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REGULATION OF AMINOPHOSPHOLIPID ASYMMETRY IN MURINE FIBROBLAST PLASMA MEMBRANES BY CHOLINE AND ETHANOLAMINE ANALOGUES

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Summary

The regulation of the asymmetric distribution of aminophospholipids in mammalian cell plasma membranes is not understood at this time. One approach to determine the nature of such regulatory mechanisms is to attempt alteration of the plasma membrane phospholipid composition. Choline analogues such as *N,N'*-dimethylethanolamine and *N*-monomethylethanolamine lowered the quantity of phosphatidylethanolamine in the plasma membrane of LM fibroblasts grown in defined medium without serum. Ethanolamine supplementation increased the phosphatidylethanolamine content while ethanolamine analogues such as 2-amino-2-methyl-1-propanol, 2-amino-1-butanol, 1-aminopropanol, and 3-aminopropanol did not alter the aminophospholipid content significantly. The transverse distribution of aminophospholipids in the plasma membrane was determined by use of a chemical labelling reagent trinitrobenzenesulfonic acid. The percent phosphatidylethanolamine trinitrophenylated by trinitrobenzenesulfonate in the outer plasma membrane monolayer of LM cells supplemented with choline analogues was not altered. In contrast, ethanolamine analogue supplementation increased the percentage of aminophospholipid in the outer monolayer 2—3-fold. Ethanolamine analogue-containing phospholipids were distributed asymmetrically across the plasma membrane with 85 to 91% being located in the inner monolayer of the plasma membrane, a distribution similar to that of phosphatidylethanolamine. The fatty acyl composition of aminophospholipids in the outer monolayer was in all cases more saturated than in the corresponding phospholipids of the inner monolayer. However, choline analogues and especially the ethanolamine analogues reduced this difference. Thus, base analogues of choline and ethanolamine may alter the

aminophospholipid asymmetry, the surface charge, and the acyl chain asymmetry of LM cell plasma membranes

Introduction

Although numerous investigations demonstrated the existence of aminophospholipid asymmetry in mammalian or bacterial membranes, very little is known regarding regulation of this phenomenon. If transbilayer migration (flip-flop) of aminophospholipids is slow, it appears possible that serum, enriched with phosphatidylcholine and sphingomyelin, exchanges these lipids with the outer monolayer of the plasma membrane thereby causing an asymmetric distribution of lipids in mammalian surface membranes. This possibility was tested in three mammalian systems devoid of serum: (a) plasma membranes of LM cells grown in a chemically defined medium in the absence of serum [1,2]; (b) phagosomes, surface membrane derivatives, of LM cells grown in semidefined medium [3], and (c) synaptosomal plasma membranes of mouse brain, a biological membrane system which is not exposed to serum lipoproteins, since lipoproteins are not believed to cross the blood-brain barrier [4,5]. In all three cases aminophospholipid asymmetry appears to be present, indicating that serum lipids do not induce an asymmetric distribution of aminophospholipids in mammalian cell membranes. Such data are consistent with the de novo origin of aminophospholipid asymmetry. Other investigators showed the existence of a similar asymmetric distribution of phosphatidylethanolamine in bacterial membranes [6]. Thus, aminophospholipid asymmetry per se also appears to originate de novo in procaryotes.

Despite much effort in illustrating the existence of aminophospholipid asymmetry in biological membranes, very little is known regarding its structural or functional implications. Asymmetry of fluidity (vertical fluidity gradient) has been demonstrated as a structural feature of LM cell plasma membranes [7], red blood cell membranes [8], and sarcoplasmic reticulum [9]. Functional roles for asymmetry have been implicated in platelet aggregation and in pathological conditions such as sickle cell anemia and cell transformation [10—12]. One approach to study regulation of aminophospholipid asymmetry is to alter the aminophospholipid composition of the cells. Large alterations in plasma membrane lipid composition induced by media constituents may result in altered aminophospholipid asymmetry in LM cells. LM cells grown in the absence of choline (LM cells cannot synthesize choline), but supplemented with *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine readily incorporate these analogues into plasma membrane lipids (34%, 33% and 40%, respectively) [13]. The first two analogues decrease while ethanolamine increases the phosphatidylethanolamine content in LM cell plasma membranes. Aminophospholipid alterations can affect the activity of LM cell plasma membrane adenylate cyclase and thymidine transport [14—16]. In addition, normal chick embryo fibroblasts surface membrane morphology is altered by ethanolamine analogues to resemble that of virus-transformed chick embryo fibroblasts [17]. Thus it is possible that aminophospholipid asymmetry and/or vertical fluidity gradients in these membranes may be altered and these

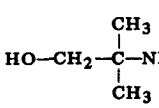
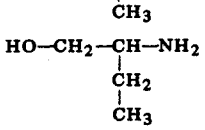
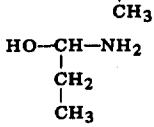
changes may in part be responsible for the different plasma membrane morphology and enzyme activities. The data presented here detail the effects of choline and ethanolamine analogues on (1) the asymmetric distribution of phosphatidylethanolamine, (2) the asymmetric distribution of fatty acyl groups, and (3) the asymmetric distribution of novel aminophospholipids in the LM cell plasma membrane.

Materials and Methods

Cell culture. LM cells, a choline-requiring strain of mouse fibroblasts, were grown in suspension culture in a serum-free, chemically defined medium as described by Schroeder et al. [13]. Cells in logarithmic growth were centrifuged at 225 × *g* for 10 min and the supernatant was decanted (all centrifugal forces refer to r_{av}). The cell pellet was resuspended at $1 \cdot 10^6$ cells/ml in choline media containing 4 μCi [^{32}P]phosphate/ml and maintained in logarithmic phase by the daily addition of the same choline-containing medium for 6 days. This procedure resulted in homogeneous labelling of all membrane phospholipids. The cells were then grown without choline but in the presence of choline analogue or ethanolamine analogue-containing media with the same specific activity of [^{32}P]phosphate for 3 days as described earlier [13]. The effect of two classes of aminophospholipid polar head group analogues was investigated. From the structures shown in Table I it is evident that group 1 bases (*N*,*N*'-dimethylethanolamine, *N*-monomethylethanolamine, ethanolamine) can be considered as analogues of choline with decreasing numbers of CH_3 groups on the nitrogen atom. However, the carbon skeleton of the base is unaltered. Group 2 analogues (2-amino-2-methyl-1-propanol, 2-amino-1-butanol, 1-amino-

TABLE I

GENERAL STRUCTURES OF PHOSPHOLIPID POLAR HEAD GROUP ANALOGUE BASES

Analogue base	Structure
Choline analogues (Group I)	
Choline	$HO-CH_2-CH_2-N^+(CH_3)_3$
N,*N*'-Dimethylethanolamine	$HO-CH_2-CH_2-NH(CH_3)_2$
N-Monomethylethanolamine	$HO-CH_2-CH_2-NH_2CH_3$
Ethanolamine	$HO-CH_2-CH_2-NH_2$
Ethanolamine analogues (Group II)	
2-Amino-2-methyl-1-propanol	
2-Amino-1-butanol	
1-Aminopropanol	
3-Aminopropanol	$HO-CH_2-CH_2-CH_2-NH_2$

propanol, and 3-aminopropanol) resemble ethanolamine in having an intact 1-amino group but altered carbon chain structure. Group 1 analogues were utilized to lower the aminophospholipid content (except for ethanolamine) while group 2 analogues were utilized to increase the aminophospholipid content of LM cell plasma membranes.

Labelling reactions. Our method of determining the asymmetric distribution of aminophospholipids in LM cell surface membranes utilized trinitrobenzenesulfonic acid, a chemical labelling reagent which binds covalently to primary amines of phospholipids and proteins. Details of the labelling procedure are provided elsewhere [2,4,5,7]. Plasma membrane, microsome, and mitochondrial fractions were isolated from cells treated with trinitrobenzenesulfonate as described previously [13]. The latter two membrane fractions were analyzed for trinitrophenylphosphatidylethanolamine content in order to determine the degree of penetration of trinitrobenzenesulfonate into the cell.

Analytical procedures. Lipid analysis was the same as described previously [2]. Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was determined as previously described [13]. Protein was measured by the method of Lowry et al. [18].

Results

Effects of trinitrobenzenesulfonate treatment on plasma membrane purification

Aminophospholipid asymmetry in LM cells was determined by trinitrophenylating aminophospholipids in the outer monolayer of the plasma membrane of intact LM cells with trinitrobenzenesulfonate. The cells were then fractionated and the plasma membrane was purified as described earlier [1,2, 13]. Trinitrobenzenesulfonate treatment of choline-fed cells decreased the activity of ($Na^+ + K^+$)-ATPase in both the cell homogenate and the purified plasma membrane by approx. 20% [1,2]. Choline analogue supplementation did not alter the plasma membrane purity [13]. As shown in Table II, trinitrobenzenesulfonate treatment lowered the ($Na^+ + K^+$)-ATPase activity of LM cell plasma membranes by approx. 20% in *N,N'*-dimethylethanolamine,

TABLE II

($Na^+ + K^+$)-ATPase ACTIVITY IN PLASMA MEMBRANES OF TRINITROBENZENESULFONATE-TREATED AND UNTREATED LM CELLS

The cells were fed choline or choline analogues as described in Materials and Methods. The ($Na^+ + K^+$)-ATPase activity of isolated plasma membranes was determined before or after labelling whole cells with 4 mM trinitrobenzenesulfonic acid, pH 8.5 and 4°C for 2 h. Values are the average of two determinations and individual values varied less than 10% from the average. Control values are from Ref. 13.

Choline substitute	nmol/mg protein per min	
	Control	Trinitrobenzene-sulfonate treated
Choline	66	53
N,N'-Dimethylethanolamine	74	66
N-Monomethylethanolamine	85	61
Ethanolamine	84	66

N-methylethanolamine and ethanolamine-supplemented cells. Similar decreases were found in whole cell homogenates (data not shown). Other amino-reactive reagents such as dansyl chloride also decrease ATPase activity by a small amount, 6% [19]. Comparison of the phospholipid composition and sterol/phospholipid ratio of the unlabelled versus trinitrobenzenesulfonate-labelled membranes showed no major differences (data not shown). These results indicate that although trinitrobenzenesulfonate labelling decreased the ATPase activity slightly it did not appear to affect membrane purification to any great extent.

Effect of choline analogue supplementation on LM cell plasma membrane aminophospholipid asymmetry

Plasma membranes from LM cells supplemented with choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, ethanolamine had 4.3 ± 0.3%, 3.5 ± 0.2%, 2.7 ± 0.2%, and 3.4 ± 0.9% ($n = 3$), respectively, of their phosphatidylethanolamine labelled by trinitrobenzenesulfonic acid. In cultures supplemented with choline analogues phosphatidylcholine, which most investigators consider to be localized mostly in the outer monolayer, was decreased 4-fold [13]. This labelling percentage was similar to that obtained with choline-fed LM cells [1,2] and is about 2—3-fold smaller than that of murine synaptosomal plasma membranes [4,5]. We showed previously that trinitrobenzenesulfonate did not penetrate the LM cells to any great extent under our labelling procedure since subcellular membranes were not labelled [1,2]. Essentially the same results were obtained with analogue supplemented cells. Thus, a constant percentage of phosphatidylethanolamine in the outer monolayer of plasma membranes from choline analogue (group I)-supplemented LM cells appeared to be reactive to trinitrobenzenesulfonic acid. Although the percentage distribution of phosphatidylethanolamine in the LM cell surface membrane was unchanged, the total quantity of phosphatidylethanolamine in the outer monolayer was altered. Approx. 1.7%, 0.7%, 0.3%, and 1.3% of the total plasma membrane phospholipids in choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine-fed cells, respectively, was in the outer monolayer as phosphatidylethanolamine. Therefore, although analogue supplementation did not change the percent distribution of phosphatidylethanolamine vertically across the plasma membrane bilayer, the actual quantity of phosphatidylethanolamine in the other monolayer varied.

Phosphatidylethanolamine acyl group asymmetry

The above studies indicated that an asymmetry distribution of aminophospholipids exists across the LM surface membrane. The phosphatidylethanolamine from the outer monolayer had a different acyl group composition than phosphatidylethanolamine from the inner monolayer [1,2,7]. Table III shows the results of fatty acid analysis of phosphatidylethanolamine and of trinitrophenylphosphatidylethanolamine from the plasma membranes of choline analogue-fed cells treated with trinitrobenzenesulfonic acid. The data indicate that the unlabelled phosphatidylethanolamine (that which we consider mostly to be in the inner monolayer of the plasma membrane bilayer) had a higher ratio of unsaturated to saturated fatty acids (U/S ratio) and a higher ratio of

TABLE III

DISTRIBUTION AND COMPOSITION OF THE PHOSPHATIDYLETHANOLAMINE ACYL GROUPS BETWEEN THE INSIDE AND OUTSIDE MONOLAYERS OF THE PLASMA MEMBRANE OF CHOLINE ANALOGUE-SUPPLEMENTED LM CELLS

Whole LM cells were reacted with 4 mM trinitrobenzenesulfonic acid, pH 8.5, and 4°C for 2 h. The fatty acid compositions was determined on phosphatidylethanolamine (PE) and trinitrophenylphosphatidylethanolamine (TNP-PE) isolated by thin-layer chromatography and analyzed by gas-liquid chromatography as described in Materials and Methods. *L/S*, the ratio of long-chain fatty acids (18 carbon long or longer) to short-chain fatty acids (14—7) carbons long). *U/S*, the ratio of unsaturated to saturated fatty acids. Values indicate the fatty acid composition (percent by wt. and represent the averages of two determinations with individual values deviating less tahn 10% from the average.

Fatty acid	Choline substitution							
	Choline *		*N,N'*-Dimethyl-ethanolamine		*N*-Monomethyl-ethanolamine		Ethanolamine	
	TNP-PE	PE	TNP-PE	PE	TNP-PE	PE	TNP-PE	PE
14 : 0	2.3	0.6	4.2	1.1	9.1	0.8	0.5	0.6
15 : 0	**	1.5	**	1.2	3.0	1.0	1.4	1.4
16 : 0	41.1	8.9	29.0	8.9	33.0	13.2	35.6	9.8
16 : 1	2.8	7.5	6.2	10.9	6.9	9.8	3.6	7.7
18 : 0	34.1	10.0	25.4	7.0	21.3	12.0	27.6	10.1
18 : 1	3.1	46.9	24.8	32.9	11.2	33.1	15.9	45.0
18 : 2	1.2	1.7	6.2	5.1	7.5	4.6	6.2	2.4
>C18	11.4	22.6	4.2	32.6	8.0	25.0	9.2	22.8
L/S	1.0	4.3	1.5	3.5	0.9	2.9	1.4	4.1
U/S	0.1	2.5	0.6	2.1	0.3	1.9	0.3	2.0

* Data are from Ref. 2.

** These fatty acids were present in quantities too low to be detected.

long-chain (18 carbons long or longer) to short-chain fatty acids (14—17 carbons long) than the trinitrophenylphosphatidylethanolamine. More specifically, phosphatidylethanolamine containing the less-fluid fatty acyl groups, 16 : 0 and 18 : 0, were located primarily in the outer monolayer of the plasma membrane bilayer. Unsaturated fatty acids, 16 : 1 and 18 : 1 were enriched in the inside monolayer. Diunsaturated fatty acids (18 : 2) were present in low amounts primarily in the inside monolayer. These differences cannot be explained on the basis of differential reactivity of saturated versus unsaturated phosphatidylethanolamine because (1) trinitrobenzenesulfonate labelling times were maximal and further labelling did not increase the percentage phosphatidylethanolamine in the outer monolayer, and (2) saturated and unsaturated phosphatidylethanolamine either of synthetic or natural origin reacted equally well with trinitrobenzenesulfonate [1,2].

Table III indicated that the unlabelled phosphatidylethanolamine of choline analogue (group 1)-supplemented cells was also more unsaturated than the labelled phosphatidylethanolamine. However, an interesting difference in 18 : 2 distribution was noted. The phosphatidylethanolamine of analogue-supplemented cells had more 18 : 2 than choline-fed cells. In addition, most of these 18 : 2 fatty acids from choline analogue-fed cells were present in the outer monolayer of the plasma membrane. Comparing the trinitrophenylphosphatidylethanolamine fatty acyl groups of choline fed cells with those of the

other analogue-fed cells the following trends in response to analogue supplementation were evident: (a) a decrease in saturated fatty acids, palmitic (16 : 0) and stearic (18 : 0), and (b) an increase in unsaturated fatty acids, palmitoleic (16 : 1), oleic (18 : 1) and linoleic (18 : 2). The changes in the unlabelled phosphatidylethanolamine (inside monolayer) acyl groups with decreasing number of methyl groups on the analogue appear to be a decrease in 18 : 1 and an increase in 18 : 2. These results can be summarized as follows: when compared to choline-supplemented LM cells, plasma membranes from cells supplemented with choline analogues (group I) had more unsaturated fatty acyl groups in phosphatidylethanolamine of the outer monolayer than in the inner monolayer. However, in all cases the inner monolayer phosphatidylethanolamine was much more unsaturated than the outer monolayer. Thus, both the quantity (but not percentage distribution) of phosphatidylethanolamine and the degree of unsaturation of phosphatidylethanolamine in the outer monolayer was affected by choline analogue (group I) supplementation.

Alteration of plasma membrane aminophospholipid asymmetry by ethanolamine analogues

Ethanolamine or ethanolamine analogue supplementation resulted in larger alterations of cell morphology, thymidine transport, hormone-stimulated adenylate cyclase activity, and physical properties than choline analogue supplementation [14—17,20—23]. Both transport and adenylate cyclase stimulation by hormones can be considered as vectorial processes across the cell membrane. Similarly the 'ruffling' of the cell surface may be due to alterations in surface area of the outer monolayer versus the inner monolayer of the plasma membrane [24]. If these processes are regulated at least in part by aminophospholipid asymmetry of the plasma membrane, then either (a) alteration of the percentage distribution of phosphatidylethanolamine across the plasma membrane bilayer, or (b) alteration of the total quantity of phosphatidylethanolamine in the outer monolayer could change these activities. Larger alterations in aminophospholipid asymmetry might be expected if ethanolamine analogues were supplemented to LM cells instead of choline analogues. Such analogues have extra methyl (2-amino-2methyl-1-propanol) or ethyl (2-amino-1-butanol-1-aminopropanol) side chains on the carbon structure of ethanolamine. Alternately, the ethanolamine carbon skeleton can be elongated (C_3 instead of C_2 in 3-aminopropanol) or shortened (C_1 instead of C_2 in 1-aminopropanol). None of the ethanolamine analogues (group II) alter the primary amino group, which reacts with trinitrobenzenesulfonic acid, of ethanolamine in any way.

Ethanolamine analogues such as 2-amino-2-methyl-1-propanol, 2-amino-1-butanol, 1-aminopropanol, and 3-aminopropanol, were incorporated into LM cell plasma phospholipids 12.4 ± 1.8%, 23.1 ± 1.8%, 18.3 ± 0.7% and 20.2 ± 2.9%, respectively, of the total phospholipids. Similar quantities were incorporated into the microsomal and mitochondrial phospholipids (data not shown). Ethanolamine analogue-supplemented cells were labelled with trinitrobenzenesulfonic acid and the plasma membranes were purified. As shown in Table IV, phosphatidylethanolamine and the other aminophospholipids were trinitrophenylated. Supplementation with 2-amino-1-butanol resulted in 12.6% of

TABLE IV

WHOLE CELL LABELLING OF AMINOPHOSPHOLIPIDS IN PLASMA MEMBRANES OF ETHANOLAMINE ANALOGUE-FED CELLS WITH TRINITROBENZENESULFONIC ACID

Analogue supplement	Percentage aminophospholipid labelled						
	Phosphatidyl-ethanolamine *	Phosphatidyl-serine	Phosphatidyl-2-amino-2-methyl-1-propanol **	Phosphatidyl-2-amino-1-butanol **	Phosphatidyl-1-amino-propanol **	Phosphatidyl-3-amino-propanol **	Total **
2-Amino-2-methyl-1-propanol	4.4 ± 1.3	4.6 ± 0.2 **	1.9 ± 0.1	—	—	—	9.0 ± 2.0
2-Amino-1-butanol	12.6 ± 2.6	3.8	—	2.8 ± 1.1	—	—	15.3 ± 3.3
1-Aminopropanol	5.5 ± 1.4	3.5	—	—	1.2 ± 0.3	—	9.8 ± 2.8
3-Aminopropanol	7.1 ± 1.1	2.1	—	—	—	2.0 ± 1.0	14.1 ± 2.6

Values represent means ± S.E., $n = 3$.

* Values represent averages ± range.

** Microsomal and mitochondrial aminophospholipids were labelled between 1 and 2% during the whole cell labelling procedure.

plasma membrane phosphatidylethanolamine becoming trinitrophenylated. Intracellular aminophospholipids were labelled only 1—2% during the trinitrobenzenesulfonic acid treatment procedure. In contrast under penetrating conditions (24°C for 2 h), 90% of all the aminophospholipids was labelled by trinitrobenzenesulfonate. This indicated that the ethanolamine analogue-containing phospholipids are just as reactive as phosphatidylethanolamine. However, approx. 10% of these phospholipids did not react with trinitrobenzenesulfonate. The total percentage aminophospholipid labelled in the ethanolamine analogue-fed cells was between 9.0 and 15.3%. The percentage of aminophospholipid labelled in LM cell plasma membranes from ethanolamine analogue-supplemented cells was therefore 2.2—3.7 times greater than in choline or choline analogue-supplemented cells.

Phosphatidylethanolamine fatty acyl chain asymmetry in ethanolamine analogue-supplemented cells

We have shown that the acyl groups of phosphatidylethanolamine labelled by trinitrobenzenesulfonic acid in the outer monolayer of plasma membranes were highly saturated while unreacted (inner monolayer) phosphatidylethanolamine acyl groups were enriched with unsaturated fatty acid (see Table III). The effect of ethanolamine analogue supplementation was tested to determine if variation in the carbon chain of the supplement would cause a compensating change in the phosphatidylethanolamine acyl chain composition or asymmetry. LM cells are fully capable of such 'homeostatic adaptation' or compensation when supplemented with base analogues [13,20—23]. As shown in Table V,

TABLE V

FATTY ACID COMPOSITION OF INNER AND OUTER MONOLAYER PHOSPHATIDYLETHANOLAMINE FROM PLASMA MEMBRANES OF ETHANOLAMINE ANALOGUE-SUPPLEMENTED LM CELLS

All procedures were performed as described in Materials and Methods and Table IV. Values indicate the fatty acid composition (percentage by wt.) and represent the means of three experiments with individual values deviating less than 10% from the average. TNP-PE, trinitrophenylphosphatidylethanolamine; PE, phosphatidylethanolamine; *L/S*, 18 carbon or longer fatty acids/14—17 carbon fatty acids; *U/S*, unsaturated/saturated.

Fatty acid	Ethanolamine substitution							
	2-Amino-2-methyl-1-propanol		2-Amino-1-butanol		1-Aminopropanol		3-Aminopropanol	
	TNP-PE	PE	TNP-PE	PE	TNP-PE	PE	TNP-PE	PE
14 : 0	1.3	1.7	1.0	1.5	0.9	2.8	1.2	0.4
15 : 0	0.1	1.5	0.2	3.5	—	1.3	0.1	1.0
16 : 0	29.6	12.0	30.9	8.5	27.8	19.0	24.4	25.7
16 : 1	0.6	1.4	0.3	3.4	—	3.4	0.3	1.7
18 : 0	28.0	31.4	24.0	16.6	15.3	21.6	20.8	19.2
18 : 1	16.6	28.8	18.1	26.5	2.6	13.0	13.1	23.1
18 : 2	—	0.4	—	1.3	—	1.4	0.2	1.0
>C18	26.0	22.4	26.6	38.4	50.1	37.2	39.7	27.6
L/S	2.4	4.6	2.2	4.8	2.1	2.7	2.8	2.4
U/S	0.4	0.9	0.6	1.3	0.7	0.8	0.7	1.0

ethanolamine analogue supplementation altered the distribution of saturated and unsaturated fatty acyl chain in phosphatidylethanolamine. Table III indicated that in choline-supplemented cells the outer monolayer phosphatidylethanolamine had a U/S (unsaturated/saturated fatty acid ratio) of 0.1 while inner monolayer phosphatidylethanolamine had a value of 2.5. The ethanolamine analogues increased the ratio of U/S of phosphatidylethanolamine in the outer monolayer to 0.4—0.7 and decreased the ratio in the inner monolayer to 0.8—1.3. Thus, the asymmetric distribution of phosphatidylethanolamine acyl groups was changed by ethanolamine analogues. This trend was evident even if U/S ratios were compared to those of phosphatidylethanolamine from ethanolamine-supplemented LM cells. Similarly the ratio of long-chain/short-chain fatty acids of the outer monolayer was approx. 2-fold greater in plasma membrane phosphatidylethanolamine of ethanolamine or ethanolamine analogue-fed cells than in choline-fed LM cells. In summary, supplementation of LM cells with ethanolamine analogues resulted in alterations in the U/S and L/S ratios of phosphatidylethanolamine in both trinitrobenzenesulfonate-reactive (outer monolayer) and unreacted phosphatidylethanolamine (inner monolayer).

Phosphatidylethanolamine analogue acyl asymmetry in ethanolamine analogue-fed cells

The previous section indicated that ethanolamine analogue supplementation did not result in a simple replacement of phosphatidylethanolamine by phosphatidylethanolamine analogue. Instead, it appeared as if either (a) selective types of phosphatidylethanolamine were replaced, or (b) the asymmetric distribution of phosphatidylethanolamine was altered. If the first possibility is true then it would seem logical that the acyl chains of phosphatidylethanolamine analogue in the outer monolayer might have a U/S ratio different from the phosphatidylethanolamine in the same monolayer. Table VI shows the acyl chain composition of trinitrobenzenesulfonate-derivatized ethanolamine analogue-containing phospholipids from plasma membranes of ethanolamine-supplemented cells. As indicated, more unsaturated fatty acids were associated with the underivatized (inner monolayer) phosphatidylethanolamine analogues. However, these phospholipids had a lower U/S ratio than choline or choline analogue-supplemented cells (0.8—0.9 versus 1.8—2.4). Their U/S ratios did resemble closely those of the corresponding phosphatidylethanolamines from the ethanolamine analogue-supplemented membranes (see Table V). Thus, possibility (a) above appears less likely than (b). Ethanolamine analogue supplementation therefore decreased the acyl chain asymmetry of aminophospholipids across the LM cell plasma membrane (as determined by U/S ratios). Similar alterations appear to occur in the L/S ratios. Thus, it appears as if ethanolamine analogues supplementation causes a more symmetric distribution of acyl chains in aminophospholipids across the surface membrane bilayer than choline analogue supplementation.

Alteration in quantity of aminophospholipids

The above results indicate that not only the percentage distribution and acyl chain composition but also the quantity of aminophospholipids in the plasma membrane was altered by base analogues. The latter data are summarized in

TABLE VI

FATTY ACID COMPOSITION OF INNER AND OUTER MONOLAYER ETHANOLAMINE ANALOGUE-CONTAINING PHOSPHOLIPID OF PLASMA MEMBRANES FROM ANALOGUE-SUPPLEMENTED LM CELLS

All procedures were performed as described in Materials and Methods and Table IV. Values indicate the fatty acid composition (percentage by wt.) and represent the means of three experiments with individual values deviating less than 10% from the mean. TNP-PAMP, trinitrophenylphosphatidyl-2-amino-2-methyl-1-propanol; PAMP, phosphatidyl-2-methyl-1-propanol; TNP-PAB, trinitrophenylphosphatidyl-2-amino-1-butanol; PAB, phosphatidyl-2-amino-1-butanol; TNP-P1-AP, trinitrophenylphosphatidyl-1-aminopropanol; P-1-AP, phosphatidyl-1-aminopropanol; TNP-P-3-AP, trinitrophenylphosphatidyl-3-aminopropanol; P-3-AP, phosphatidyl-3-aminopropanol; *L/S*, 18 carbon or longer fatty acids/14—16 carbon fatty acids; *U/S*, unsaturated/saturated.

Fatty acid	Ethanolamine substitution							
	2-Amino-2-methyl-1-propanol		2-Amino-1-butanol		1-Aminopropanol		3-Aminopropanol	
	TNP-PAMP	PAMP	TNP-PAB	PAB	TNP-P-1-AP	P-1-AP	TNP-P-3-AP	P-3-AP
14 : 0	1.9	1.0	0.4	0.9	6.0	1.2	0.9	1.1
15 : 0	1.0	0.9	0.6	1.0	0.2	1.1	0.2	1.4
16 : 0	23.6	25.0	32.3	25.8	49.3	16.8	37.0	23.9
16 : 1	0.9	1.7	—	1.6	—	2.3	—	2.4
18 : 0	23.9	23.2	22.1	23.3	16.7	26.8	28.1	23.7
18 : 1	13.8	15.5	11.9	14.4	2.7	21.3	3.6	25.5
18 : 2	—	0.4	—	0.9	0.3	2.2	0.2	2.5
>C18	34.9	32.4	33.4	31.9	24.9	20.3	30.1	19.8
L/S	2.6	2.8	2.1	2.4	0.8	3.2	1.6	2.5
U/S	0.5	0.9	0.6	0.9	0.4	0.8	0.4	0.8

Table VII. *N,N'*-Dimethylethanolamine and *N*-monomethylethanolamine decreased the percentage of aminophospholipids in plasma membranes from 39% to 30% and 24%, respectively. In contrast ethanolamine supplementation increased the percentage of aminophospholipid from 39% to 51%. Ethanolamine analogues such as 2-amino-2-methyl-1-propanol, 2-amino-1-butanol, 1-aminopropanol and 3-aminopropanol did not change the percentage of

TABLE VII

ALTERATION IN TOTAL QUANTITY OF AMINOPHOSPHOLIPID IN PLASMA MEMBRANES OF CHOLINE AND ETHANOLAMINE ANALOGUE-SUPPLEMENTED LM CELLS

Values represent means ± S.E., $n = 3$.

Analogue supplement	% aminophospholipid in total phospholipid
Choline	39.7 ± 2.3
N,N'-Dimethylethanolamine	30.2 ± 2.6
N-Monomethylethanolamine	24.5 ± 1.9
Ethanolamine	51.1 ± 3.0
2-Amino-2-methyl-1-propanol	37.9 ± 2.1
2-Amino-1-butanol	36.5 ± 2.5
1-Aminopropanol	41.4 ± 4.4
3-Aminopropanol	37.6 ± 2.5

aminophospholipid significantly as compared to choline-fed cells. Therefore, since choline analogues (group I) did not change the percentage phosphatidylethanolamine found in the outer monolayer (while, in contrast, ethanolamine analogues increased this percentage 2—3-fold, then the total amount of net negatively charged (acidic) lipids could be considerably altered in the outer monolayer of the plasma membrane. As shown in Table VIII, the ratio of zwitterionic to acidic phospholipids is decreased from 1.07 to 0.37, 0.53 and 0.71, respectively, by *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine supplementation. Ethanolamine analogues also decrease this ratio from 1.07 to 0.68 ± 0.06. These results indicate that since the percentage aminophospholipid is increased by some of these analogues and since the ratio of acidic to zwitterionic (reciprocal of zwitterionic/acidic) phospholipids increases, then the surface charge of the LM cell plasma membrane may also be altered unless some other compensatory change were taking place.

Homeostatic responses to ethanolamine analogue supplementation

Large alterations in phospholipid composition can modify the physical properties of membranes, the activities of membrane-bound enzymes, and other biological processes. We have previously shown that LM cells can isothermally adapt to choline analogue supplementation [13,20—23]. However, it is not known if ethanolamine analogue incorporation into LM cells can lead to compensation or adaptation to the expected increases in membrane rigidity resulting from increased aminophospholipid content [25,26]. The data in this section are provided to clarify this point. The phospholipid composition of plasma membranes from LM cells grown in the presence of ethanolamine analogues is shown in Table IX (percentage phosphatidylethanolamine analogue is given in Table IV). Choline-supplemented cells contain 37.8% and 29.6% of their plasma membrane phospholipid as phosphatidylcholine and phosphatidylethanolamine, respectively [13]. The ethanolamine analogues decrease both the

TABLE VIII

EFFECT OF SUPPLEMENTING LM CELLS WITH ETHANOLAMINE ANALOGUES OF CHOLINE ON RATIO OF ZWITTERIONIC TO AMINO PLASMA MEMBRANE PHOSPHOLIPIDS

[Zwitterionic/amino] = (PC + SP + CPC)/(PE + PS + PX + PG + PA + CL + PI) where X = AMP, AB, 1-AP, or 3-AP. Values represent the means ± S.E., n = 3.

Supplement	[Zwitterionic/acidic] phospholipids
Choline	1.07 ± 0.12
N,N'-Dimethylethanolamine *	0.37 ± 0.05
N-Monomethylethanolamine *	0.53 ± 0.08
Ethanolamine	0.71 ± 0.09
2-Amino-2-methyl-1-propanol	0.63 ± 0.11
2-Amino-1-butanol	0.73 ± 0.09
1-Amino-2-propanol	0.62 ± 0.16
3-Amino-1-propanol	0.71 ± 0.13

* Phosphatidyl-*N,N'*-dimethylethanolamine and phosphatidyl-*N*-monomethylethanolamine are omitted in this calculation since the net charge of these polar head groups is not known. However, if these phospholipids are assumed to be zwitterionic with no net negative change the zwitterionic/acidic phospholipid ratios would be essentially the same as that of choline-supplemented LM cells.

TABLE IX

EFFECT OF ETHANOLAMINE ANALOGUE SUPPLEMENTATION ON COMPOSITION OF LM CELL PLASMA MEMBRANE PHOSPHOLIPIDS

Composition excludes analogue-containing phospholipids. Values represent the means ± S.E., $n = 3$.

Analogue supplement	Phospholipid composition (percentage)							
	Choline	Ethanol-amine	Inositol	Serine	Sphingo-myelin	Glycerol	Cardio-lipin	Other
2-Amino-2-methyl-1-propanol	20.44 ± 0.96	22.46 ± 1.71	6.25 ± 1.11	3.02 ± 0.63	4.76 ± 1.35	13.48 ± 1.16	—	17.20 ± 2.7
2-Amino-1-butanol	25.99 ± 0.50	11.31 ± 1.01	7.40 ± 0.97	2.04 ± 0.72	4.03 ± 0.72	10.74 ± 1.49	—	15.4 ± 2.0
1-Aminopropanol	22.8 ± 2.1	20.3 ± 1.2	4.1 ± 1.0	2.6 ± 0.6	3.0 ± 1.0	12.1 ± 2.5	—	16.9 ± 2.8
3-Aminopropanol	22.6 ± 1.8	15.2 ± 1.1	5.0 ± 0.7	2.2 ± 0.9	4.7 ± 1.1	13.5 ± 0.8	—	16.6 ± 2.9

percentage phosphatidylcholine and the percentage phosphatidylethanolamine by as much as 45% and 62%, respectively. Phosphatidylcholine is more fluid than phosphatidylethanolamine with the same acyl chain composition [20,21]. Therefore, if the ethanolamine analogue phospholipids resemble phosphatidylethanolamine in their physical properties and since the phosphatidylcholine content is decreased, the membrane phospholipids would be expected to be more rigid. The decreased phosphatidylethanolamine content may partially compensate for this since the total percentage aminophospholipid is not altered significantly by ethanolamine analogues (see Table VII). Thus adaptation by lowering of phosphatidylethanolamine appears to occur. In contrast the total percentage aminophospholipid of the mitochondria of ethanolamine analogue-supplemented cells was increased 1.5—1.7-fold as compared to mitochondria of choline-fed controls. Therefore, in the mitochondria adaptation by decreased phosphatidylethanolamine content would not appear to be an adequate compensatory mechanism.

Increased lysophospholipid can also fluidize membranes [27]. LM cells appear to compensate to increased fluidity due to choline analogue supplementation by increased lysophosphatidylcholine but not lysophosphatidylethanolamine content [13]. The lysophosphatidylcholine content of LM cell

TABLE X

EFFECT OF SUPPLEMENTING ETHANOLAMINE ANALOGUES ON LM CELL PLASMA MEMBRANE LYSOPHOSPHOLIPID COMPOSITION

Each value represents mean ± S.E., $n = 3$.

Analogue supplement	Percentage of total phospholipid	
	Lysophosphatidyl-choline	Lysophosphatidyl-ethanolamine
2-Amino-2-methyl-1-propanol	10.5 ± 2.1	1.1 ± 0.3
2-Amino-1-butanol	9.2 ± 1.2	0.8 ± 0.4
1-Aminopropanol	8.0 ± 2.1	0.6 ± 0.1
3-Aminopropanol	11.2 ± 2.2	0.6 ± 0.1

TABLE XI

FATTY ACID COMPOSITION OF PLASMA MEMBRANE PHOSPHOLIPIDS FROM ETHANOLAMINE ANALOGUE-SUPPLEMENTED LM CELLS

All procedures were performed as described in Materials and Methods and Table VII. Values indicate the fatty acid composition (percentage by wt.) and refer to mean. Individual values deviated less than 10% from the mean. PE, phosphatidylethanolamine; PC, phosphatidylcholine; P-AMP, phosphatidylaminomethylpropanol; P-3-AP, phosphatidyl-3-aminopropanol.

Fatty acid	Ethanolamine substitution											
	2-Amino-2-methyl-1-propanol			2-Amino-1-butanol			1-Aminopropanol			3-Aminopropanol		
	PE	P-AMP	PC	PE	PAB	PC	PE	P-1-AP	PC	PE	P-3-AP	PC
14 : 0	1.7	1.0	0.5	1.4	0.8	1.8	2.7	1.2	3.5	0.5	1.0	2.1
15 : 0	1.5	0.9	1.7	3.0	1.0	1.5	1.3	1.1	0.9	0.9	1.4	2.1
16 : 0	12.8	24.9	33.6	11.5	25.9	31.7	19.5	17.2	41.8	25.7	24.2	33.5
16 : 1	1.4	1.6	0.9	3.0	1.5	0.5	3.3	2.3	0.6	1.6	2.4	0.6
18 : 0	31.4	23.1	23.0	17.6	23.3	20.8	21.3	26.7	19.4	19.4	23.7	21.9
18 : 1	28.3	15.4	10.3	25.0	14.3	13.3	12.5	12.1	7.3	22.4	25.1	13.9
18 : 2	0.4	0.4	1.7	1.2	0.8	1.0	1.3	2.2	0.9	1.0	2.5	1.3
>C18	22.6	32.8	28.1	37.1	31.9	29.2	38.4	28.3	25.6	28.5	20.0	24.6
L/S	4.7	2.5	1.7	4.2	2.4	1.8	2.7	3.6	1.1	2.5	2.8	1.6
U/S	0.9	0.8	0.5	1.2	1.0	0.6	0.8	0.8	0.4	1.0	0.8	0.5

plasma membranes and L cell plasma membranes is about 6% [13,28]. As shown in Table X, ethanolamine analogues increased the lysophosphatidylcholine content of LM cell plasma membranes to 8—11%. Mitochondrial lysophosphatidylcholine content also increased 2 or 3% (data not shown).

Alterations in the sterol/phospholipid ratio can alter membrane fluidity by increasing or decreasing the molecular area of the phospholipids [25]. Choline analogues decreased the desmosterol/phospholipid ratio in LM cell plasma membranes from 0.65 in choline-fed cells to 0.48 and 0.44 in *N,N'*-dimethylethanolamine and *N*-monomethylethanolamine-fed cells, respectively [13]. The ethanolamine analogues 2-amino-2-methyl-1-propanol, 2-amino-1-butanol, 1-aminopropanol, and 3-aminopropanol decreased this ratio even further to 0.17 ± 0.05, 0.14 ± 0.03, 0.16 ± 0.05, and 0.19 ± 0.05, respectively. This would further tend to fluidize the plasma membranes. The desmosterol/phospholipid molar ratio of the microsomes and mitochondria was similar to those of control choline-fed cells.

Lastly, it is possible that variations in acyl chain composition can compensate for alterations in membrane fluidity. As shown in Table XI, the U/S and L/S ratios of the acyl groups from phosphatidylethanolamine analogue lipids were usually intermediate between phosphatidylethanolamine and phosphatidylcholine from the same membrane. These results indicated that the acyl chain of the ethanolamine analogue phospholipids are not the same as those of phosphatidylethanolamine, the aminophospholipid which it displaces. Such intermediate acyl chain composition may result in phospholipids of intermediate physical properties, thereby compensating for alterations in the polar head group.

Discussion

Several observations regarding regulation of asymmetric distribution of aminophospholipids in surface membranes of LM fibroblasts may be made from the data presented here: (1) the transbilayer distribution of aminophospholipids can be altered by supplementing the cells with a phospholipid polar head group analogue; (2) the acyl chain asymmetry can also be altered; (3) both types of changes can be accomplished in the absence of serum. Since the amount of aminophospholipid labelled by trinitrobenzenesulfonate in the plasma membrane is small, several criteria indicate that these conclusions are valid: (a) The fold purification of plasma membranes from analogue-supplemented cells labelled with trinitrobenzenesulfonate was similar to that obtained with unlabelled cells. (b) As shown earlier at 4°C under the reaction conditions used herein the trinitrobenzenesulfonate did not appear to penetrate the cell [1,2]. In addition, fluorescence data also are consistent with little penetration of trinitrobenzenesulfonate. If trinitrobenzenesulfonate penetrated the LM cell and labelled the plasma membrane on both sides then 90—95% of incorporated β-parinarate fluorescence was quenched [7]. However, if the cells were labelled under our reaction conditions, then only 45% of the fluorescence was quenched [7]. If trinitrobenzenesulfonate had penetrated then greater than 50% quench would be expected. These results are consistent with the interpretation that little trinitrobenzenesulfonate labelling, if any, occurred on the

inner monolayer of the plasma membrane. (c) Both saturated and unsaturated aminophospholipids of synthetic or biological origin reacted equally well with trinitrobenzenesulfonate [1,2]. (d) Essentially all of the aminophospholipid should react with trinitrobenzenesulfonate under penetrating conditions. This result was not obtained herein (85—90% labelling) nor by any of the other major asymmetry measuring methods (chemical labelling, phospholipases, or phospholipid exchange proteins) without damaging the membrane. In fact, it has been shown that phosphatidylserine appears to form a non-exchangeable annulus surrounding the sarcoplasmic reticulum Ca^{2+}-ATPase [28].

Both choline and ethanolamine analogue supplementation appeared to alter the asymmetric distribution of aminophospholipids in LM cell plasma membranes. Choline analogues (except for ethanolamine) decreased the content of aminophospholipid but not the percentage distribution across the membrane. Ethanolamine analogues did not increase the content of aminophospholipids but did increase the percentage found in the outer monolayer. The net result of these alterations was (1) an increase in the ratio of acidic/zwitterionic phospholipids in the plasma membranes; (2) choline analogues such as *N,N'*-dimethylethanolamine and *N*-monomethylethanolamine appear to decrease the amount of negatively charged lipids in the outer monolayer and (3) ethanolamine and its analogues increased the quantity of negatively charged phospholipids in the outer monolayer of the plasma membrane.

Both choline and ethanolamine analogues appear to alter the acyl chain asymmetry of the aminophospholipids in the plasma membranes (ethanolamine analogues had the greater effect). In all cases, the unsaturated/saturated fatty acid ratio of the outer monolayer aminophospholipids was lower than that of the inner monolayer aminophospholipids. Analogue supplementation, especially with ethanolamine analogues, decreased this difference. An asymmetric distribution of acyl groups in aminophospholipids has been demonstrated in LM fibroblast plasma membranes [1,2], synaptosomal plasma membranes [29] and erythrocyte membranes [30]. An asymmetric distribution of acyl chains has also recently been shown in vesicular stomatitis virus and *Acholeplasma laidlawii* [31,32]. Our results are consistent with those obtained in the above investigations: unsaturated phospholipid species are enriched in the inner monolayer of membrane bilayers. Our results do not agree with those obtained with model membrane studies which show that upon sonication, single bilayer vesicles of limiting radii of curvature have saturated phospholipids primarily on the inner monolayer [31]. However, these data were obtained with a model system in which an asymmetric distribution appears only after sonication. In addition, it has been shown that the asymmetric distribution of dimyristoylphosphatidylethanolamine between the two monolayers of small single-walled vesicles is the result of the combined properties of the acyl chain and polar head group composition [33]. In contrast to the asymmetric distribution of acyl chains in phosphatidylethanolamine and other aminophospholipids of isolated plasma membranes from LM cells shown here, phagosomes derived from LM cells, did not have an acyl chain asymmetry [3]. Phagosomes obtained from several systems differ significantly in enzyme composition and other factors from the rest of the plasma membrane surface and may therefore represent specialized membrane regions [34,35].

In summary, it appears that choline and ethanolamine analogues may alter or in part regulate the aminophospholipid asymmetry of LM cell plasma membranes.

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