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LIPID COMPOSITION AND PHYSICAL PROPERTIES OF MEMBRANES FROM C-6 GLIAL CELLS WITH ALTERED PHOSPHOLIPID POLAR HEADGROUPS

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Growth of C-6 glial cells in media enriched in the polar headgroup precursors *N*,*N*-dimethylethanolamine, *N*-monomethylethanolamine or ethanolamine for 24 h resulted in the accumulation of the corresponding phospholipids to about 30% of total membrane phospholipid. Under these conditions the cholesterol to phospholipid ratios were unaffected. With the exception of arachidonic acid, which was significantly reduced in the lipids from cells grown in the presence of *N*-monomethylethanolamine, the fatty acid composition of cells grown under the various conditions was identical. The physical properties of membranes prepared from these cells were compared by electron spin resonance spectroscopy using spin-labelled stearic acid. Modifications in cellular phospholipid composition did not affect either the order parameter or the correlation time of fatty acid spin labels. Since there are no significant effects on the other membrane lipids and since the physical properties of the membranes are maintained, these modifications in phospholipid composition provide a valuable means for studying the role of phospholipid polar headgroups in the function of membrane-bound enzymes and hormone receptors in C-6 cells.

Introduction

The functions of membrane-bound enzymes and hormone receptors can be influenced strongly by their lipid environment [1]. There are two main strategies by which the lipid requirements of membrane proteins can be assessed. The protein may be purified in a delipidated form and subsequently reconstituted with defined lipids. Alternatively membrane lipid may be modified in vivo. This is done most conveniently in cultured cells by the inclusion of fatty acids [2] or precursors of phospholipid polar headgroups [3] in the growth medium. We have adopted the latter approach in order to study how the polar headgroups of phospholipids influence hormone receptor function in C-6 cells. Enrichment of C-6 cell membranes in phosphatidyl-*N*,*N*-dimethylethanolamine has already been shown by Finkel and Volpe [4] to diminish the rate of cholesterol biosynthesis by inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase. An altered membrane phospholipid composition in mouse LM cells stimulates the enzyme adenylate cyclase [5] and inhibits stearoyl-CoA desaturase [6]. In the same cell line endocytosis is also impaired by changes in membrane phospholipid composition [7]. In another type of cell, MOPC-31C plasmacytoma cells, intracellular transport and secretion of immunoglobulins is inhibited by changes in membrane phospholipid composition caused by the inclusion of choline analogues in the growth medium [8]. Enrichment of the membranes of mouse myeloid leukemia cells with phosphatidyl-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

N-monomethylethanolamine or phosphatidyl-*N*,*N*-dimethylethanolamine by the same technique induces these cells to differentiate into macrophages [9].

In principle, changes in phospholipid composition could alter the physical properties of the membrane and might therefore be accompanied by changes in the fatty acyl chain or cholesterol content of the lipid bilayer. A decrease in the phosphatidylcholine to phosphatidylethanolamine ratio in mouse liver membranes has already been shown to cause a significant change in membrane physical properties as judged by the increased fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene [10]. By contrast, the physical properties of membranes from LM cells are unaffected by the incorporation of large amounts of phosphatidyl-*N*-monomethylethanolamine or phosphatidyl-*N*,*N*-dimethylethanolamine [11,12]. In this study we have characterised the lipid composition and physical properties of C-6 glial cell membranes that have been enriched in phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine or phosphatidylethanolamine. We show that the phospholipid content of these membranes can be modified extensively without affecting their cholesterol to phospholipid ratios, fatty acyl chain composition or their physical properties.

Materials and Methods

Cell culture. C-6 cells were obtained from Flow Laboratories (Irvine, Ayrshire) and maintained at 37°C as monolayers with Dulbecco's Modified Eagle's Medium supplemented with 5% foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Flow Laboratories). *N*-Monomethylethanolamine (Fluka), *N*,*N*-dimethylethanolamine and ethanolamine (both from British Drug Houses Ltd.) were dissolved in distilled water and, after the pH was adjusted to 7.2, the solutions were sterilized by autoclaving. The final concentration of the choline analogues in the growth medium was 5 mM. After incubation with choline analogues, the cells were washed twice with Dulbecco's phosphate-buffered saline (Flow laboratories) and scraped off the petri dishes into the same medium. All cells used in these experiments were between passages 46 and 52.

Membrane preparation. Cells were homogenized in Dulbecco's phosphate-buffered saline by sonication with a probe sonifier (M.S.E. Instruments) for 15 s. The homogenate was centrifuged at 35 000 × *g* for 20 min at 4°C and the membrane pellet was resuspended in the homogenization buffer at a concentration of 10 mg protein/ml.

Lipid analysis. Lipid extracts were prepared and washed by the method of Folch et al. [13]. Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel 60 (Merck) that contained Florisil (1.7% w/w) (Merck). Plates were activated at 100°C for 1 h immediately before use. The solvent in the first dimension was chloroform/methanol/35% ammonia/water (58 : 35 : 2.5 : 5, v/v) and in the second dimension was propionic acid/*n*-propanol/chloroform/water (2 : 2 : 1 : 1, v/v). Lipids were visualized by brief exposure to iodine vapour and were identified by comparison with phospholipid standards (Sigma). The phosphorus content of each spot was determined by the method of Rouser et al. [14].

Cholesterol was determined in membrane lipid extracts by an enzymic method [15].

Fatty acid analysis on lyophilized membranes was done by a modification of the method of Nordoy and Lund [16]. Fatty acid methyl esters were prepared by the addition of methanol (4 ml) containing H_2SO_4 (1.5%, v/v) and butylated hydroxytoluene (0.01%, w/v) to a dry membrane sample (1.5–2 mg protein). After incubation at 80°C for 1h under argon, the fatty acid methyl esters were extracted three times with 3 ml of light petroleum (b.p. 35°–60°C) containing butylated hydroxy toluene (0.01%, w/v). The pooled extracts were washed twice with 2 ml of $KHCO_3$ solution (2%, w/v). The extract was then dried with anhydrous Na_2SO_4 and evaporated under argon. The methyl esters were dissolved in diethyl ether and identified by chromatography on a Pye Model 104 gas-liquid chromatograph equipped with a column containing 15% EGSS-Y on Diatomite CQ (Field Instruments Co. Ltd., Middx) at 194°C or one packed with 15% EGSS-X on Diatomite CQ (Field Instruments Co. Ltd., Middx) at 178°C. The fatty acid methyl esters were identified by comparison of their retention times with those of standards (Chromatography Services, Merseyside).

Protein content. Protein concentrations were

measured by the method of Lowry et al. [17].

Electron spin resonance measurements. Stearic acid with the 4,4-dimethyl-3-oxazolidynyloxy (doxyl) group attached to either carbon 5 (5-doxylstearic acid) or 16 (16-doxylstearic acid) was purchased from Syva Assoc., CA. Stock 0.86 mM solutions were prepared in ethanol. Spin-labelled stearic acid was incorporated into membrane preparations by first drying the spin label (4.4 nmol) at the bottom of a small glass test-tube to which was added 100 μl of membrane suspension (1 μmol phospholipid). After incubation for 10 min at room temperature the membranes were removed and centrifuged at 35000 × *g* for 30 min. The membrane pellet was resuspended in 50 μl of 100 mM NaCl/1 mM EDTA/2 mM Hepes (pH 7.4) and transferred to a capillary for electron spin resonance (ESR) measurements.

ESR spectra were recorded with a JEOL JES-PE-1X or a Varian E3 spectrometer equipped with a nitrogen flow temperature control system. The temperature of the sample was monitored with a thermocouple placed close to the sample. The order parameter (S) can be calculated for 5-doxylstearic acid undergoing rapid anisotropic motion from the equation $S = (T_{\|} - T_{\perp})/(T_{zz} - T_{xx})$ where $T_{\|}$ and $T_{\perp}$ are half the separation of the outer and inner hyperfine splittings, respectively, and T_{zz} and T_{xx} are the hyperfine splitting tensors in the z and the x direction, respectively. A value of 26.3G was taken for $T_{zz} - T_{xx}$ [18]. The correlation time (τ) was calculated for 16-doxylstearic acid undergoing rapid isotropic motion from the equation

$$\tau = 6.5 \cdot 10^{-10} W_0 \left((h_0/h_{-1})^{1/2} - 1 \right)$$

where W_0 is the peak to peak width and h_0 is the height of the centre line and h_{-1} is the height of the high-field line [19].

Results

Effect of choline analogues on lipid composition

After 24 h of growth of C-6 cells in the presence of *N,N*-dimethylethanolamine the amount of phosphatidyl-*N,N*-dimethylethanolamine (PDME) had almost reached its maximum value, therefore this incubation time was adopted throughout.

The changes in cellular phospholipid composition produced by the incorporation of choline analogues are shown in Table I. *N*-Monomethylethanolamine and *N,N*-dimethylethanolamine were incorporated into the corresponding phospholipids phosphatidyl-*N*-monomethylethanolamine (PME) and PDME, respectively. These modifications were accompanied by decreases in the amounts of both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Under these conditions there were no changes in the amounts of the minor phospholipids, sphingomyelin, phosphatidylserine and phosphatidylinositol. By contrast the increased amount of PE in the mem-

TABLE I

PHOSPHOLIPID COMPOSITION AND CHOLESTEROL TO PHOSPHOLIPID RATIO IN C-6 CELLS GROWN IN THE PRESENCE OF CHOLINE ANALOGUES

C-6 cells were grown for 24 h in the presence of choline analogues (5 mM). Phospholipid analyses were performed on total cellular lipid extracts and cholesterol to phospholipid ratios were measured in membrane lipid extracts as described in the Materials and Methods. All values are the means of three separate experiments; n.d., none detectable.

Supplement	Phospholipid composition (mol% of total phospholipid)				Ratio (mol/mol)
	PC	PDME	PME	PE	Cholesterol / Phospholipid
None	49.4	n.d.	n.d.	27.4	0.40
N,N-Dimethylethanolamine	27.5	32.6	n.d.	17.8	0.38
N-Monomethylethanolamine	27.7	n.d.	31.0	16.8	0.37
Ethanolamine	46.5	n.d.	n.d.	37.1	0.40

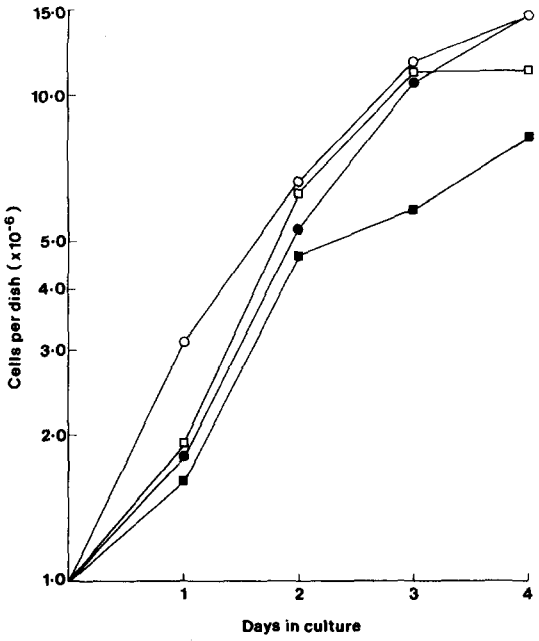

Fig. 1. Growth rates of C-6 cells in the presence of 5 mM choline analogues. ○——○, Control; ●——●, *N,N*-dimethylethanolamine; □——□, *N*-monomethylethanolamine; ■——■, ethanolamine. Cells were grown in 20 cm^2 dishes and harvested after trypsinization. Viable cells were counted using a haemocytometer.

branes as a result of supplementation of the growth medium with ethanolamine was achieved at the expense of PC and each of the minor phospholipids. Table I also shows that modification of phospholipid composition caused little change in cholesterol to phospholipid ratios.

Effect of choline analogous on cell growth

In order to ensure that C-6 cells were not damaged by alterations to their phospholipid content we measured their growth rates under the various conditions. Fig. 1 shows that although growth in the presence of choline analogues caused an initial slight depression in the rate of cell growth relative to the control, this lower growth rate was only maintained by the cells grown in the presence of ethanolamine.

Effect of choline analogues on fatty acid composition

The replacement of phosphatidylcholine by phospholipids that contain fewer methyl groups on the nitrogen of the polar head groups might be expected to have a rigidifying effect on the lipid bilayer [20]. One way of compensating for such an effect would be for the cells to modulate the degree of unsaturation of the fatty acyl chains of their phospholipids. Table II shows the amounts of the major fatty acyl chains present in the membrane lipids of cells grown under the different conditions. With the sole exception of a lower level of arachidonic acid in the fatty acyl chains from cell enriched in PME, there were no other significant changes in the fatty acid profiles. This prompted us to determine how these alterations in phospholipid composition of C-5 cell membranes might have influenced the physical properties of the membranes.

Physical properties of modified cell membranes

Schreier et al. [21] have stressed that the degree

TABLE II

FATTY ACID COMPOSITION OF C-6 CELLS GROWN IN THE PRESENCE OF DIFFERENT CHOLINE ANALOGUES

Fatty acid methyl esters were prepared from cell membranes and analyzed on an EGSSY column as described in the Materials and Methods. Values are the means ± S.E. for three different experiments. *, $p < 0.005$.

Supplement	Fatty acid composition (% by weight)				
	16:0	16:1	18:0	18:1	20:4
None	20.9 ± 1.6	8.8 ± 1.0	16.6 ± 0.5	38.2 ± 2.2	5.7 ± 1.7
N,N-Dimethyl-ethanolamine	19.8 ± 0.8	8.1 ± 0.7	16.7 ± 1.7	33.2 ± 1.1	3.8 ± 0.4
N-Monomethyl-ethanolamine	23.3 ± 0.4	11.7 ± 0.9	16.3 ± 1.7	39.1 ± 1.0	1.4 ± 0.1 *
Ethanolamine	21.0 ± 2.5	7.8 ± 0.9	16.2 ± 1.2	37.3 ± 1.0	3.0 ± 0.6

TABLE III

ORDER PARAMETERS AND CORRELATION TIMES FOR SPIN-LABELLED FATTY ACIDS INCORPORATED INTO MEMBRANES WITH A MODIFIED PHOSPHOLIPID COMPOSITION

The order parameters for 5-doxylstearic acid and the correlation times for 16-doxylstearic acid in membranes from cells grown in the presence of choline analogues were measured at 37°C as described in the Materials and Methods. All values are for means of four separate experiments ± S.E.

Supplement	Order parameter	Correlation time (s) ($\times 10^{10}$)
None	0.59 ± 0.01	5.44 ± 0.30
N,N-Dimethylethanolamine	0.59 ± 0.02	5.72 ± 0.28
N-Monomethylethanolamine	0.59 ± 0.01	5.68 ± 0.17
Ethanolamine	0.58 ± 0.01	5.76 ± 0.10

of molecular order of a lipid bilayer and the rates of lipid motion are quite distinct parameters. When identifying possible changes in the fluidity of a membrane it is therefore necessary to consider effects on both the order parameter and the correlation time of bilayer lipid. Electron spin resonance spectroscopy of appropriate spin-labelled fatty acids that have been incorporated into membrane provides a convenient method for measuring both order parameters (with 5-doxylstearic acid) and correlation times (with 16-doxylstearic acid). Table III shows that lipid modification had no effect on either the order parameter or the correlation time of the fatty acid spin labels when measured at 37°C. A more extensive study of the temperature dependence of both order parameters and correlation times between 15 and 40°C revealed no changes in the physical properties of membranes with an altered phospholipid composition when compared to controls (data not shown).

Discussion

The data presented here show that it is possible to make extensive modifications to the phospholipid composition of C-6 cell membranes without affecting either the amount of cholesterol in the membranes or the overall fatty acyl group content of the membrane lipids. Finkel and Volpe [4] showed that enrichment of C-6 cell membranes with phosphatidyl-*N,N*-dimethylethanolamine causes a reduction in the rate of cholesterol biosynthesis. Therefore it would seem that in our experiments the cells do not depend on endogenous synthesis of cholesterol but rather they obtain adequate supplies from lipoproteins in the medium. The fact that the cholesterol to phospholipid ratios were unaffected by phospholipid modification agrees with similar studies on Ml neuroblastoma cells [22] although growth of LM cells in the presence of choline analogues does reduce the levels of membrane sterol [3].

Schroeder [7] has made a detailed study of the effect of phospholipid modification in LM cells on the fatty acyl groups of membrane lipids. Our results agree with his in that there is no overall change although he did find a significant change in the distribution of fatty acyl groups amongst the various phospholipid upon enrichment of the membranes with phosphatidyl-*N,N*-dimethylethanolamine, phosphatidyl-*N*-monomethylethanolamine or phosphatidylethanolamine. In these experiments there were no changes in the physical properties of the LM cell membranes as measured by the fluorescence polarization of β-parinaric acid. Similarly we have shown by ESR that the physical properties of C-6 cell membranes are not perturbed by extensive phospholipid substitution. This argues against one suggestion made by Finkel and Volpe [4] that the decrease in membrane-bound 3-hydroxy-3-methylglutaryl-CoA reductase activity that accompanies enrichment with phosphatidyl-*N,N*-dimethylethanolamine might be a means of homeoviscous adaptation to a change in membrane fluidity.

In the absence of any changes in cholesterol content or fatty acyl group composition, substitution of phosphatidylcholine with phospholipids that are less methylated in their polar headgroups would tend to have a rigidifying effect on the lipid bilayer [11,12]. Our results demonstrate that a major reason for the maintenance of a constant membrane fluidity when cells are grown in the presence of *N,N*-dimethylethanolamine or *N*-monomethylethanolamine must be the decrease in the amounts of phosphatidylethanolamine since the other phospholipids are unaffected. When cells are enriched in phosphatidylethanolamine there are compensatory reductions in all of the other

phospholipids. The results of these experiments allow us to investigate the effects of polar headgroup substitution on a variety of membrane-bound enzymes and hormone receptors. These studies will be assisted by the knowledge that the alterations in phospholipid content are without effect on either the other lipid components of the membrane or the physical properties of the lipid bilayer matrix.

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