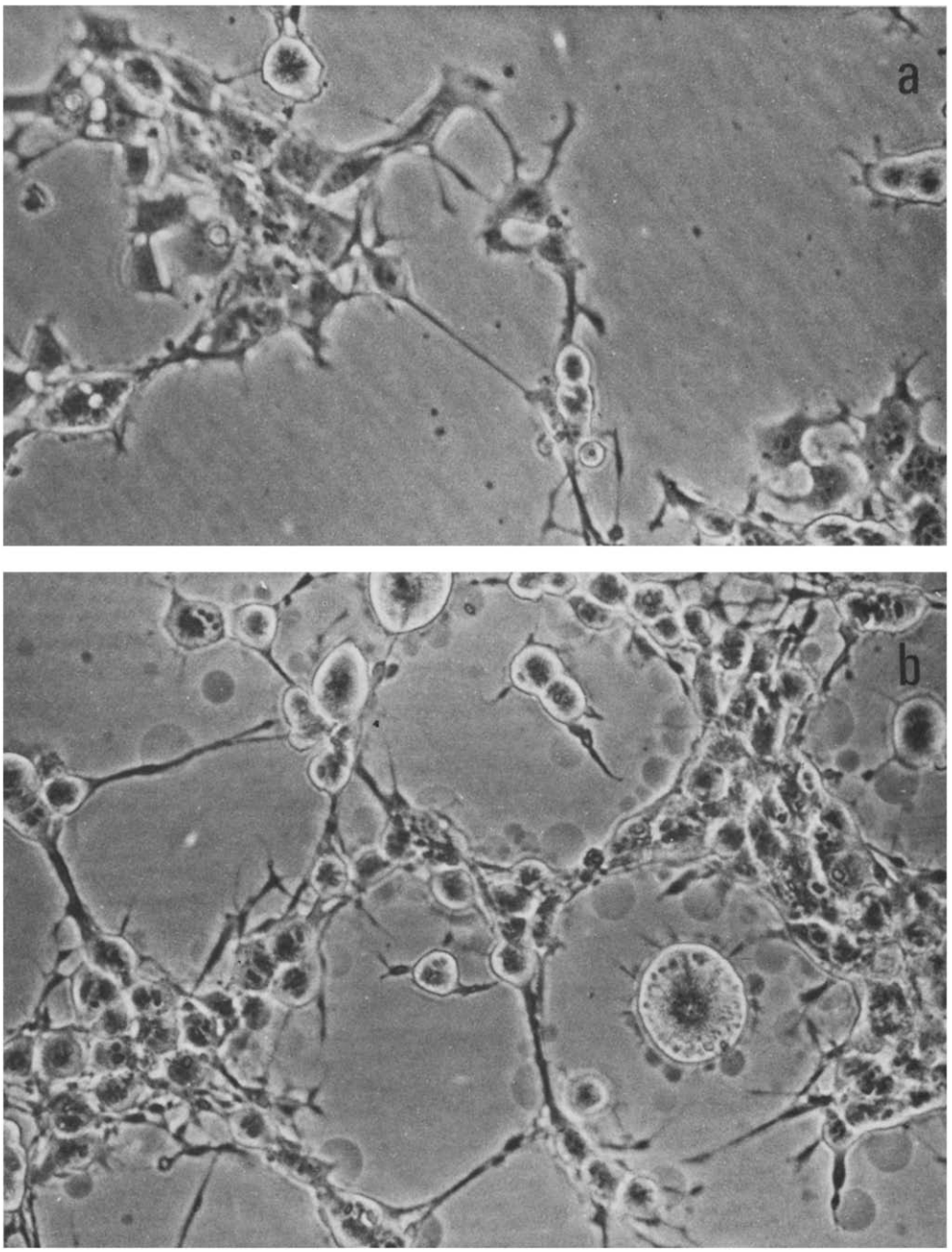

Fig. 1. Phase contrast micrographs of cells before (a) and after (b) formaldehyde treatment. Note the appearance of vesicles of varying dimensions in close apposition to the cell body and the neuritic extension. Magnification, X100.

lowing exposure to formaldehyde, illustrating both cells and adjacent plasma membrane vesicles is shown in Fig. 1. The majority of microscopically visible vesicles budding both from the cell body and neuritic extensions were firmly attached to the surface. The process of vesicle formation was time and temperature dependent and was most prominent in the presence of Ca^{2+} at a physiological pH as noted by Scott for other cell systems [15]. Vesicle formation was also found in the absence of dithiothreitol, although the yield was considerably lower. When 155 mM formaldehyde was used, shedding of vesicles was maximal between 1.5 and 2 h after the addition of the cross-linking reagent. No secondary vesicle bursting occurred when fixed cells were repeatedly treated with fresh formaldehyde. At 10—20 mM formaldehyde, vesicle formation was equally efficient, but was accompanied by marked cell detachment; hence these concentrations were not routinely used. Highly enriched vesicle preparations could be separated from the bulk of fixed cells by using a moderately alkaline buffer solution. The suspension thus obtained was freed of cell contaminants by low speed centrifugation and contained about 5.6% of the cellular phospholipids and 4.8% of the cellular proteins and essentially no DNA as shown in Table I. A two-fold enrichment of cholesterol was obtained in the isolated vesicle. When pelleted at 10 000 × *g* for 1 h, the vesicle yield, as assessed by the phospholipid and cholesterol content decreased 30%. This was accompanied by a greater loss of proteins and as a result the phospholipid/protein ratio in vesicles increased more than three-fold.

Analysis of the phospholipid composition in vesicles indicated significant changes compared to those seen in whole cells, as evident from Table II. A relative enrichment of phosphatidylcholine and a two-fold increase in the content of vesicle sphingomyelin was evident. In contrast, the relative amount of serine phosphoglycerides and acid-labile (1-alkenyl, 2-acyl) and acid-stable (1,2-diacyl) phosphatidylethanolamine was greatly reduced in vesicles as opposed to non-treated cells. From Table II, it is also evident that the ethanolamine phospholipid species isolated from formaldehyde-treated cells was 37%

TABLE I

PHOSPHOLIPIDS, CHOLESTEROL, PROTEIN AND DNA CONTENT AT DIFFERENT STEPS OF PLASMA MEMBRANE VESICLES ISOLATION

Formaldehyde-induced plasma membrane vesicles were prepared as described under Materials and Method. The 100 × *g* vesicle suspension was dialysed for 24 h against two changes 0.5 l each of 0.8% NaCl and 20 mM Tris-HCl (pH 8.5) and centrifuged for 1 h at 10 000 × *g*. Values represent a typical preparation from three dishes. The variation of lipid phosphorus and cholesterol content among different batches was about 5 and 10%, respectively. For estimation of DNA in vesicles cells were prelabeled with [^{3}H]thymidine and the counts residing in vesicles was divided by the cellular DNA specific radioactivity.

Purification steps	Cholesterol (nmol)	Lipid P_i (nmol)	Protein (mg)	DNA (μg)	Cholesterol/ lipid P_i (molar ratio)	Lipid P_i/ protein (nmol/mg)
Cells	619	2940	17.4	1640	0.21	169
Vesicles						
Supernatant (100 × *g*)	69.3	165	0.83	1.6	0.42	199
Dialysed	69.3	165	0.80	1.6	0.42	206
Pellet (10 000 × *g*)	48.5	116	0.20	1.6	0.42	580

TABLE II

DISTRIBUTION OF PHOSPHOLIPIDS IN NG 108-15 CELLS AND MEMBRANE VESICLES

Experimental details were described under Materials and Methods. Values expressed as percent of lipid phosphorus represent averages ± S.E. from duplicate cultures of three separate experiments.

Phospholipid class	Cells (mol%)		Vesicles (mol%)
	Non-treated	Formaldehyde treated	
Phosphatidylinositol	6.8 ± 0.2	9.0	5.9 ± 0.6
Phosphatidylserine	4.5 ± 0.4	4.4	2.3 ± 0.1
Sphingomyelin	4.4 ± 0.2	5.1	10.6 ± 1.0
Phosphatidylcholine *	58.6 ± 4.1	64.6	70.6 ± 3.5
Phosphatidylethanolamine			
Acid labile	9.4 ± 0.4	5.1	3.8 ± 0.4
Acid stable	9.9 ± 0.6	7.0	4.5 ± 0.4
Cardiolipin	2.5 ± 0.1	2.4	0

* Including acid labile.

lower than that observed in non-treated cultures. Preliminary studies suggest that part of the phosphatidylethanolamine was not extracted by chloroform/methanol. The absence of cardiolipin in the isolated vesicles most probably indicates that no mitochondrial lipid contamination was present.

Time-dependent course of dimethylethanolamine- and choline-phosphoglycerides accumulation in plasma membrane vesicles

In a recent study we have demonstrated that incorporation of nitrogen base analogs such as monomethyl- and dimethylethanolamine in neuroblastoma cell cultures proceeded entirely via a phosphate-mediated pathway [13]. As a result of nitrogen base competition, presumably on similar enzymes the newly synthesized phospholipids accumulated rapidly in cells and displaced the preexisting phospholipids. This process could be reversed by adding the counterpart nitrogen base at appropriate concentrations. It was therefore of interest to investigate the rate of the appearance of lipid analogs in vesicles and compare it to that of intact cells in order to probe the mechanism by which lipid components are conveyed to, and subsequently distribute into the plasma membrane. For this study the nitrogen base pair, choline and dimethylethanolamine was chosen, since phosphatidylcholine accounted for the bulk of the vesicle phospholipids.

Table III shows the time-dependent accumulation of phosphatidyldimethylethanolamine in whole cells and purified plasma membrane vesicles. With the exception of the first time point studied, it became evident that the content of phosphatidyldimethylethanolamine increased both in the vesicles and whole cells in a parallel manner and by 24 h reached a value slightly greater than that of phosphatidylcholine. Since the latter phospholipid was proportionally reduced, the molar ratio of the sum of the two phospholipids to cholesterol remained practically constant throughout the experiment, in accord with our recent observations [22].

Similar results were obtained when phosphatidyldimethylethanolamine-

TABLE III

CHANGES IN THE MAJOR CELLULAR AND PLASMA MEMBRANE VESICLE PHOSPHOLIPIDS AFTER ADDITION OF DIMETHYLETHANOLAMINE

About 48 h after seeding, cells were incubated with regular medium containing 5 mM dimethylethanolamine and 15 μCi $^{32}P_i$/dish. At times designated the experimental medium was removed and plasma membrane vesicles prepared as described under Materials and Methods. Vesicles and harvested cells pooled from 3—4 petri dishes were subjected to lipid extraction and thin-layer chromatography separation, as detailed under Materials and Methods. Values given as mol lipid phosphorus/mol cholesterol.

Time in presence of dimethylethanolamine (h)	Vesicles (molar ratio)		Cells (molar ratio)	
	Phosphatidyl-choline	Phosphatidyl-analog	Phosphatidyl-choline	Phosphatidyl-analog
1.5	1.80	0.06	3.75	0.17
4.0	1.70	0.14	3.50	0.31
8.0	1.49	0.31	3.42	0.66
24.0	0.85	0.95	1.96	2.03

modified cells were supplemented with 5 mM choline, as shown in Fig. 2. The rates of phosphatidylcholine appearance in purified vesicles paralleled the rates of accumulation in whole cells. This was also evident from the similarities of the specific radioactivity values of ^{32}P-labeled phosphatidylcholine isolated from cells and vesicles (not shown in the figure). These observations suggest a rapid transport of the novel phospholipid from its site of synthesis to the plasma membrane compartment. The kinetics of phosphatidylcholine appearance in vesicles was fitted using the equation $([a_\infty] - [a]) = e^{-kt}$, where $[a_\infty]$ is the steady-state concentration of phosphatidylcholine and [a] is the concentration observed at time t. The data conformed to a straight line on a semi-

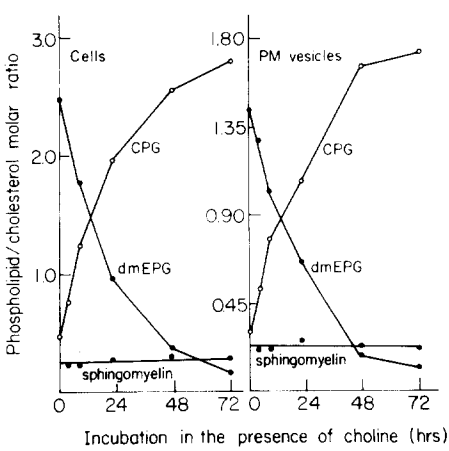

Fig. 2. Time-dependent concentration of phosphatidylcholine in whole cells and plasma membrane vesicles, following addition of choline. Cells were cultured for one week with 5 mM dimethylethanolamine and thereafter replated and grown in the presence of 5 mM choline. At times designated cells were treated with formaldehyde and plasma membrane vesicles isolated as described under Materials and Methods. Lipids were extracted from cells and vesicles and lipid phosphorus of phosphatidyldimethylethanolamine (dmEPG), phosphatidylcholine (CPG) and sphingomyelin was determined. Values expressed as mol phosphorus/mol cholesterol represent pooled lipid extracts from three cultures.

logarithmic plot, the slope of which suggested a doubling time for vesicle phosphatidylcholine pool of 12 ± 1 h.

Phospholipid analog translocation

In order to measure the relative rate of appearance of the lipid analog at the outer surface, vesicles were exposed to phospholipase degradation [23]. The agarose-linked phospholipase A_2 appeared to us a proper tool for such studies for (a) its ability to catalyse equally effectively the cleavage of the 2-acyl position of both choline- and dimethylethanolamine phosphoglycerides as suggested from its minimal substrate requirement considerations [24] and (b) its large molecular weight which may reduce its penetrability. When treating plasma membrane vesicles obtained from control cultures with phospholipase A_2 about 60% of the total phosphatidylcholine was degraded during the first hour of incubation as seen in Fig. 3. Extending the incubation time up to 4 h did not result in more than a moderate increase in the amount of degraded substrate. A significant portion of phosphatidylcholine was unavailable to the enzyme presumably by being located on the interior surface of the vesicle thus supporting a bilayered structure for the vesicle membrane. As also illustrated in Fig. 3, after 4 h of incubation, about half of the inositol- and ethanolamine-containing phospholipids (the latter not drawn) were degraded. Therefore incubations of shorter duration were undertaken to minimize the degradation of internalized phospholipids.

Fig. 4 shows an experiment in which vesicles with varying amounts of ^{32}P-labeled phosphatidylcholine and phosphatidyldimethylethanolamine species were treated with phospholipase A_2 for a period of 20 min, and the resulting phospholipids separated by thin-layer chromatography. It is evident that the amount of radioactivity in the dimethylethanolamine lipid analogs (spots

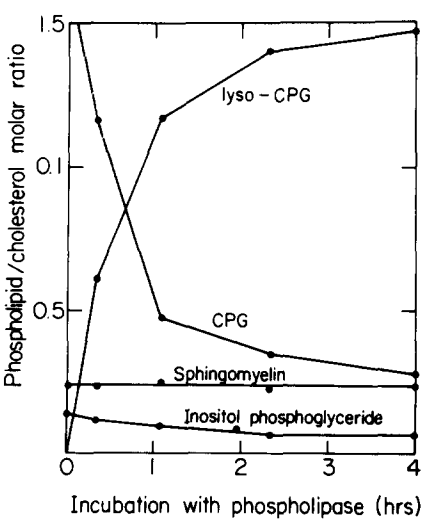

Fig. 3. Time-dependent degradation of major vesicle phospholipids by phospholipase A_2. Preparation of vesicles and incubation conditions have been described under Materials and Methods. CPG, phosphatidylcholine.

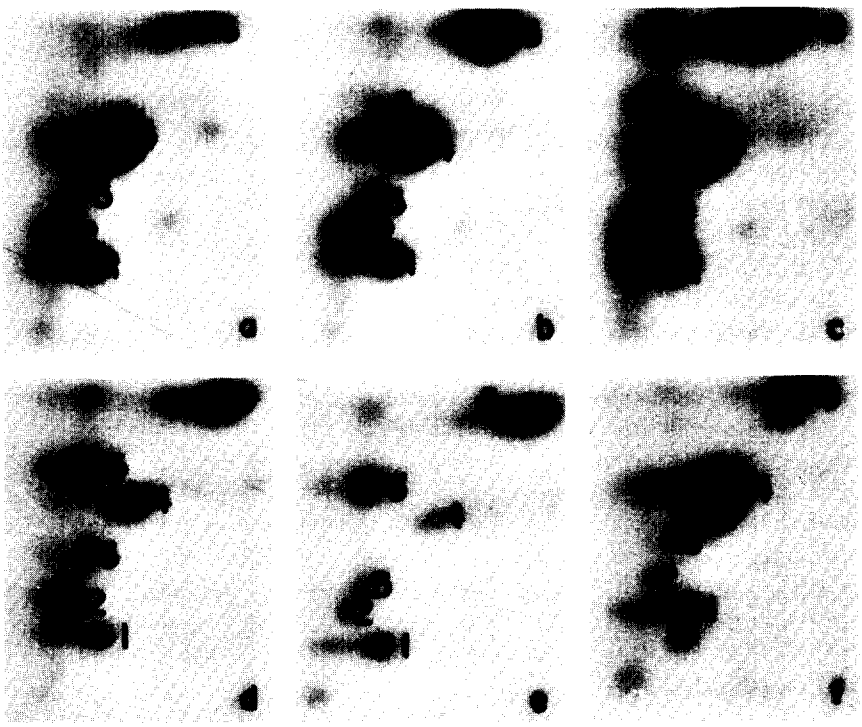

Fig. 4. Relative migration of ^{32}P-labeled phospholipids isolated from vesicles exposed to phospholipase A_2 cleavage. Incubation conditions with dimethylethanolamine and $^{32}P_i$ were as described for Table IV. Plasma membrane vesicles isolated from cells after 1.5, 4, 8, 24 and 72 h (a—e), respectively, were stored in NaCl/Tris-HCl buffer at 4°C as described under Materials and Methods. At the completion of the metabolic experiment vesicles from each time point were then treated for 20 min with phospholipase A_2 and phospholipids extracted, separated and radioactivity and P_i content measured. The numbers on the autoradiographs denote the migration of phosphatidylinositol (1), lyso phosphatidylcholine (2) sphingomyelin (3), phosphatidylcholine (4), lysophosphatidyldimethylethanolamine (5), and phosphatidyldimethylethanolamine (6). Phospholipase A_2—untreated vesicle phospholipid pattern after 1.5 h exposure to dimethylethanolamine as in (a) is depicted in the lower right-hand corner (f).

Nos. 5 and 6) increased whereas that of choline-containing phosphoglycerides (spots Nos. 2 and 4) decreased as the analog became the predominant vesicle phospholipid. This observation suggests that under these conditions phosphatidylcholine pool could not be replenished by any cellular biosynthetic mechanisms. Analysis of the various phospholipid pools of the vesicles after enzymatic hydrolysis (Table IV) indicate increasingly amounts of phosphatidyldimethylethanolamine which become susceptible to phospholipase A_2 degradation. Thus after 8 h exposure of cells to dimethylethanolamine the percent of phospholipase-susceptible analog fraction is less than 15% whereas by 24 h the amount of the lyso analog accounted already for about 30%. At this time both the analog and phosphatidylcholine were present in almost equimolar concentrations. The principal findings apparent from this experiment can be summarized as follows: (a) For about 4 h after supplementing cells with dimethylethanolamine, the resulting phospholipid analog was undetected at the exterior surface of the vesicle suggesting either its absolute internalization or

TABLE IV

MASS DISTRUBITION OF MAJOR VESICLE PHOSPHOLIPIDS FOLLOWING PHOSPHALIPASE A_2 TREATMENT

For details see Fig. 4. Values expressed as mol phosphorus/mol cholesterol represent pooled vesicles from three cultures.

Lipid class	Time of exposure to dimethylethanolamine (h)						
Phosphatidylcholine	1.28	1.30	1.22	1.01	0.43	0.28	0.16
Lysophosphatidylcholine	0.47	0.50	0.48	0.48	0.42	0.33	0.15
Phosphatidyldimethylethanolamine	0	0.06	0.12	0.27	0.70	0.75	0.92
Lysophosphatidyldimethylethanolamine	0	0	0.02	0.04	0.25	0.49	0.63

being beyond detection levels by the phospholipase because of substrate competition constraints. (b) The relative fraction of phosphatidylcholine susceptible to phospholipase cleavage increased as the amount of vesicle phosphatidylcholine decreased. (c) A greater proportion of phospholipid was degraded in phosphatidyldimethylethanolamine-enriched vesicles (see value at 72 h) than in control vesicles (time zero). A possible explanation for the enhanced hydrolysis is that in contrast to choline, the dimethylethanolamine-containing vesicles may be better exposed to phospholipase degradation. (d) The cross-linking reagent had induced plasma membrane vesicles which presumably contain the correct 'right-side out' topology as inferred from the gradual appearance of the lyso analog.

These observations enabled us to measure the time-dependent partition of the lipid analog between the inner and outer leaflet of the vesicle as shown in Fig. 5. Thus when plotting the logarithmic value of the phospholipase-susceptible lipid analog fraction out of the total lyso products formed; log([lyso analog]/[lyso analog] + [lysophosphatidylcholine]) against the time of incubation in the presence of the base analog, a straight line with a discontinuity at about 24 h was evident. From the resulting slopes a fast (α_f) and a slow (α_s) component with apparent doubling times of 7 and 43 h, respectively, could be estimated. These slopes intuitively represent the apparent rates of lipid analog

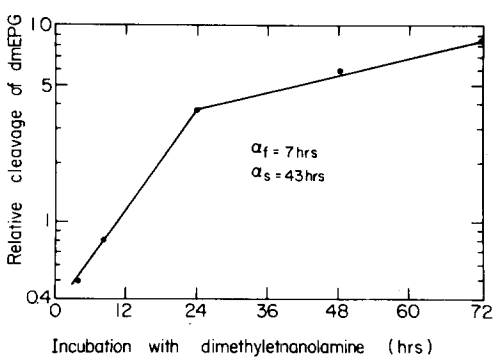

Fig. 5. Phospholipase-susceptible lipid analog fraction of vesicles obtained from cells exposed to dimethylethanolamine for different lengths of time. The experimental conditions as described for Fig. 4.

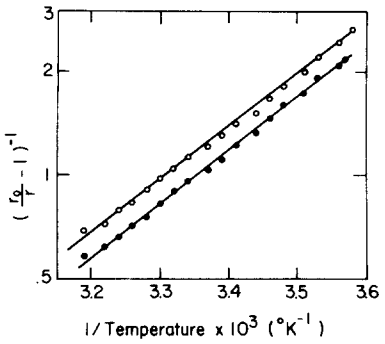

Fig. 6. Temperature dependence of fluorescence polarization in vesicles prepared from control (•) and dimethylethanolamine-supplemented (○) cultures. r_0 and r, the limiting and the measured fluorescence anisotropies, respectively [21,36].

translocation in the native membrane. Not unlikely is also the possibility that there are two distinctly compartimentalized analog pools, on the same leaflet, each expressing a different susceptibility to the phospholipase degradation. Thus in view of the limitations of the phospholipase A_2, in particular in its capacity to generate lysophosphoglycerides which may themselves modify the transbilayer lipid asymmetry, other complementary probes to test these possibilities are in order.

Fluorescence polarization measurements

The relative contribution of the dimethylethanolamine phospholipid analog to lipid fluidity was determined by the fluorescence polarization of the hydrocarbon fluorophore diphenylhexatriene in isolated vesicles. As illustrated in Fig. 6, the temperature profile of vesicles consisting of 54.5 and 4.1% acid-stable and acid-labile phosphatidyldimethylethanolamine, respectively, and 12.2% phosphatidylcholine was compared to that of vesicles in which phosphatidylcholine was the bulk phospholipid (70 mol%). When plotting the logarithm of the parameter $((r_0/r) - 1)^{-1}$ which is a first approximation of the lipid microviscosity [21] against the reciprocal of the absolute temperature, straight lines without discontinuities were obtained for both types of vesicles. This would most probably indicate a monophasic state of these membrane vesicles, in accordance with previous observations for other membrane preparations [25–27]. As is also evident from Fig. 6, the $((r_0/r) - 1)^{-1}$ value in the analog-enriched vesicles was significantly lower than that observed in control vesicles suggesting the latter to be more rigid. Since the content of cholesterol and other minor phospholipids are practically identical in both types of vesicles, the difference in the fluidity appears to be related to the number of methyl groups. Using the same fluorescence probe Esko et al. [26] reported a greater microviscosity in isolated plasma membranes of LM cells enriched with phosphatidylethanolamine.

Discussion

Formaldehyde-induced vesicles as plasma membrane derivatives

Several lines of evidence supporting the derivation of the formaldehyde-

induced vesicles from the cell surface rather than from internalized cell membranes were already presented by Scott [15]. These include the presence of antigenic determinants [28] membrane-associated enzymatic activities, ultrastructural characteristics and enrichment of certain lipid components. The higher content of cholesterol and sphingomyelin and the complete absence of cardiolipin, an exclusively mitochondrial lipid marker, in these vesicles in comparison with whole cells (Table I) are in notable agreement with this concept. However, the increase in the cholesterol/phospholipid and sphingomyelin/phospholipid molar ratios observed in our preparations are only moderate when compared to those of Scott.

From a number of considerations these vesicles seem to be appropriate for studying certain aspects of membrane lipid components assembly. First, they appear to be relatively stable sealed structures, as is evident from their light and electron microscopy appearance and ability to retain radionuclides such as rubidium and chromium (unpublished observations).

Second, the rather simplistic procedures for obtaining these preparations lends itself to measuring relatively fast membrane-associated metabolic events. Third, unlike particle-mediated plasma membrane fragments [29,30] or other conventional plasma membrane preparations which involve disruption of cell integrity, these vesicles apparently maintain the correct 'right-side out' sidedness. This may be attributed to cell fixation which predates vesicle shedding. Most of the released vesicles are not empty membranous structures but rather enclose constituents of the cytosol. This suggests their being formed by exocytosis and therefore bearing a 'right-side out' topology. Fourth, the asymmetric distribution of the lipid analog is preserved in the vesicles for considerable lengths of time (3 days and longer) which would suggest, in analogy to synthetic liposomes [31,32] that the rates of flip-flop are extremely slow. Hence they could be safely employed for lipid asymmetry studies since no lipid mixing occurred prior to phospholipase treatment.

Dimethylethanolamine-induced phospholipid asymmetry and its dissipation

The advantage of creating a transient state of asymmetry using a metabolic probe almost identical to the native one has been noted. Dimethylethanolamine, a close analog of choline, is a relatively non-perturbing probe, and we were able to follow its rate of translocation in its lipid-integrated form from one side to the other side of the plasma membrane. The fast component of translocation observed at early times after addition the nitrogen base analog (Fig. 5) presumably represents the initial velocity rate at which the lipid analog is translocated through to the outer leaflet of the lipid bilayer. As the concentration of the lipid analog increases and becomes equally partitioned on both sides of the plasma membrane (as seen after 24 h), the rate of translocation becomes significantly lower. In spite of the fact that both the fast and the slow translocation components behave in an apparently linear manner it is not unlikely that they are composed of multiple, exponentially decreasing rates as the asymmetry is dissipated. This possibility should be anticipated because in addition to the asymmetric distribution of the phospholipid analog at early times, non-steady-state equilibrium considerations such as synthesis, degradation and outward-inward translocation of membrane phospholipid may affect

the observed rates of incorporation of phosphatidyldimethylethanolamine. Thus, on one hand, phosphatidylcholine is constantly depleted from the membrane because of a lack of choline to maintain its metabolic turnover while on the other hand the newly synthesized analog is continuously translocated to maintain membrane phospholipid levels. These complex non-steady-state kinetic aspects are currently under investigation.

The apparent value measured for the initial rate of translocation of phospholipid from the inner to the outer segment of the membrane bilayer is generally compatible with that observed for other mammalian cells in which asymmetries have been introduced by isotopic means (for review see Ref. 33). However, unlike isotopic asymmetries which require the assumption of isotopic equilibrium and pool homogeneity, in our experimental system there is no preexisting analog pool at the initiation of the experiment. The fact that the analog pool is built up and conveyed very rapidly to the plasma membrane before the appearance of phospholipase A_2-sensitive phospholipid analog (Figs. 2 and 4) suggests that both transport to the membrane and translocation through it may be coupled phenomena. The possibility that phospholipid exchange proteins [34,35] may be involved in these reactions needs further investigation.

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