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ISOTHERMAL REGULATION OF MEMBRANE FLUIDITY IN MURINE FIBROBLASTS WITH ALTERED PHOSPHOLIPID POLAR HEAD GROUPS

FRIEDHELM SCHROEDER

Department of Pharmacology, University of Missouri Medical School, Columbia, Mo. 65212 and Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Mo. 63110 (U.S.A.)

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Summary

Fatty acyl chain and polar head group metabolism in a transformed murine fibroblast cell line, LM cells, may be linked and provide a mechanism for homeoviscous adaptation. Fluorescence polarization studies with β-parinaric acid, 1,6-diphenylhexatriene, and *N*-phenylnaphthylamine indicated that the microenvironments reported by each probe were different in plasma membranes isolated from LM cells grown in the presence of choline. In addition, each fluorescence probe molecule had markedly different polarization values in the plasma membranes, microsomes, and mitochondria. β-Parinaric acid incorporated into plasma membranes, microsomes, or mitochondria isolated from LM cells grown in the presence of *N*,*N*'-dimethylethanolamine or *N*-monomethylethanolamine had essentially the same polarization values as in the corresponding membranes from choline-grown cells. However, polarization values of β-parinaric acid in microsomes and mitochondria from ethanolamine supplemented LM cells were markedly higher.

These differences in polarization of the fluorescence probes in the isolated membranes may in part be due to a relationship between phospholipid polar head group and acyl group metabolism by which isothermally grown cells attempt to maintain the fluidity of their membranes. Data from choline-grown cells indicated that: (a) The fatty acid composition of phosphatidylcholine varied with respect to chain length and degree of unsaturation in the three membrane fractions. (b) The acyl chain composition of the other phospholipid species differed markedly from phosphatidylcholine in each membrane fraction and also exhibited major differences in chain length and degree of unsaturation depending on the membrane source. (c) The fatty acid composition of the zwitterionic phospholipids with a no net charge (phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine) as well as the negatively charged phospholipids (phosphatidylethanolamine, phosphatidylserine, phosphatidyl-

inositol, and cardiolipin) increased or decreased in degree of unsaturation and/or chain length depending on the phospholipid species as well as the membrane source. Secondly, supplementation of the culture medium with the choline analogues *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine resulted in incorporation of the bases into membrane phospholipids and in alteration of acyl chain distribution: (a) Newly synthesized phosphatidyl-*N,N'*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine from all three LM cell membrane fractions had fatty acid composition, intermediate between those of the phosphatidylcholine and phosphatidylethanolamine in that membrane fraction. (b) The choline analogues also affected the acyl chain composition of the other membrane phospholipid species, e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin. Phosphatidylcholine, phosphatidylethanolamine and cardiolipin acyl groups became more unsaturated in microsomes and mitochondria of analogue supplemented cells grown in suspension. (c) Neither of the alterations in a or b occurred to the same extent and in the same direction in all three membrane fractions.

Introduction

Although mammals can readily synthesize choline from ethanolamine, analogues of choline can be incorporated into plasma membrane phospholipids of mammalian cells [1—4]. High levels of choline analogues such as *N,N'*-dimethylethanolamine or *N*-monomethylethanolamine (30—40%) can be incorporated into membrane phospholipids of a transformed mouse fibroblast cell line, LM cells [2]. The LM fibroblasts cannot methylate ethanolamine and therefore require choline for growth. Thus tissue cultured LM fibroblasts grown in suspension culture incorporated these analogues at the expense of choline. These analogue-containing phospholipids were also found in microsomal and mitochondrial membrane constituents [1,2]. The functional significance of such large alterations in membrane lipid composition can be partially explained by the physicochemical properties of the analogue-containing phospholipids. Biophysical studies utilizing differential scanning calorimetry [5] or fluorescence probes [6,7] indicated that such alterations in polar head group composition of the phospholipids caused large changes in physical properties of the phospholipid vesicles when the acyl chain composition was constant. Therefore, in vivo alterations in phospholipid composition of mammalian membranes would also be expected to alter drastically the physical properties of the membranes [8—11,3] and thereby interfere with functional processes such as transport or otherwise alter the activities of some membrane bound enzymes of LM cells (refs. 2 and 3, and Doi, O., Doi, F., Schroeder, F., Alberts, A.W. and Vagelos, P.R., unpublished). The above expectations rely on the assumption that the acyl chain composition of the phospholipid remained the same and that no other compensatory alterations were occurring.

Of the nine membrane processes studied in the LM cell system in which the phospholipid polar head group composition was manipulated in vivo, only thymidine transport and adenylate cyclase activity were altered in whole cells

(refs. 2 and 3, and Doi, O., Doi, F., Schroeder, F., Alberts, A.W. and Vagelos, P.R., unpublished). The largest deviations were noted when ethanolamine was supplemented to the cells. The activities of seven other membrane-bound lipid and/or polar head group-dependent enzymes from purified plasma membranes, microsomes or mitochondria were essentially unaltered with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine, supplementation [2].

These results led to the interesting hypotheses that, since the tested functional processes were largely unaltered, perhaps the physical properties were also unchanged. Unaltered physical properties could only arise by some sort of compensatory mechanism in LM cells [2,8—10]. Such a process called "homeoviscous adaptation" exists in *Escherichia coli* and in *Tetrahymena* and occurs primarily by alterations in phospholipid acyl group metabolism [12,13]. Some evidence suggests that this self-regulating process is due to a direct modulation of fatty acid desaturase activity governed by membrane fluidity [13]. In the present study we report the results of investigations to determine if alterations in polar head group metabolism of LM cells can result in altered phospholipid acyl chain composition such that homeoviscous adaptation occurs.

Materials and Methods

Materials

N,N'-Dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine were obtained from Eastman Organic Chemicals, Rochester, New York. Choline was obtained from Sigma Chemical Co., St. Louis, Mo. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Aldritch Chemical Co., Milwaukee, Wisc. β-Parinaric acid was from Molecular Probes, Inc., Roseville, Minn. and *N*-phenyl-1-naphthylamine was from Eastman Organic Chemicals.

Methods

Cell culture and growth conditions. LM cells were obtained from the American Type Culture collection (CCL 1.2) and cultured in suspension with the serum-free lipid-free medium of Higuchi [14] as modified by Schroeder et al. [2]. Choline analogue supplementation was carried out by adding 40 μg/ml analogue to medium instead of choline. The cells were grown, and harvested as described earlier [2].

Membrane isolation. Plasma membranes, microsomes, and mitochondria were isolated from LM suspension cells by modification of two established procedures as described by Schroeder et al. [2].

Lipid determination. All organic solvents were glass distilled and all glassware was sulfuric acid-dichromate washed before use. Membranes were resuspended in phosphate-buffered saline made up without magnesium or calcium [15] and lipids were extracted by the method of Bligh and Dyer [16] as described by Ames [17]. Neutral lipids and phospholipids were separated by silicic acid chromatography using Unisil (100—200 mesh, Clarkson Chemical Co., Williamsport, Pa.). The phospholipids were labeled with [^{32}P]phosphate as described and separated by preparative two-dimensional thin-layer chromato-

graphy and visualized by radioautography using silica gel G plates (500 μm thick, Analtech. Inc., Newark, Del.) that had been preactivated with acetone [2]. Phospholipid standards were used for co-chromatography and identification of unknown phospholipids: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine, and cardiolipid were obtained from Serdary Research Labs., London, Ontario, Canada; phosphatidylglycerol and sphingomyelin were from Applied Science Labs., State College, Pa.; lysophosphatidylcholine was from Supelco, Bellefonte, Pa.; phosphatidyl-*N*,*N'*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine were generously provided by Dr. M. Glaser, Department of Biochemistry, University of Illinois, Urbana, Ill. Fatty acid methyl esters were prepared from phospholipids and neutral lipids as described previously [2]. The methyl esters were identified by comparison of their retention times with standards on 3% SE-30 (on Gas Chrom Q, Analabs, Newark, N.J.) at 185°C and on 10% DEGS (on Anachrom SD, 60—70 mesh, Analabs, Newark, N.J.) at 165°C. An Aerograph Model 2100 (Varian Aerograph, Inc., Walnut Creek, Calif.) gas chromatograph equipped with flame ionization detectors was used for the gas chromatographic identifications. In addition, the fatty acid methyl esters were identified by combined gas-liquid chromatography/mass spectrometry by comparison with standards run on the same instrument. The mass spectra were obtained by Mr. Hank Holland, Department of Biological Chemistry and Psychiatry, Washington University School of Medicine, St. Louis, Mo., on a $\frac{1}{4}$ inch, 6 ft, 3% SE-30 column with Gas Chrom Q support (Analabs, Newark, N.J.) and Model 9000 LKB Gas Chromatograph Mass Spectrometer (LKB Instruments, Inc., Bromma, Sweden).

Fluorescence polarization studies. β-Parinaric acid was incorporated into isolated membranes as described previously [8,9]. *N*-phenyl-1-naphthylamine was incorporated similarly. 1.6-Diphenyl-1,3,5-hexatriene was added to membranes as follows. A stock 1,6-diphenyl-1,3,5-hexatriene solution was prepared in tetrahydrofuran (1 mg/ml) and diluted 1 : 100 with tetrahydrofuran to give a working solution. 10-μl aliquots were added to 2 ml of membrane in phosphate-buffered saline (50 μg protein/ml) to give a final 1,6-diphenyl-1,3,5-hexatriene concentration of 0.1 μg/ml. The membrane suspension was then vortexed for 3 min at 37°C, followed by incubation at 37°C for an additional 20 min. All polarization readings were performed at 24°C with a computer-centered spectrofluorimeter previously described [8,9]. The incident light was polarized with a Glan-Thompson polarizer. The fluorescence intensities were observed with two Glan-Thompson polarizers independently oriented parallel and perpendicular at 90°C to the incident beam. Light scattering was reduced by cut-off filters. Polarization values were obtained with an on-line computer (PDP-8E, Digital Equipment Company, Maynard, Mass.) such that each determination was the average of several hundred readings taken in a few milliseconds. Precision in polarization measurements was ±0.001. β-Parinaric acid, *N*-phenyl-1-naphthylamine, and 1,6-diphenyl-1,3,5-hexatriene were excited at 313, 343, and 362 nm, respectively, while emission was measured at 415, 425, and 430 nm, respectively.

Results

Fluorescence polarization studies of LM cell membranes

Fluorescent molecules such as β-parinaric acid, *N*-phenyl-1-naphthylamine, and 1,6-diphenyl-1,3,5-hexatriene can be incorporated into mammalian cell membranes. The probes can report on variations or differences in their microenvironment by alterations in spectral parameters such as polarization (*P*). Polarization values can be directly related to microviscosities, $\overline{\eta}$, by the Perrin equation [18]. However, we report only *P* values here rather than calculate $\overline{\eta}$ since the probes may not be located in a homogeneous lipid phase of the membrane. The probes may be reporting an average of several environments. Fatty acid probes such as β-parinaric acid are believed to be oriented with their carboxyl groups at the polar interface of membranes and the acyl chain in the hydrocarbon core [19]. This probe is sensitive to both polar head group and acyl chain vaiations in membranes [6—10]. In contrast, 1,6-diphenyl-1,3,5-hexatriene appears to be located in the hydrocarbon core of the membrane and is primarily sensitive to packing constraints. *N*-Phenyl-1-naphthylamine is sensitive to both polarity and packing constraints and may be oriented at protein-lipid interfaces or regions intermediate between β-parinaric acid and 1,6-diphenyl-1,3,5-hexatriene [20]. Thus, these probes would be expected to report on three different microenvironments of the cell membrane. As shown in Table I, the polarization values of β-parinaric acid, 1,6-diphenyl-1,3,5-hexatriene, and *N*-phenyl-1-naphthylamine in the three membrane fractions from LM cells grown with choline are not identical. Generally, *N*-phenyl-1-naphthylamine had the lowest polarization value (less than 0.200) while β-parinaric acid had the highest (0.325). A comparison of β-parinaric acid and 1,6-diphenylhexatriene indicates that in the plasma membrane they reside in environments with the same average polarization (and microviscosity) while in the microsomes and mitochondria they do not. Only *N*-phenyl-1-naphthylamine indicates that the mitochondria may have regions more rigid than the plasma membrane (0.199 vs. 0.137). Thus, qualitatively these data indicate a great heterogeneity in microenvironment "fluidity" of LM cell subcellular membranes. As indicated in subsequent sections this may be the result of the different lipid composition of these membranes.

LM cells were also supplemented with choline analogues which altered the

TABLE I

FLUIDITY OF LM CELL MEMBRANE LIPID CORES AS REPORTED BY MICROENVIRONMENT OF THREE FLUORESCENCE PROBES

All conditions described in Materials and Methods. Each value represents the mean ± S.E. of three experiments.

Membrane fraction	Polarization		
	β-Parinaric acid	Diphenylhexatriene	*N*-Phenyl-1-Naphthylamine
Plasma membranes	0.325 ± 0.005	0.328 ± 0.005	0.137 ± 0.003
Microsomes	0.311 ± 0.006	0.285 ± 0.006	0.139 ± 0.005
Mitochondria	0.277 ± 0.005	0.307 ± 0.006	0.199 ± 0.004

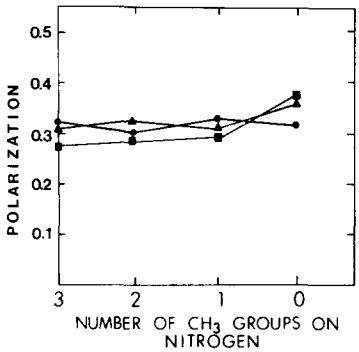

Fig. 1. Effect of phospholipid polar head group manipulation on membrane fluidity. Polarization of β-parinaric acid in membranes isolated from LM cells supplemented with choline analogues was measured as described in Materials and Methods. The ratio of β-parinaric acid to membrane lipid was 1 : 100. The number of CH_3 groups refers to the number of methyl groups or the nitrogen atom of the choline analogue supplemented to the cell (3 = choline; 2 = *N,N'*-dimethylethanolamine; 1 = *N*-monomethylethanolamine; 0 = ethanolamine). ●———●, plasma membrane; ▲———▲, microsomes; ■———■, mitochondria.

phospholipid composition of the membrane [2]. The altered lipid composition was expected to make the membranes more rigid. However as shown by the polarization values in Fig. 1, there was no trend toward increasing polarization of β-parinaric acid in the plasma membranes as the membranes were enriched in phospholipids that were expected to be more rigid by 10—20° if the acyl chain composition was unaltered [8—10]. Essentially similar results were obtained with microsomes and mitochondria except with ethanolamine supplemented cells. The latter had polarization values much higher than those of choline fed cells (0.360 vs. 0.310 in microsomes and 0.375 vs. 0.275 in mitochondria of ethanolamine- vs. choline-fed cells). These results indicate that the LM cells must be compensating for the rigidifying effects of the altered polar head groups. One such adaptive mechanism would be to alter the degree of unsaturation and/or chain-length of the phospholipid acyl groups.

Fatty acid composition of analogue phospholipid species whose polar head groups were derived from the culture medium

It has previously been shown that the physical "transition" temperatures of a series of phospholipids containing the same acyl chains, but having polar head groups differing only in the number of methyl groups on the nitrogen atom of the base, increased 6—10° with each additional methyl group [5—7]. LM cells cannot synthesize choline by methylation of ethanolamine, *N*-monomethylethanolamine, or *N,N'*-dimethylethanolamine precursors. Such base analogues were incorporated into LM suspension cell phospholipid of plasma membranes, microsomes, and mitochondria [2]. Four possibilities for base analogue incorporation may be postulated. (1) New polar head groups are added via turnover of pre-existing polar head groups without subsequent acyl group compositional changes. (2) Same as possibility 1 but with subsequent acyl group compositional changes. (3) Synthesis from newly synthesized diglyceride without subsequent acyl group compositional changes. (4) Same as possibility 3 but with subsequent acyl group compositional changes. Since neither the degree of unsaturation nor content of short chain fatty acids

increased in total phospholipid extracts from such membranes [2], it seemed probable that the maintenance of physical characteristics was not due to alterations in fatty acid composition. However, it was possible that the analogue phospholipid species of individual membrane fractions did not have the same acyl chain composition as phosphatidylcholine. Whole cell data on LM cells grown in monolayer with choline analogues had indicated that phosphatidyl-*N*,*N*′-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine had fatty acid composition intermediate between those of phosphatidylcholine and phosphatidylethanolamine [15]. Whole cell data, however, represent the average fatty acid composition of each phospholipid species from all cellular membranes. In addition considerable differences in lipid metabolism between suspension and monolayer grown LM cells have been noted [2]. Therefore the fatty acid composition of phospholipid species from individual membrane fractions isolated from LM suspension cells were determined.

As shown in Table II, the fatty acyl groups present in largest quantity in plasma membranes, microsomes, and mitochondria were 16 : 0, 16 : 1, 18 : 0, and 18 : 1. Smaller amounts of 14 : 0 and 15 : 0 and trace quantities of 17 : 0 and 17 : 1 fatty acids were also detected. The presence of diunsaturated fatty acids such as 18 : 2 was unusual since most mammalian cells grown in the absence of serum cannot synthesize polyunsaturated fatty acids de novo [21]. The ability to biosynthesize polyunsaturated fatty acids seems to be a biochemical characteristic lost by most cells after prolonged periods in culture [22]. Indeed, most cell lines do not appear to have a significant requirement for polyunsaturated fatty acids of any type [23]. However, diunsaturated fatty acids can be synthesized de novo by skin epithelium [24], Ehrlich ascites carcinoma cells [25], and possibly some transformed cells and tumor lines [26]. The polyunsaturates found in LM cells were not medium components and were shown to be synthesized de novo from acetate [2,35]. The fatty acids of chain lengths longer than 18 carbons were comprised primarily of 20 : 0, 22 : 0, 24 : 0, and 26 : 0 with smaller amounts of their corresponding monounsaturates and some diunsatures. Small quantities of odd chain length fatty acids (19 : 0, 21 : 0, 23 : 0, and 25 : 0) were also detected. Generally less than 4% of the phospholipid acyl groups were polyunsaturates (Table II).

As shown in Table II, after analogue supplementation two trends were evident in the fatty acid composition of phospholipids. (1) In all three membrane fractions (plasma membranes, microsomes, and mitochondria) the ratio of unsaturated fatty acids to saturated fatty acids varied inversely with the degree of methylation of the nitrogen atom of the base supplement incorporated into the phospholipid species. In addition, the unsaturated/saturated fatty acid ratio was generally of the order mitochondria > microsomes > plasma membranes. (2) The ratio of fatty acid with chain lengths $C_{18} - C_{26}$/ fatty acids with chain lengths $C_{14} - C_{17}$ (or long chain/short chain) varied inversely with the degree of methylation of the nitrogen atom of the base supplement. The latter changes were accounted for primarily by decreases in 16-carbon fatty acids and increases in 18 or longer carbon fatty acids. Thus, phosphatidyl-*N*,*N*′-dimethylethanolamine, and phosphatidyl-*N*-monomethylethanolamine had fatty acid compositions intermediate between those of

TABLE II

FATTY ACID COMPOSITION OF PHOSPHOLIPID SPECIES WHOSE POLAR HEAD GROUPS HAVE BEEN SUPPLEMENTED IN THE MEDIUM OF LM SUSPENSION CELLS

Individual phospholipid species were isolated by two-dimensional thin-layer chromatography and their fatty acid compositions were determined as described in Methods. Values represent the mean of three experiments with individual values deviating less than 10% from the mean. Long/short refers to the ratio of long chain length fatty acids (18—26 carbons) to short chain length fatty acids (14—17 carbons). Unsaturated/saturated refers to the ratio of unsaturated to saturated fatty acids. Plasma membranes, microsomes, and mitochondria were isolated from LM suspension cells as previously described [2].

Supplement	Phospholipid species	Fatty acid composition (percent by weight)								Long/Short	Unsaturated/Saturated
		14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes											
Choline	Phosphatidylcholine	0.1	0.1	22.4	6.6	8.0	46.9	2.2	13.7	2.42	1.50
N,N'-Dimethylethanolamine	Phosphatidyl-*N,N'*-dimethylethanolamine	2.5	0.5	12.0	6.8	10.9	47.3	4.4	15.7	3.61	1.80
N-Monomethylethanolamine	Phosphatidyl-*N*-monomethylethanolamine	0.7	0.5	8.1	10.1	12.3	50.0	4.8	13.5	4.15	2.38
Ethanolamine	Phosphatidylethanolamine	0.5	1.3	9.0	5.7	11.0	47.9	2.4	21.4	4.78	2.00
Microsomes											
Choline	Phosphatidylcholine	1.3	0.7	25.3	12.2	7.0	36.3	3.7	13.5	1.53	1.52
N,N'-Dimethylethanolamine	Phosphatidyl-*N,N'*-dimethylethanolamine	1.1	0.6	11.3	10.7	14.6	54.9	tr.	6.8	3.22	2.02
N-Monomethylethanolamine	Phosphatidyl-*N*-monomethylethanolamine	0.1	1.0	7.3	7.2	18.1	56.5	tr.	9.8	5.41	2.33
Ethanolamine	Phosphatidylethanolamine	0.9	0.7	7.7	5.7	8.2	64.7	1.7	10.4	5.67	3.61
Mitochondria											
Choline	Phosphatidylcholine	0.3	0.2	20.3	10.3	7.0	52.9	4.0	4.9	2.21	1.87
N,N'-Dimethylethanolamine	Phosphatidyl-*N,N'*-dimethylethanolamine	tr.	tr.	11.2	7.6	13.9	56.3	5.0	6.0	4.32	2.42
N-Monomethylethanolamine	Phosphatidyl-*N*-monomethylethanolamine	0.1	0.3	5.8	9.2	19.8	62.1	tr.	2.8	5.54	2.66
Ethanolamine	Phosphatidylethanolamine	0.2	0.5	3.5	5.5	11.5	69.1	tr.	9.6	9.20	3.90

tr., trace.

phosphatidylcholine and phosphatidylethanolamine. These results support possibilities 2 and 4 above. Therefore, the phospholipid analogues as well as phosphatidylcholine did not have the same degree of unsaturation or (as indicated by the long chain/short chain fatty acid ratio) fatty acid chain length in each of the three membrane fractions. However, the two trends described above were evident in each membrane fraction obtained from analogue-supplemented cells.

Thus, it appears that the phospholipid analogue species had fatty acid compositions that differed from phosphatidylcholine. This could account, in part, for maintenance of the physicochemical environment of LM cell membranes.

Effect of supplementation with choline analogues on fatty acid composition of zwitterionic neutral phospholipid species

It has been shown that a vertical asymmetry of distribution of phospholipid species exists across red blood cell, rat liver, and hepatoma plasma membranes [27,28]. Zwitterionic neutral phospholipids such as phosphatidylcholine and sphingomyelin are generally believed to be located on one side (usually the outside) of bilayer membranes while negatively charged phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are located on the outer (usually the cytoplasmic side or inside) of the bilayer membrane. In our studies it is possible that analoque supplementation may have affected the fatty acid composition of individual phospholipid species and may have thereby altered the vertical distribution of unsaturated or short chain length fatty acids. This could be achieved either by altering the asymmetry of phospholipid species distribution in LM cells [36] and/or by altering the fatty acid composition of the other phospholipid species. In this section are presented the effects of base analogue supplementation on the choline-containing phospholipid species of subcellular membranes from LM suspension cells, while that of the negatively charged species is detailed in the following section.

The fatty acid composition of phosphatidylcholine from plasma membranes, microsomes, and mitochondria varied as a function of base analogue supplementation as shown in Table III. As indicated by the unsaturated to saturated fatty acid ratio, the degree of fatty acid unsaturation of phosphotidylcholine decreased slightly in plasma membranes, while it increased in the microsomes and mitochondria, as a function of decreasing analogue supplement nitrogen methylation. The chain length ratio increased in plasma membranes and microsomes, but remained essentially constant in the mitochondrial membrane fraction. Thus, in contrast to results obtained with whole cells, the fatty acid compositions of phosphatidylcholine from membranes of analogue-supplemented LM cells were altered, but not in the same manner for each membrane fraction. Such differences were obscured in whole cell data, which represented an average fatty acid composition of phosphatidylcholine from all membrane sources [2,15]. The degree of unsaturation of phosphatidylcholine in the three membrane fractions was usually mitochondria > microsomes > plasma membranes.

The fatty acid composition of the other choline-containing phospholipids, sphingomyelin and lysophosphatidylcholine is given in Table IV. The ratio

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE FROM MEMBRANES OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes										
Choline	0.1	0.1	22.4	6.6	8.0	46.9	2.2	13.7	2.42	1.50
N,N'-Dimethylethanolamine	3.0	3.0	15.8	6.2	10.0	10.7	1.1	49.3	2.54	1.14
N-Monomethylethanolamine	1.1	3.0	14.1	11.1	12.4	25.6	tr.	33.4	2.44	1.24
Ethanolamine	0.3	0.3	15.9	9.0	11.2	23.3	0.8	39.0	2.89	1.20
Microsomes										
Choline	1.3	0.7	25.3	12.2	7.0	36.3	3.7	13.5	1.53	1.52
N,N'-Dimethylethanolamine	2.1	1.0	15.0	14.7	10.2	33.3	3.2	20.5	2.05	1.78
N-Monomethylethanolamine	1.0	1.0	14.2	14.5	10.5	25.5	tr.	33.3	2.26	1.67
Ethanolamine	1.0	0.8	11.1	10.4	10.7	43.6	tr.	22.4	3.29	1.99
Mitochondria										
Choline	0.3	0.2	20.3	10.3	7.0	52.9	4.0	4.9	2.21	1.87
N,N'-Dimethylethanolamine	1.2	0.8	13.3	12.0	7.3	44.4	2.0	19.1	2.68	2.07
N-Monomethylethanolamine	1.1	0.7	15.6	13.9	9.8	46.9	tr.	12.0	2.19	2.27
Ethanolamine	0.9	0.3	13.4	12.1	6.6	55.9	tr.	8.0	2.56	3.42

tr., trace.

TABLE IV

FATTY ACID COMPOSITION OF SPHINGOMYELIN + LYSOPHOSPHATIDYLCHOLINE FROM MEMBRANES OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes										
Choline	0.5	0.4	40.7	5.4	6.1	5.3	1.7	39.0	1.11	0.56
N,N'-Dimethylethanolamine	0.5	0.2	36.5	3.8	8.3	4.3	1.2	44.8	1.43	0.57
N-Monomethylethanolamine	0.5	1.5	44.0	6.9	6.3	3.4	tr.	36.0	0.84	0.45
Ethanolamine	0.8	0.7	45.6	7.3	4.3	1.4	tr.	40.1	0.84	0.41
Microsomes										
Choline	1.4	0.5	28.0	21.1	6.0	12.2	4.2	26.5	0.96	1.22
N,N'-Dimethylethanolamine	2.6	2.7	19.0	17.6	10.1	13.9	2.4	31.8	1.39	1.12
N-Monomethylethanolamine	2.2	2.2	29.0	20.2	3.8	9.2	tr.	29.0	0.78	1.00
Ethanolamine	1.3	0.7	40.1	19.5	4.1	3.2	0.2	30.9	0.62	0.83
Mitochondria										
Choline	1.1	0.7	36.5	10.3	4.5	31.6	0.7	14.5	1.06	1.04
N,N'-Dimethylethanolamine	0.5	0.3	30.8	8.8	12.4	14.3	2.7	30.5	1.48	0.81
N-Monomethylethanolamine	1.6	1.3	32.1	6.7	9.1	14.8	tr.	34.5	1.40	0.70
Ethanolamine	2.0	0.5	41.0	11.9	5.7	17.3	tr.	21.6	0.81	0.49

tr., trace.

of unsaturated to saturated fatty acid varied directly with the degree of analogue supplement methylation in all three membrane fractions. The unsaturated fatty acid content was much lower than in phosphatidylcholine. This result would be expected if the lysophosphatidylcholine was derived from phosphatidylcholine which has lost the acyl group at the number two carbon atom of glycerol which usually contains most of the unsaturated acyl groups of phospholipid species. In addition, sphingomyelin is generally not very unsaturated [27]. The chain length ratio of these phospholipids did not appear to vary systematically with base analogue methylation. Unlike phosphatidylcholine, the unsaturation of these phospholipids in the three membranes varied with microsomes > mitochondria > plasma membranes. In summary, the zwitterionic choline-containing phospholipid species varied considerably in their degree of unsaturation and chain length as a function of base supplement methylation. However, these variations depended upon the membrane fraction and on the choline-containing phospholipid species. Generally, increases in unsaturation occurred in phosphatidylcholine of microsomes and mitochondria. The mitochondria had the highest level of choline analogue incorporation into phospholipids (up to 50%). The higher unsaturated/saturated fatty acid ratio would be expected to help maintain the physicochemical environment of these membranes by compensating for the less fluid phospholipids one might expect due to polar head group alteration alone.

Effect of supplementation with choline analogues on fatty acid composition of anionic phospholipid species.

As opposed to the choline-containing phospholipids, the negatively charged phospholipids (phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and cardiolipin) all bear net negative charges at neutral pH and appear to be localized primarily on the inner surface of biological bilayer membranes. Phosphatidylethanolamine had longer chain length and more unsaturated fatty acids than did phosphatidylcholine (compare Table V with Table III). However, the trends in unsaturated/saturated fatty acid ratios and in chain length ratios of the plasma membranes, microsomes and mitochondria were similar to those noted with phosphatidylcholine. Phosphatidylglycerol had a very high content of long chain fatty acids in all three membrane fractions (Table VI), but no definite trends as a function of base supplement methylation were evident. However, the unsaturated to saturated fatty acid ratio varied with the degree of base supplement methylation in all three membranes. Phosphatidylinositol and phosphatidylserine had fairly constant ratios of unsaturated to saturated fatty acids (Table VII). The content of unsaturated fatty acids in these phospholipid species was also very similar and relatively invariant in all three membrane fractions. The chain length ratio in plasma membranes and microsomes varied directly with base supplement methylation, but remained fairly constant in mitochondria.

Cardiolipin (diphosphatidylglycerol), the phospholipid found exclusively in the mitochondria, showed direct correlation in unsaturated/saturated fatty acid ratio and inverse correlation in chain length ratio with the degree of base supplement methylation (Table VIII). These trends were not similar to those of phosphatidylglycerol from mitochondria. The mitochondrial cardiolipin was

TABLE V

FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINE FROM MEMBRANES OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes										
Choline	0.6	1.6	9.9	6.5	9.0	47.9	1.7	21.0	3.90	2.40
N,N'-Dimethylethanolamine	1.5	1.2	8.4	9.9	8.7	31.9	5.3	31.1	3.35	2.14
N-Monomethylethanolamine	0.8	1.5	12.2	10.8	11.0	31.7	4.5	26.3	2.77	1.82
Ethanolamine	0.5	1.3	9.0	6.7	11.0	47.9	2.4	21.4	4.31	2.00
Microsomes										
Choline	0.9	1.6	10.9	9.4	11.2	42.2	4.3	20.3	3.42	2.27
N,N'-Dimethylethanolamine	1.4	2.0	11.7	11.9	10.2	36.2	5.6	17.8	2.32	2.00
N-Monomethylethanolamine	tr.	tr.	9.7	8.8	8.9	42.9	2.0	23.3	3.34	2.68
Ethanolamine	0.9	0.7	7.7	5.7	8.2	64.7	1.7	10.4	5.67	3.61
Mitochondria										
Choline	0.3	1.2	6.7	6.2	15.4	57.5	0.3	12.5	5.95	2.44
N,N'-Dimethylethanolamine	tr.	1.0	3.0	5.1	15.8	59.9	3.9	10.0	8.45	2.88
N-Monomethylethanolamine	0.3	0.7	3.6	7.6	17.2	61.2	tr.	9.6	7.21	3.26
Ethanolamine	0.2	0.5	3.5	5.5	11.5	69.1	tr.	9.6	9.30	3.90

tr., trace.

TABLE VI

FATTY ACID COMPOSITION OF PHOSPHATIDYLGLYCEROL FROM MEMBRANES OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes										
Choline	2.0	1.3	8.2	4.5	5.0	21.8	15.1	42.0	5.21	2.66
N,N'-Dimethylethanolamine	4.4	4.5	11.6	7.1	8.6	12.1	3.6	48.1	2.62	1.02
N-Monomethylethanolamine	2.0	3.2	20.5	10.1	8.1	16.3	tr.	37.9	1.65	1.31
Ethanolamine	0.5	0.7	11.7	2.0	5.4	11.3	1.8	65.8	5.30	0.91
Microsomes										
Choline	1.2	1.6	9.0	2.9	4.1	17.6	16.4	47.3	5.81	3.65
N,N'-Dimethylethanolamine	6.6	2.3	17.2	7.2	16.3	4.6	3.3	42.5	2.00	0.56
N-Monomethylethanolamine	1.6	4.0	17.8	8.0	8.1	17.5	tr.	39.7	1.88	0.72
Ethanolamine	3.8	3.8	14.4	6.7	5.1	14.2	1.3	50.7	2.48	0.98
Mitochondria										
Choline	1.1	1.3	17.9	9.0	9.9	25.8	1.3	33.7	2.41	1.19
N,N'-Dimethylethanolamine	2.7	1.5	11.6	5.6	9.1	28.3	3.2	38.0	3.67	0.91
N-Monomethylethanolamine	0.1	0.1	19.2	8.0	8.8	25.7	tr.	34.4	2.22	0.81
Ethanolamine	1.7	2.5	44.3	5.5	8.8	6.9	0.4	26.4	0.74	0.41

tr., trace.

TABLE VII

FATTY ACID COMPOSITION OF PHOSPHATIDYLINOSITOL + PHOSPHATIDYLSERINE FROM MEMBRANES OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Plasma membranes										
Choline	0.3	0.1	4.0	2.3	32.0	36.6	3.2	22.4	14.06	1.13
N,*N*′-Dimethylethanolamine	1.6	1.7	7.8	6.7	20.0	19.0	2.1	41.2	4.62	1.12
N-Monomethylethanolamine	0.3	1.4	9.3	7.0	24.5	19.4	tr.	37.7	4.46	0.94
Ethanolamine	0.3	0.2	10.2	6.1	28.0	18.0	0.7	35.9	4.72	1.14
Microsomes										
Choline	0.7	0.9	8.3	6.5	23.9	35.7	5.7	18.3	5.09	1.63
N,*N*′-Dimethylethanolamine	1.7	2.0	11.0	9.0	15.0	27.3	2.8	31.2	3.22	1.12
N-Monomethylethanolamine	0.1	1.7	9.6	8.8	18.8	27.6	tr.	24.2	3.46	1.13
Ethanolamine	1.9	1.8	9.5	8.7	25.2	32.7	0.9	19.3	3.57	1.21
Mitochondria										
Choline	0.8	0.2	5.6	3.3	27.3	47.3	0.5	15.1	9.10	1.69
N,*N*′-Dimethylethanolamine	0.4	0.5	6.5	3.3	22.3	40.7	7.7	19.0	8.38	1.95
N-Monomethylethanolamine	0.3	0.2	6.6	4.0	28.9	46.5	tr.	13.3	7.99	1.55
Ethanolamine	0.4	0.2	5.9	4.3	30.0	45.6	4.1	9.5	8.26	1.50

tr., trace.

TABLE VIII

FATTY ACID COMPOSITION OF CARDIOLIPIN FROM MITOCHONDRIA OF LM CELLS GROWN IN SUSPENSION WITH ANALOGUES OF CHOLINE

All methods as in Table II.

Supplement	Fatty acid composition (percent by weight)								Long / Short	Unsaturated / Saturated
	14 : 0	15 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	$>C_{18}$		
Choline	0.8	0.4	18.2	6.2	4.9	46.1	0.9	22.4	2.89	1.63
N,*N*'-Dimethylethanolamine	1.5	0.8	23.7	6.1	7.2	43.9	3.0	13.9	2.13	1.69
N-Monomethylethanolamine	1.5	1.0	25.8	8.4	3.9	50.4	tr.	9.0	1.72	1.84
Ethanolamine	1.7	0.6	16.1	11.0	3.3	55.5	6.2	5.7	2.41	2.95

tr., trace.

generally more unsaturated and had a longer fatty acid chain length ratio (due to the high content of oleic acid, 18 : 1, rather than increased content of fatty acids of carbon chain length greater than 18) as contrasted to phosphatidylglycerol. The fatty acid composition of the mitochondrial cardiolipins did not appear to be the same as that of any of the corresponding phosphatidylglycerol species shown in Table V. Thus, it seems unlikely that these phosphatidylglycerols were the immediate precursors of the acyl groups in mitochondrial cardiolipin. Rather, subsequent acyl group turnover may be responsible for the differences in fatty acid composition.

The negatively charged phospholipid species varied in complex fashion, depending on membrane source and on individual species, as a function of base supplement methylation. The increases in unsaturation of phosphatidylethanolamines may be important in maintaining the fluidity of microsomes and mitochondria from analogue supplemented LM cells.

Discussion

Homeoviscous adaptation or self-regulation of membrane fluidity may be present in the transformed cell line, LM cells. The data presented here with β-parinaric acid, a naturally occurring fluorescent molecule used as a probe, indicate that in the plasma membranes the LM cells are able to maintain their fluidity in the presence of altered phospholipid polar head group composition. Similar results were obtained with microsomes and mitochondria from *N,N'*-dimethylethanolamine- and *N*-monomethylethanolamine-supplemented cells. Only ethanolamine supplementation significantly increased the polarization of β-parinarate in microsomes and especially in mitochondria. This indicates that homeoviscous adaptation may not overcome the effect of head group alteration by ethanolamine supplementation in microsomes and mitochondria. Other physical parameters also indicate the existance of homeoviscous adaptation [2—11]. For example, the characteristic transition temperatures of the intact membranes or their isolated lipids were measured with β-parinaric acid and were unaltered [8—10]. These transitions reflect alterations in lipid phase. However relative fluorescence efficiency, a parameter sensitive to charge and polarity effects, indicated that the probe environment was dependent on the polar head group composition of the membrane phospholipids in a manner that was not reflected by the characteristic temperatures or the polarization values presented here. The fluorescence efficiency of β-parinaric acid varied with each subcellular membrane fraction studied, depending on the lipid composition as well as the membrane source. Relative fluorescence efficiency values of β-parinaric acid in plasma membranes of LM cells decreased with ethanolamine supplementation [9]. Fluorescence efficiency of a probe molecule is dependent on dielectric as well as viscosity effects. β-Parinaric acid, which would be oriented with its carboxyl group at the polar surface may be much more sensitive to these alterations than diphenylhexatriene, which would be expected to be localized entirely in the hydrocarbon core of the bilayer.

Alterations in fluorescence probe behavior are also significantly dependent upon probe location. When diphenylhexatriene was used as a probe for the membrane interior, increases in the microviscosity of the plasma membrane

core from LM cells supplemented with ethanolamine were found relative to choline-supplemented cells, especially at 10°C [3,11]. However, at more physiological temperatures (25 and 37°C) the polarization and fluorescence lifetime values of diphenylhexatriene in plasma membranes from choline- versus ethanolamine-supplemented cells appeared, within experimental error, to be very similar. These results appear consistent with the polarization of β-parinarate in the LM cell membranes as shown herein. However, fatty acid alterations in the phospholipids did occur with choline analogue supplementation and some fluorescence parameters of this surface-sensitive probe were shown to be altered [8,9]. Diphenylhexatriene did not indicate such differences. Other probes such as *N*-phenyl-1-naphthylamine and pyrene (both are assumed to be located in the hydrophobic core of membranes) indicated opposite alterations in membrane microviscosity in response to experimental manipulation of BHK cells [20]. These data indicate the existence of multiple hydrophobic environments within the cell membranes and indicate serious deficiencies in extrapolation of polarization to microviscosity data from one type of probe molecule alone.

Many factors contribute to the apparent microviscosity, hence extrapolations from microviscosity measurements to mechanism of alteration might be inappropriate. Microviscosities are calculated from polarization, lifetime, and volume values. Recent data of Chen et al. [29] indicate that microviscosities measured with diphenylhexatriene assuming only singly exponential decay may have to be re-evaluated. Thus, if the probe exists in multiple compartments within a membrane and if the decay times are not equivalent, microviscosity measurements calculated from steady-state fluorescence polarization would not be meaningful. This possibility has recently been confirmed by Kawato et al. [30] and Veatch and Stryer [31].

The bulk of the biophysical evidence indicated that the LM cells somehow maintained a relative constancy of certain physicochemical and biochemical features of their membranes. Several compensatory mechanisms were proposed that would maintain the constancy of the physicochemical environment of LM cell membranes, but only some of these mechanisms were shown to be operative [2,30]. The simplest mechanism whereby such compensation could arise was expected to be via alteration of the degree of fatty acyl group unsaturation and/or chain length. Unexpectedly, Schroeder et al. [2] found that the total phospholipid fraction of membranes of LM cells grown in suspension with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, of ethanolamine did not contain higher percentages of unsaturated fatty acids or shorter chain length fatty acids. These data did not, however, preclude the possibility that the distribution of fatty acids among the various phospholipid species from plasma membranes, microsomes, or mitochondria might have changed. In this paper we have presented the results of investigations intended to clarify these possibilities.

In earlier work we have demonstrated a metabolic regulatory relationship between phospholipid polar head groups and neutral lipid alkyl ether groups as well as phospholipid alkyl ether groups [32]. The data presented here indicate that phospholipid polar head group and phospholipid acyl group composition may also be regulated by polar head group metabolism.

The data indicate that the fatty acid composition of individual phospholipid species from LM cell plasma membranes, microsomes, and mitchondria varied in degree of fatty acid unsaturation and in the ratio of long chain to short chain fatty acids in response to base analogue supplementation. Three types of acyl chain alterations in response to polar head groups were noted: (1) Newly synthesized analogue-containing phospholipids such as phosphatidyl-*N,N'*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine of plasma membranes, microsomes, and mitochondria had fatty acid compositions intermediate between those of phosphatidylcholine and phosphatidylethanolamine from the same organelles. These results were predicted by earlier workers using whole LM monolayer cells [15] instead of membrane isolates from suspension cells as used herein. (2) The acyl chain composition of the same phospholipid species (e.g. phosphatidylcholine) was not the same in all the membrane fractions. (3) The acyl chain composition of each phospholipid species varied in response to base analogue supplementation. However, this effect was larger in some membranes than others or in a different direction. For example, the unsaturated fatty acid to saturated fatty acid ratio increased in microsomes and mitochondria from analogue-supplemented cells. With ethanolamine supplementation the increase was greatest (2.1-fold) in mitochondria and even decreased by 20% in plasma membranes. Similar results were obtained with the phosphatidylethanolamine unsaturated/saturated fatty acid ratio. The latter points were not predicted by earlier results of others [15]. In fact the unsaturated/saturated fatty acid ratio of phosphatidylethanolamine from whole LM monolayer cells decreased by 34% in analogue-supplemented cells. No increase as noted herein with microsomes and mitochondria could have been forcast by whole cell data alone. No data on the effect of polar head group manipulation on acyl chain composition of the other individual phospholipid species (phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, and cardiolipin) has previously been presented. Alteration in the long chain fatty acid/short chain fatty acid ratio also occurred with choline analogue supplementation and the direction of the changes in phosphatidylcholine and phosphatidylethanolamine were generally similar to those noted above for the unsaturated/saturated fatty acid ratio.

A second major finding of this investigation is that alterations in the phosphatidylcholine and phosphatidylethanolamine fatty acyl groups of individual membrane fractions occurred. These changes could help to maintain a constancy of the membrane physicochemical environment. Alterations in phospholipid fatty acid composition had previously been shown in phospholipids of choline-deficient insects where compensatory changes in fatty acids of the phospholipids were noted in choline- and ethanolamine-containing phospholipids [33]. Thus it appears that compensatory changes in fatty acid composition of individual phospholipid species might account in part for the observed constancy in some physicochemical properties of LM suspension cell membranes such as characteristic transition temperatures [8,9] even though these compensatory alterations were not apparent from fatty acid compositions of whole phospholipid extracts of LM cell membranes [2] or whole LM cells grown in monolayer [15].

It has been shown that L-fibroblast phospholipids undergo deacylation-reacylation reactions, but the acyl chains did not equilibrate with either extracellular or intracellular pools of unesterified fatty acid [34]. If this also occurred in LM suspension cells, then extentsive "reshuffling" of pre-existing acyl groups between phospholipid species may be occurring during analogue supplementation as well as de novo synthesis of analogue-containing phospholipids. The "reshuffling" may be primarily responsible for changes in acyl composition of phosphatidylcholine and phosphatidylethanolamine. However, extensive acyl group retention does not occur in LM cells supplemented with choline, but may occur in LM cells supplemented with choline analogues, especially ethanolamine [35].

Thus, an interrelation of polar head group and acyl chain metabolism is indicated by each of the major membrane fractions of LM cells and this relationship varies with the type of membrane studied. In addition, it appears that alterations in fatty acid composition of individual membrane phospholipid species may be an important mechanism whereby LM suspension cells can compensate for expected alterations in physical properties in response to base analogue supplementation.

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