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Structure and dynamics of the phosphatidylcholine and the phosphatidylethanolamine head group in L-M fibroblasts as studied by deuterium nuclear magnetic resonance

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Mouse fibroblast L-M cells were grown in tissue culture medium containing selectively deuterated choline or ethanolamine. Both compounds were incorporated into the corresponding phospholipids at levels > 50% thus leading to a selective deuteration of these phospholipid head groups. Choline and ethanolamine were labeled at either the α- or the β-carbon atom and well-resolved deuterium and phosphorus n.m.r. spectra were obtained from intact cells, crude plasma membranes and lipid extracts, leading to the following conclusions. (i) A large fraction, if not all, of the phospholipds in the intact L-M cell membranes were organized in a liquid crystalline bilayer. (ii) The phosphoethanolamine and the phosphocholine head group conformation were found to be remarkably similar in pure lipid bilayers and in intact L-M cell membranes with the head group dipoles being oriented parallel to the membrane surface. (iii) The deuterium T_1 spin lattice relaxation times fell in the range of 7–25 ms and were similar in intact L-M cells and in pure lipid model membranes, suggesting that the two head groups are not involved in strong interactions with membrane proteins. The rotational diffusion rate of the two head groups was reduced by at least a factor of 10 compared to molecules of the same size in aqueous solution. (iv) The phosphocholine head group was sensitive to the size and sign of membrane surface charges as verified in mixing experiments with charged lipids. In L-M cell membranes the phosphocholine appeared to sense an electrically neutral environment in spite of the fact that L-M cell membranes contain 10–20% negatively charged lipdis.

Key words: phosphatidylcholine/phosphatidylethanolamine/n.m.r./L-M fibroblasts

Introduction

Lipids are the essential building blocks of biological membranes, forming stable, but nevertheless very thin and fluid-like separating walls between aqueous compartments. Compared to other biological molecules, lipids are unique in their property of adopting quite different supramolecular organizations such as bilayers, hexagonal and cubic phases and micellar structures. The chemical structure of most lipids – leaving aside the glycolipids – is generally quite simple and both the chemical synthesis and the biosynthesis of lipids are well-understood. In spite of these deceptively simple chemical and physical features very fundamental problems in lipidology and membrane research are still unresolved. First and above all, no unifying concept has emerged to explain the enormous diversity of lipid species. If, as according to many biochemical textbooks, the role of lipids is limited to providing a 'grease' for the protein machinery this certainly could be achieved with a much reduced number of lipid species. Lipid fluidity alone would also not explain the experimental fact of membrane asymmetry, i.e. the observation that biological bilayer membranes carry different lipids in the two monolayer leaflets. Another argument which is often advanced to explain lipid diversity associates an enzyme–substrate relationship between membrane-bound proteins and lipids. However, the lipid specificity of most reconstituted membrane proteins is rather broad, i.e. their activity can be restored with quite different lipids.

We therefore feel that specific, co-factor type of interactions are rather rare and that lipids instead act collectively by modulating and amplifying the physicochemical properties of biological membranes. One interesting parameter in this respect is the membrane suface potential and, as the molecular basis of the latter, the distribution of electric charges at the membrane surface. Since these charges act at short distances even small variations suffice to trigger conformational changes of nearby proteins. We have investigated the membrane surface potential previously in connection with the Ca^{2+} binding to membrane surfaces (Altenbach and Seelig, 1984; Macdonald and Seelig, 1987a,b). In the present work, we address a related problem, namely the orientation of the phosphocholine and the phosphoethanolamine head groups in biological membranes. Both head groups are zwitterionic; however, depending on their orientation quite different electric effects are generated. If the head group dipoles are oriented upright, i.e. pointing perpendicular to the surface of the membrane, molecules approaching the membrane surface will see a positive potential; in contrast, molecules embedded in the hydrophobic interior encounter a negative dipole field. This may be compared to an in-plane orientation of the lipid head groups which would create an electrically neutral membrane surface.

In fact, relatively little is known about the orientation of the major phospholipid head groups with respect to the membrane surface. X-ray structural analysis has been performed on single crystals of phosphatidylethanolamine (PE) (Hitchcock *et al.*, 1974), phosphatidylcholine (PC) (Pearson and Pascher, 1979) and phosphatidylglycerol (PG) (Pascher *et al.*, 1987) and the polar groups were found to be aligned approximately perpendicular to the long axis of the hydrocarbon chains. In parallel, fully hydrated phospholipid bilayers in the gel state and the fluid state have been investigated with neutron diffraction techniques (Büldt *et al.*, 1978, 1979; Büldt and Seelig, 1980; Mischel *et al.*, 1987) and deuterium n.m.r. (Gally *et al.*, 1975; Seelig and Gally, 1976; Wohlgemuth *et al.*, 1980). In pure lipid model membranes the head groups of PC, PE and PG were found to be aligned parallel (within 30°) to the plane of the membrane both in the gel state and in the liquid crystalline state.

Little information exists also concerning the lipid head group orientation in intact biological membranes. Generally, the complex biochemical composition of biological membranes precludes the observation of individual lipid classes *in situ*. However, by combining n.m.r. with biosynthetical isotopic labeling individual

phospholipid head groups can be singled out and become amenable to a conformational analysis. Using deuterium n.m.r. and supplying selectively deuterated glycerol to the growth medium of an *Escherichia coli* mutant it was possible, for example, to elucidate the phosphoglycerol head group conformation in *E. coli* cell membranes (Gally *et al.*, 1981; Borle and Seelig, 1983). Likewise, Chinese hamster ovary (CHO) cells were grown on ^{13}C-labeled choline and the observed n.m.r. coupling constants were used to obtain conformational information on the phosphocholine group in the cell membranes (London *et al.*, 1979). γ-Deuterated choline [$(CD_3)_3NCH_2CH_2I^-$] has been incorporated biosynthetically into the L-M cell line (Arvidson *et al.*, 1975; Oldfield *et al.*, 1976). The same molecule has been fed also to rats and the deuterium n.m.r. spectra of several excised rat tissues were obtained (Curatolo *et al.*, 1985). Unfortunately, the observed deuterium n.m.r. spectra were not very informative and did not provide insight into the head group conformation.

Our aim in the present work was to provide a structural and dynamic analysis of the phosphocholine and phosphoethanolamine head groups in intact eukaryotic cell membranes. A strain of mouse fibroblasts, L-M cells, derived from the connective tissue of mouse was used and the polar head groups of the phospholipids were manipulated by adding either deuterated choline or deuterated ethanolamine to a serum-free tissue culture medium (Higuchi, 1970). We obtained ^2H- and ^{31}P-n.m.r. spectra from lipid extracts, crude plasma membrane preparations, and intact cells and compared them with those of synthetic lipid model membranes. A comparison of ^2H- and ^{31}P-n.m.r. spectra allowed a quantitative estimate of the extent of deuterium labeling. The measurement of deuterium spin lattice relaxation times of cells and lipid extracts provided information on the rate of head group reorientation. In addition, in order to explain the influence of lipid heterogeneity on the polar head group structure, a synthetic head group deuterated lipid (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, POPC) was mixed with various proportions of zwitterionic and negatively charged phospholipids. At least up to lipid concentrations of 30 mol% the head group splittings were found to vary linearly with the electric surface charge density.

Results

Cell viability

L-M mouse fibroblasts were chosen because they can be cultured in a chemically defined, serum-free medium (Higuchi, 1970). Choline is an essential ingredient of the growth medium and has been replaced by deuterated choline in the present experiments. As a further advantage the cells can be grown in suspension culture facilitating large-scale preparations of cell membranes and lipids. The phospholipid composition of L-M cells can be manipulated and has been studied in detail (Glaser *et al.*, 1974; Schroeder *et al.*, 1976; Schroeder, 1984; Rujanavetch and Silbert, 1986).

The metabolic state of L-M cells under the conditions of the n.m.r. experiment was assessed with high resolution ^{31}P-n.m.r. Figure 1A shows a time-course of ^{31}P-n.m.r. spectra of L-M cells centrifuged at 100 *g* and transferred into a 10 mm n.m.r. tube. Initially, the cells were rich in ATP and low in inorganic phosphate. Within 2 h (at 10°C) after the start of the experiment the ATP reservoir was almost completely exhausted while the inorganic phosphate concentration increased concomitantly. At 20 and 30°C the ATP decay occurred more rapidly (within 30 min). Nevertheless, most of the cells were still intact and viable

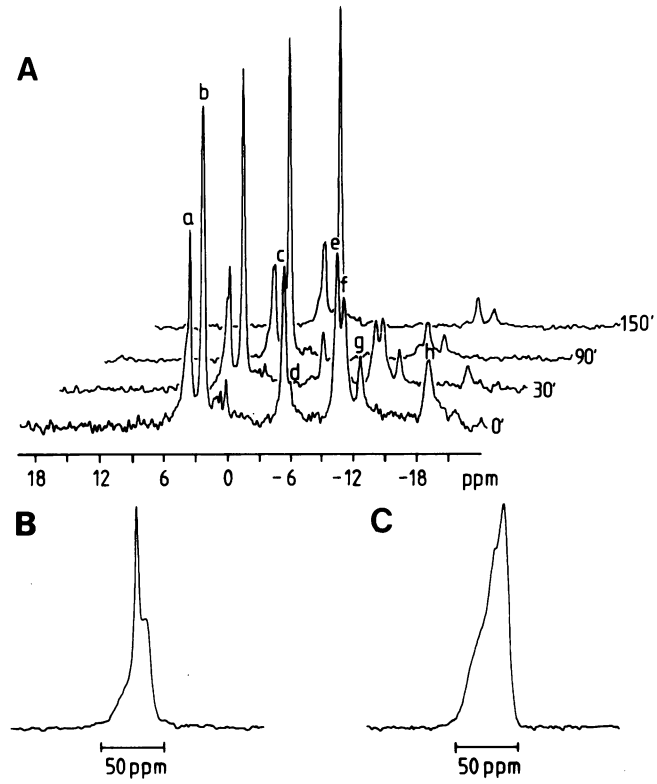

Fig. 1. Phosphorus n.m.r. spectra at 121.48 MHz of intact L-M cells and membrane preparations. **A:** high resolution n.m.r. spectra of $\sim 6\times10^8$ cells in buffer at 10°C. Each spectrum required 22 min of accumulation. Resonance assignments: a, α-AMP and various sugar phosphates; b, inorganic phosphate; c, γ-ATP; d, β-ADP; e, α-ATP and α-ADP; f, nicotinamide adenine dinucleotides; g, uridine diphosphoglucose, and h, β-ATP (assignments referred to external 85% H_3PO_4). **B:** solid-state ^{31}P-n.m.r. spectrum of cell membrane preparation ($\sim$350 mg wet weight, 1250 scans corresponding to $\sim$50 min measuring time). **C:** solid-state ^{31}P-n.m.r. spectrum of large multilamellar vesicles formed from L-M cell lipid extract ($\sim$75 mg) lipid, $\sim$200 scans, $\sim$9 min) at 37°C.

as judged by the behavior of the cells after re-incubation with fresh culture medium at 37°C. Furthermore, viability tests with trypan blue (Phillips and Terryberry, 1957) also indicated an intact plasma membrane and were consistent with the re-incubation experiments. When tested at the end of the more stringent deuterium n.m.r. measurements (packed cells, 60–90 min measuring time, and 36°C measuring temperature) the viability of the L-M cells was found to be 40–60%.

Deuterium and phosphorus n.m.r. spectra of L-M cells and model membranes

L-M cells contain on average 48% PC and 28% PE as the major lipids. In addition, small amounts of sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS), PG and cardiolipin (CL) account for a total of $\sim$20% of the phospholipids (Schroeder *et al.*, 1976).

Wide-line ^{31}P-n.m.r. spectra of L-M plasma membrane preparations and model membranes composed of lipid extracts are displayed in Figure 1B and C, respectively. Both spectra exhibit the characteristic line shape of fluid-like lipid bilayers (Seelig, 1978). The chemical shielding anisotropy is -30 p.p.m. to -35 p.p.m. for the plasma membrane and -50 p.p.m. for the lipid extract. The latter value is in agreement with other phospholipid model membranes, e.g. bilayer dispersions, of POPC (Tamm and Seelig, 1983). Individual lipid species cannot

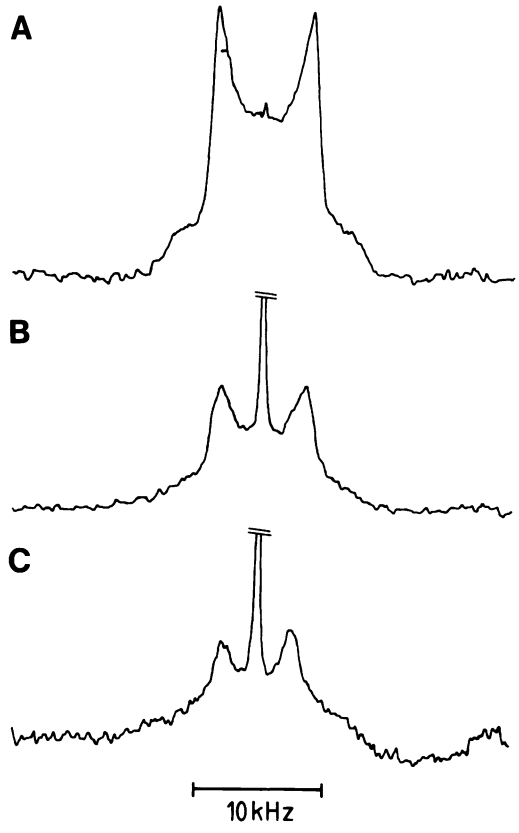

Fig. 2. Deuterium n.m.r. spectra at 46.1 MHz of L-M fibroblasts grown on α-deuterated choline [$(CH_3)_3\overset{+}{N}CH_2CD_2OH\ I^-$]. **A:** multilamellar liposomes formed from extracted lipids (~75 mg lipid, 10 000 scans, corresponding to 40 min measuring time, 37°C) **B:** crude plasma membrane preparations of L-M cells (~350 mg wet weight, 20 000 transients, ~90 min). **C:** intact L-M cells ($\sim 5\times10^8$) corresponding to ~6 mg labelled phosphatidylcholine (20 000 scans, 90 min).

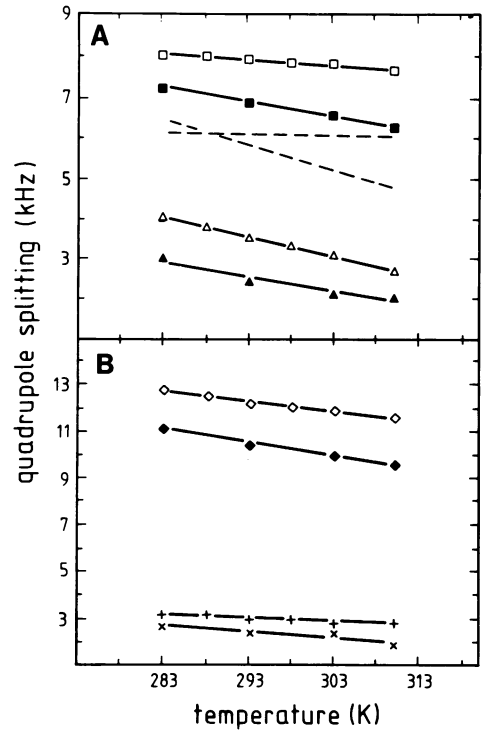

Fig. 3. Quadrupole splittings of plasma membranes and multilamellar liposomes as a function of temperature. **A:** L-M cells grown on deuterated choline. □ Lipid extract with α-deuterated phosphatidylcholine, ■ crude plasma membrane preparation with α-deuterated phosphatidylcholine, △ lipid extract with β-deuterated phosphatidylcholine, ▲ crude plasma membrane preparation with β-deuterated phosphatidylcholine. (---) Dashed line: synthetic α-(upper) or β-deuterated (lower) POPC (Tamm and Seelig, 1983). **B:** L-M cells grown on deuterated ethanolamine. ◇ Lipid extract with α-deuterated phosphatidylethanolamine, ◆ crude plasma membrane preparation with α-deuterated phosphatidylethanolamine, + lipid extract with β-deuterated phosphatidylethanolamine, × crude plasma membrane preparation with β-deuterated phosphatidylethanolamine.

be distinguished by ^{31}P-n.m.r. but the spectra demonstrate that almost all phospholid is organized in a bilayer structure. The sharp resonance at ~0 p.p.m. (Figure 1C) represents only a minor fraction of the total lipid (< 5%) and is tentatively assigned to non-bilayer structures (micelles) and small lipid vesicles.

In order to simplify the discussion of the deuterium spectra the following nomenclature of the deuterated head group segments was used:

$$\underset{\alpha\ \beta}{POCH_2CH_2\overset{+}{N}H_3}$$ phosphoethanolamine

$$\underset{\alpha\ \beta}{POCH_2CH_2\overset{+}{N}(CH_3)_3}$$ phosphocholine

We first discuss the results obtained with choline-labeled cell membranes. The synthesis of PC in eukaryotic cells involves the sequential activation of choline to choline phosphate and CDP choline followed by the transfer of choline from CDP choline to diacylglycerides. Providing deuterated choline to L-M cells thus leads to a labeling of the PC head group. In addition, SM will become deuterated too, since its biosynthesis also depends on the availability of choline.

Figure 2 reproduces ^{2}H-n.m.r. spectra obtained with lipid extracts, plasma membranes, and L-M cells. The cells were grown for two doublings (~70 h) on the choline-deficient synthetic medium of Higuchi (1970), supplemented with α-deuterated choline. The division rate of the cells was between 20 and 30 h in the suspension culture with an initial lag period of ~24 h.

Figure 2A represents the ^{2}H-n.m.r. spectrum of the total lipid extract dispersed in buffer. 70–80 mg lipid was obtained from $\sim 3\times10^9$ cells, containing ~25 mg deuterated PC. From a quantitative comparison of the areas of ^{31}P- and ^{2}H-n.m.r. spectra and calibration with synthetic compounds of known deuterium content it could be estimate that ~70% of the total PC was deuterated. This was in agreement with PC turnover studies on CHO cells (Esko and Matsuoka, 1983) which seem to have a similar regulation mechanism for PC synthesis (Sleight and Kent, 1983).

The lineshape of Figure 2A is characteristic of a random distribution of fluid-like bilayers (Seelig, 1977) and similar lineshapes were obtained for crude plasma membranes (Figure 2B) and intact cells (Figure 2C). The isotropic resonance in the latter two spectra can be explained by the natural abundance of deuterium in water. Compared to pure phospholipid dispersions the intrinsic linewidths of the plasma membrane and the cell spectra were broader, which may be traced back to the chemical heterogeneity of the biological membranes and, perhaps, to lipid–protein interactions (cf. Tamm and Seelig, 1983). Similar results were obtained when β-deuterated choline was added to the growth medium (spectra not shown).

The separation of the most intense peaks in the ^{2}H-n.m.r. spectra is the so-called residual deuterium quadrupole splitting, $\Delta\nu_Q$ which is a well-defined physical quantity and related to the orientation and average fluctuations of the labeled segment. In

Table I. ^2H quadrupole splittings $\Delta\nu_Q$ and the ^2H spin lattice relaxation times T_1 of the choline and ethanolamine head group segments in lipid extracts and intact cells at 10°C

	$\Delta\nu_Q$ (kHz)		T_1 (ms)	
Choline	α-segment	β-segment	α-segment	β-segment
Cells	~ 6	~4	10.5	11.5
Lipid extracts	8.0	4.0	9.9 ± 0.4	9.7 ± 0.1
Ethanolamine	α-segment	β-segment	α-segment	β-segment
Cells	~11	~3	8.8	10.0
Lipid extracts	12.8	3.2	7.8 ± 0.4	6.9 ± 0.2

the following, $\Delta\nu_Q$ will be used as a purely empirical parameter. Figure 3 then compares the quadrupole splittings of the plasma membranes and lipid extracts as a function of head group labeling and temperature. Also indicated are the quadrupole splittings of head group labeled synthetic POPC. The main features of Figure 3A may be summarized as follows. (i) At temperatures between 25 and 37°C the two choline head group segments are characterized by quite different quadrupole splittings. (ii) Small but systematic differences exist depending on the type of membranes investigated. (iii) The quadrupole splitting decreases with temperature. This effect is small for the α-segment and somewhat more pronounced for the β-segment.

The quadrupole splittings from intact cells are summarized in Table I and are compared with those from corresponding lipid extracts. Analogous studies were performed with deuterated ethanolamine. When ethanolamine instead of choline was supplied to the growth medium the production of PE was enhanced at the expense of PC (Glaser *et al.*, 1974; Schroeder *et al.*, 1976). Unfortunately, the cells lost their ability to grow and divide, leading to rather inefficient incorporation of deuterated ethanolamine. We therefore modified the growth medium and supplied deuterated ethanolamine together with non-labeled choline. In this fashion good incorporation of deuterated ethanolamine (~50% of the total PE was deuterated) was obtained without affecting cell proliferation. Figure 4 summarizes representative deuterium n.m.r. spectra of cells and plasma membranes grown on α- and β-deuterated ethanolamine. Since the sensitive volume in the n.m.r. experiment was constant (≈ 1 ml) the quality of the spectra decreased in parallel with the absolute amount of deuterated lipid, i.e. in the order multilamellar liposomes (spectra not shown) > plasma membrane fraction > intact cells. Even at the highest possible packing density (~200 g), the percentage of water in intact cells was at least 90% of the total sample weight, explaining the low signal-to-noise ratio of the latter spectra. Nevertheless, the quadrupole splittings were clearly resolved in all systems investigated. The variation of the quadrupole splittings with temperature is plotted in Figure 3B.

The α-segments of PC and PE exhibit quadrupole splittings of quite different separations and the two species can thus easily be differentiated. In certain systems a second pathway for the formation of PC, i.e. methylation of PE, has been found (Maeda *et al.*, 1981). The absence of any PC resonance in lipid extracts grown on α-deuterated ethanolamine excludes the possibility that the latter pathway is active to any appreciable extent in L-M mouse fibroblasts, in agreement with the biochemical evidence (Maeda *et al.*, 1981).

In order to understand the differences between lipid model

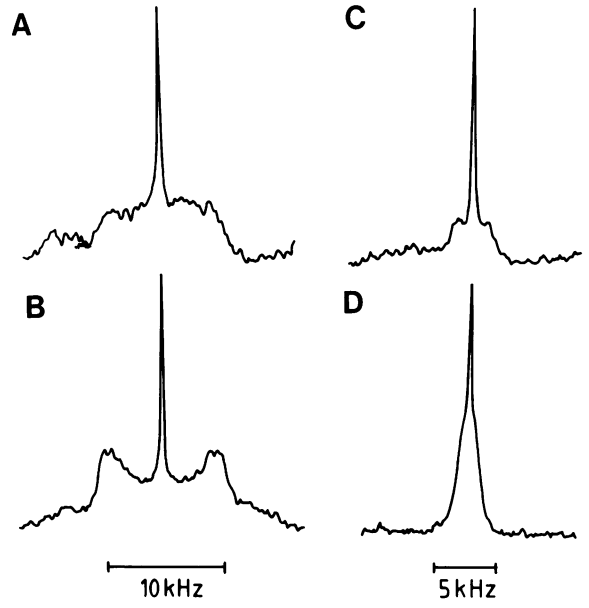

Fig. 4 Deuterium n.m.r. spectra of L-M cells and crude plasma membrane preparations containing deuterated phosphatidylethanolamine at 36°C. **A:** cells grown on α-deuterated ethanolamine ($^+NH_3CH_2CD_2OH\ I^-$), spectrum required 90 min of accumulation. **B:** crude plasma membrane preparation, corresponding to **A** (50 000 transients, 3.5 h), **C:** cells grown on β-deuterated ethanolamine ($^+NH_3CD_2CH_2OH\ I^-$), **D:** crude plasma membrane preparation corresponding to **B**. Same measuring conditions as in **A** and **B**, respectively.

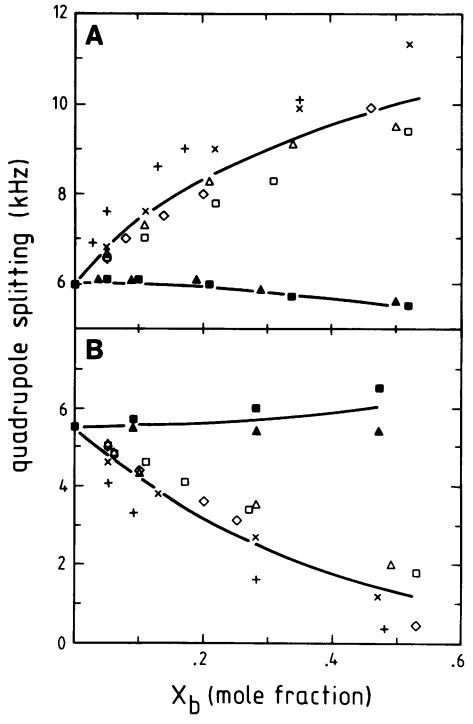

Fig. 5. Influence of neutral and charged lipids on the head group splittings of POPC at 25°C. **A:** binary mixtures with α-deuterated POPC. **B:** binary mixtures with β-deuterated POPC. Assignments: PE (■), SM (▲), PS (□), PI (◇), CL (+), PA (×) and PG (△).

membranes and intact cells we have investigated the effect of lipid charge and heterogeneity on the phosphocholine head group. To this purpose, α- and β-deuterated POPC were mixed in varying proportions with neutral lipids (SM, PE) and negatively charg-

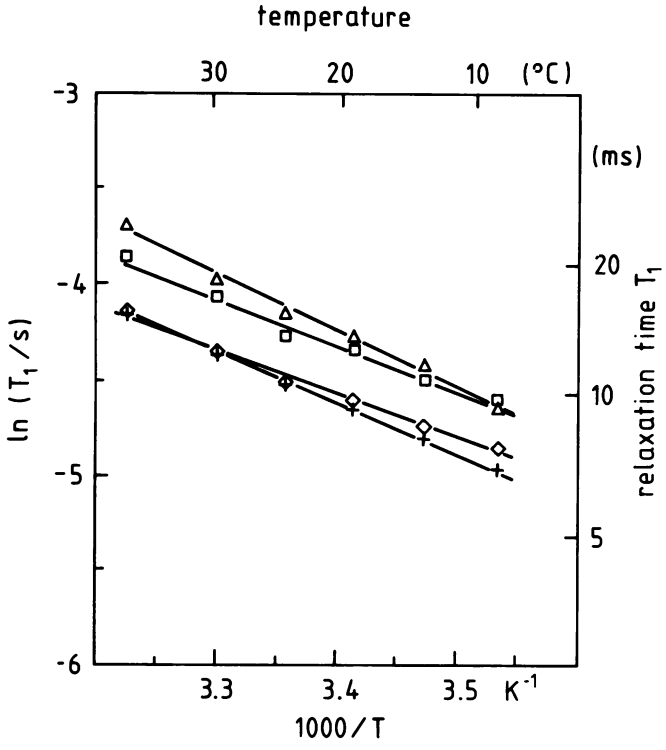

Fig. 6. Variation of the deuterium spin-lattice relaxation times with temperature. Phospholipid extracts from L-M cells. □ α-deuterated PC (E_a = 21 KJ/mol), △ β-deuterated PC (E_a = 25 KJ/mol), ◇ α-deuterated PE (E_a = 20 KJ/mol), + β-deuterated PE (E_a = 22 KJ/mol).

ed lipids [phosphatidic acid (PA), PG, PS, PI, CL]. The variation of the quadrupole splittings of α- and β-CD_2-POPC with the mole fraction of added lipid (at 25°C) is shown in Figure 5. The zwitterionic lipids SM and PE showed only a small influence on the choline head group whereas quite distinct changes were noted upon addition of negatively charged lipids. The quadrupole splitting of the α-segment increased (Figure 5A), that of the β-segment decreased (Figure 5B) with increasing mole fraction, X_b, of negatively charge lipids.

Closer inspection of Figure 5 reveals a linear variation of the quadrupole splittings with X_b at low mole fractions of negatively charged lipids. Comparing the initial slopes of the $\Delta\nu_Q$ versus X_b curves demonstrates that the slope of CL-containing membranes is exactly twice as large as that of singly charged lipids whereas PA falls in between. The intermediate slope of PA can be linked to the dissociation behavior of this lipid which has two dissociable protons the second being released between pH 6.5 and 9.5 (Träuble and Eibl, 1974). At pH 7.4 and 0.15 M NaCl, i.e. the conditions of the present experiments, we expect 1.3 – 1.5 electronic charges per PA molecule. PA is therefore more effective in changing the phosphocholine dipole than the monovalent lipids PG, PS and PI but less effective than the doubly charged CL.

Deuterium spin lattice relaxation times T_1

^{2}H spin-lattice relaxation times, T_1, were measured for lipid extracts between 10 and 37°C and for L-M cells at 10°C only (cf. Table I). The recovery of the magnetization after a 180° pulse followed a single exponential in all samples measured. The variation of the T_1 relaxation time with temperature is shown in Figure 6 in the form of an Arrhenius plot. The T_1 relaxation times were generally quite short and varied between 7 and 25 ms. For a given headgroup the T_1 relaxation times of the α- and β-segment were approximately equal. Likewise, almost no differences were observed between pure lipid membranes and intact cells. The relaxation times of the ethanolamine head group were found to be shorter than those of the choline head group indicating a somewhat slower reorientation rate of the latter. The T_1 relaxation rates increase with increasing temperatures which is typical for the fast-correlation time regime. From the slopes of the Arrhenius plots apparent activation energies of 20–25 kJ/mol were derived for all four head group segments.

Discussion

Orientation and dynamics of the phosphocholine and phosphoethanolamine head group in cell membranes

Deuterium n.m.r. is a non-perturbing method to study specific lipids or proteins in an environment as complex as a biological membrane. In the present study, we have succeeded in obtaining ^{2}H-n.m.r. spectra of L-M mouse fibroblasts with biosynthetically labeled ethanolamine and choline head groups. To our knowledge, this is the first report on the ethanolamine head group in an intact membrane although investigations of the choline head group have been attempted before (Arvidson *et al.*, 1975; Oldfield *et al.*, 1976; London *et al.*, 1979; Curatolo *et al.*, 1985). The ^{2}H-n.m.r. spectra of L-M cells exhibit just one quadrupole splitting for a given headgroup segment. This is quite a surprising result since membranes are not only multicomponent systems but may also be characterized by lateral heterogeneity and transverse asymmetry. Moreover, intracellular membranes may have different physical properties from the plasma membrane. None of these effects is reflected however in the deuterium n.m.r. spectra of intact cells. The rather constant quadrupole splittings provide evidence for unique and stable ethanolamine and choline head group conformations which are little influenced by the membrane composition.

As mentioned above, X-ray diffraction, neutron diffraction and deuterium n.m.r. studies have shown that the phosphocholine and phosphoethanolamine dipoles are aligned approximately parallel to the surface of pure lipid model membranes. The quadrupole splittings observed in intact L-M cells and plasma membranes are in quantitative agreement with those observed in the earlier ^{2}H-n.m.r. studies. Since ^{2}H-n.m.r. is extremely sensitive to even small conformational changes we conclude from the consistent nature of the quadrupole splittings that the orientation of the n.m.r.-visible phosphoethanolamine and phosphocholine head groups in intact L-M mouse fibroblasts and isolated membranes are also parallel to the membrane surface.

The ethanolamine and choline head groups are not static but undergo conformational transitions. This is indicated by the observation of a single quadrupole splitting for the two deuterons of the α- and β-segments of each head group. The simplest model to explain the n.m.r. equivalence of the two deuterium bond vectors is to assume rapid transitions between two mirror-like conformations of the ethanolamine and choline head groups. Such motions produce the same average orientation of the two deuterium bonds with respect to the bilayer normal (Seelig and Gally, 1976; Seelig *et al.*, 1977). In fact, two mirror-like head group conformations have been observed in model compounds and, in part, in phospholipid single crystals.

The measurement of the deuterium spin-lattice relaxation time provides information on the dynamics of the phosphocholine and phosphoethanolamine head groups. Since the experimental T_1 times fall into the fast correlation time regime it is possible to

calculate a correlation time τ_c according to (Abragam, 1961):

$$(1/T_1) = (3\pi^2/2)(e^2qQ/h)^2\ \tau_c \qquad \text{(Equation 1)}$$

where (e^2qQ/h) is the static quadrupole splitting ($\approx$ 170 kHz for aliphatic C-D bonds). The correlation time τ_c is a molecular parameter which depends on the effective volume, V_{eff}, of the probe and the microviscosity η:

$$\tau_c = V_{eff}\eta/(kT) \qquad \text{(Equation 2)}$$

The α- and β-segments of the ethanolamine and choline head group are characterized by T_1 relaxation times in the range of 25−7 ms (37 − 10°C), corresponding to correlation times of 100 − 300 ps. In contrast, small molecules of comparable size to the lipid polar groups exhibit T_1 relaxation times in aqueous solution which are 1−2 orders of magnitude longer (corresponding to shorter τ_c values and faster motions) (Mantsch *et al.*, 1977). For example, the relaxation time of selectively deuterated glycerol (10 wt%) in aqueous solution was found to be 200 ms at 25°C (Borle and Seelig, 1983). Hence, it is safe to conclude that the motions of the choline and ethanolamine segments are dynamically restricted at the membrane surface. The apparent viscosity is intermediate between that of a paraffin oil and an aqueous solution. This result is in agreement with deuterium T_1 relaxation time measurements of *E. coli* cell membranes (Borle and Seelig, 1983) and phosphorus-31 T_1 relaxation time studies on sarcoplasmic reticulum membranes (Seelig *et al.*, 1981). As to the molecular origin of this enhanced surface viscosity the most obvious explanation is to assume an extensive hydrogen bonding network comprising lipid polar groups as well as proteins and water.

Table I demonstrates only minor differences between the T_1 relaxation times of intact cells and lipid model membranes, which is again consistent with previous studies on *E. coli* membranes (Borle and Seelig, 1983) and reconstituted cytochrome *c* oxidase model membranes (Tamm and Seelig, 1983). The influence of membrane proteins on the fast head group motions must therefore be considered as almost negligible. However, as is obvious from Figures 2 and 4, the intrinsic linewidth in the membrane spectra is distinctly larger than in the lipid model membranes. This difference has been observed previously and is still not well understood. It is generally explained by the onset of some slow motions induced by lipid−protein interactions.

Finally it may be noted for a given head group and temperature, that the T_1 relaxation times of the α- and β-segment are practically identical. This is indicative of a concerted motion of the two segments and excludes the possibility of one segment moving considerably faster than the other.

Electrostatic interactions at the membrane surface

The interaction of PC with PG, PS and PE has been studied before employing synthetic lipids with two myristic acid chains (Sixl and Watts, 1982, 1983). It was proposed that the introduction of a negatively charged lipid would alter the hydrogen bonding or modify the water layer, or both, resulting in a conformational change of the choline head group.

The more extensive set of data shown in Figure 5, together with results obtained on synthetic bilayers composed of either positively charged dimethyldialkylammonium salts or negatively charged dialkylphosphates (P.Scherer and J.Seelig, in preparation), suggest, however, that essentially an electrostatic explanation should be advanced. Firstly, we note from Figure 5 that the quadrupole splittings depend on the amount and valence of the added lipid. At low concentrations the quadrupole splitting varies linearly with the mole fraction X_b and the initial slopes increase in proportion to the valence, z, of the lipid, i.e. in the order PE = SM (z=0) << PG $\approx$ PS $\approx$ PI (−1) < PA (−1.3) < CL (−2). Secondly, exactly opposite changes were observed when positively charged, bilayer-forming dimethyldialkylammonium salts were added to POPC bilayers. In the latter experiments the positive surface charge decreased the splitting of the α-segment and increased that of the β-segment, the variation being again linear with the mole fraction of positive lipid (P. Scherer and J. Seelig, in preparation). Thirdly, the electrostatic model leads to a consistent explanation of previous findings on metal ions (Akutsu and Seelig, 1981; Altenbach and Seelig, 1984; Macdonald and Seelig, 1987a,b), hydrophobic ions (Altenbach and Seelig, 1985) and local anesthetics (Boulanger *et al.*, 1981; Kelusky and Smith, 1984; P. Allegrini, A. Seelig and J. Seelig, in preparation). The present results, together with those obtained for synthetic bilayer membranes, provide strong evidence that it is the sign and the size of the electric charge at the membrane surface which determines the direction and the extent, respectively, of the conformational change of the phosphocholine head group.

We may now proceed to a comparison of pure PC membranes with PC in L-M cell membranes and lipid extracts. The choline α-segment (β-segment) in membranes formed from L-M lipid extracts exhibits a larger (smaller) quadrupole splitting than in pure POPC membranes (Figure 3). This difference can now be explained as a result of the negatively charged lipids in the L-M extract. At a mole fraction of ~20% negatively charged lipids the observed quadrupole splittings correspond quantitatively to those in mixed model membranes (Figure 5). Surprisingly, the influence of the negative charge appears to be eliminated in the intact L-M cell membrane. As a simple compensation mechanism we may assume positive charges on the protein surface, located in the plane of the lipid polar groups, which balance the negative lipid charges. Alternatively, the membrane proteins could specifically bind negatively charged phospholipids. Whichever mechanism applies, as a result the membrane surface appears to be electrically neutral.

In conclusion, we have demonstrated that the phosphocholine and phosphoethanolamine dipoles in L-M mouse fibroblast membranes are oriented essentially parallel to the membrane surface. Both head groups undergo fast rotational and flexing motions but the microviscosity at the membrane surface is larger by at least a factor of 10 than the bulk viscosity of the surrounding aqueous solution. The phosphocholine head group conformation is sensitive to the sign, the extent, and the location of electric charges at the membrane surface. In the case of L-M fibroblast membranes the phosphocholine and phosphoethanolamine head groups appear to sense an electrically neutral environment in spite of the fact that the cell membranes contain 10−20 mol% of negatively charged lipids.

Materials and methods

Synthesis of deuterated head group precursors and phospholipids

α-Deuterated choline and α- and β-deuterated ethanolamines were synthesized as described before (Gally *et al.*, 1975; Seelig and Gally, 1976). β-Deuterated choline was synthesized from the corresponding ethanolamine by methylation (Enz and Leuenberger, 1946). POPC was prepared from the corresponding PA (Avanti Polar Lipids, USA) following the method of Harbison and Griffin (1984).

Growth of L-M cells

A strain of mouse fibroblasts, L-M cells (CCL 1.2) was obtained from the American Type Culture Collection (ATCC) in passage 122. The cells were grown at 37°C in monolayer culture with the original medium M199 with Hank's salts

(Gibco) + 0.5% bacto-peptone (Difco) in 75 cm^2 tissue culture flasks (Falcon).

In suspension cultures, the cells were grown in the chemically defined medium of Higuchi (1970) modified as follows: the medium contained 20 mM Hepes (Sigma) and 1 g/l methylcellulose (Fulka, Switzerland) to avoid cell aggregation. The initial concentration of suspension culture was $\sim 5 \times 10^5$ cells/ml. The cells were kept in suspension either by shaking in Erlenmeyer bottles with a Lab-shaker or by stirring in spinner flasks up to a concentration of $2-3 \times 10^6$ cells/ml.

Cells were tested for mycoplasma contamination according to Chen (1977) by using the fluorescent dye Hoechst 33258 or 33342 (Serva). The tests were always negative.

Incorporation of ^2H-label

40 mg/l deuterated choline was added to the medium of Higuchi (1970). The cells were kept in this medium for about two doublings (~ 70 h), including a lag-period of ~ 24 h with the initial concentration of $\sim 5 \times 10^5$ cells/ml. In order to label the ethanolamine lipids, 40 mg/l deuterated ethanolamine was supplied to the Higuchi medium without choline. In this case the initial concentration was $\sim 2 \times 10^6$ cells/ml and the cells were kept in this medium for ~ 24 h. In a second approach 40 mg/l deuterated ethanolamine was added to the original medium containing also non-deuterated choline. The efficient incorporation of deuterated ethanolamine was quite unexpected since the conventional PE synthesis proceeds via decarboxylation of PS (Voelker, 1984; Merrill and Wang, 1986). In agreement with this pathway we observed both PS and PE synthesis when deuterated D,L-serine was added to the growth medium. Addition of ethanolamine in the presence of deuterated D,L-serine appeared to inhibit PS synthesis (in preparation).

Mixtures of headgroup deuterated POPC and negatively charged lipids

PS (bovine brain), PI (bovine heart), CL (bovine heart), SM (bovine brain), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA) were obtained from Avanti Polar Lipids, (Birmingham, AL, USA) and were used without further purifications. Mixtures of these phospholipids with deuterated POPC were prepared by pipetting corresponding volumes of concentrated stock solutions (10 mg/ml in dichlormethane/methanol) in n.m.r. measuring tube. The solvent was evaporated and the lipids were dried by high vacuum (under P_2O_5) for several hours. The dried lipids were weighed for exact determination of lipid content. The phospholipids were dispersed at room temperature in 20 mM Hepes (pH 7.4) buffer containing 150 mM NaCl. The buffer was prepared with deuterium-depleted water to minimize the isotropic ^2H-n.m.r. signals due to the natural abundance of deuterium in water. The ratio lipid/buffer was in the range 1:2 (%w/%v).

Lipid extracts

Total phospholipid extracts were prepared by chloroform/methanol extraction of washed cells (Bligh and Dyer, 1959) and further purified by acetone precipitation (Kates, 1972). Bivalent cations were removed by washing with 100 mM Na_2EDTA in saturated NaCl solution at pH 8.5. The yield was between 10 and 12 mg purified phospholipids per 1 g packed cells (centrifugation for 10 min at 200 *g*) and 1 g packed cells correspond to $\sim 4 \times 10^8$ cells.

Plasma membrane preparation

Plasma membranes were isolated according to Maeda *et al.* (1983) with the following modifications. In order to avoid any interference with the ^{31}P-n.m.r. spectra of phospholipids the sodium phosphate buffer was replaced by 20 mM Hepes. In addition, $MgCl_2$ was omitted because of the potential binding of Mg^{2+} to lipids. The homogenization of $6-8 \times 10^9$ cells was performed by sonification (tip sonifier) for 30 s and subsequent centrifugation at 1000 *g* in order to remove the heavy particles (e.g. cells, nuclei, etc.). The isolated membranes were twice washed with normal buffer and additionally washed with 3 ml buffer composed of 20 mM Hepes (pH 7.4), 150 mm NaCl and deuterium-depleted water. After centrifugation at 100 000 *g* for 30 min the wet pellet was used for the measurement (yield $\sim 200-400$ mg).

Cells for n.m.r. measurement

Approximately $4-8 \times 10^8$ cells were harvested in the logarithmic phase of growth, washed twice at 4°C with buffer (20 mM Hepes, 150 mM NaCl, pH 7.4), and transferred into a n.m.r. measuring tube. Cells for ^{31}P high resolution n.m.r. were prepared as for ^2H-n.m.r. except that the measuring buffer contained D_2O. The viability of the cells were determined before and after measurement (Phillips and Terryberry, 1957).

N.m.r. measurement

All measurements were made with a Bruker-Spektrospin CXP 300 spectrometer operating at 46.1 MHz for ^2H and 121.48 MHz for ^{31}P. The ^2H-n.m.r. spectra were measured by using the quadrupole echo technique (Davis *et al.*, 1976). Pulses (90°) of 3.5–4.0 μs were used with an echo separation of 30 μs. The spectra were recorded with 4 K data points, quadrature phase detection, a spectral width of 50 kHz and a recycle delay of at least 7 T_1. ^2H spin lattice relaxation times were measured by the inversion recovery sequence followed by the quadrupole echo sequence. Spectra were acquired with phase cycling. ^2H-n.m.r. spectra of whole cells were recorded with the inversion-recovery sequence by using a τ of 75 ms in order to suppress the isotropic ^2H signal of water. The solid-state ^{31}P-n.m.r. spectra were recorded with the spin echo sequence and proton broadband decoupling during the acquisition time. Pulses (90°) of 3–3.5 μs were used with an echo separation τ of 50 μs. The spectral width was 50 kHz and the recycle delay was 2 s. The high resolution ^{31}P-n.m.r. spectra were recorded in a 10 mm high resolution n.m.r. test tube. A 90° pulse (14–15 μs) was used with phase cycling and a recycle delay of 5 s.

For T_1 determination at least eight experiments with a delay varying from 1 to 250 ms were performed. The experiments were evaluated by a non-linear three parameter fit (Marquardt fit) on the average height of the two most intense peaks of the ^2H powder spectra according to the equation described by Brown *et al.* (1979). The error limits given in the table refer to the standard deviation of the mean.

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