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EVIDENCE FOR ISOTROPIC MOTION OF PHOSPHOLIPIDS IN LIVER MICROSOMAL MEMBRANES

A ^{31}P NMR STUDY

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Summary

1. The motional properties of phospholipids in bovine and rat liver microsomes and aqueous dispersions of the extracted lipids have been investigated employing ^{31}P NMR techniques.

2. The ^{31}P NMR spectra obtained from the microsomes indicate that a considerable portion of the constituent phospholipids experience isotropic motion on the NMR timescale (10^{-5} s). This is in strong contrast to the spectra obtained from aqueous dispersions of the extracted lipids, which display the characteristic lineshape associated with liquid crystalline phospholipids in (large) bilayer structures, which experience restricted anisotropic motion.

3. Evidence is presented which strongly suggests that the isotropic motion of microsomal phospholipids does not arise from tumbling of the microsomal vesicles or from lateral diffusion of phospholipids around these vesicles.

4. These results are discussed in terms of possible transitory formation of intramembrane non-bilayer lipid configurations, with which the bulk (bilayer) phospholipids are in rapid exchange.

Introduction

Possible relations between the physical properties of lipids in biomembranes and biological membrane function is a topic of major interest in membrane research. If such relationships exist it may be considered likely that they would be most obviously expressed in very metabolically active membranes. The liver endoplasmic reticulum is such a membrane system. Associated enzymes are involved in mixed function oxidation of drugs and other organic compounds, the oxidative desaturation of fatty acids [1], the synthesis and transport of

various proteins (including glycoproteins [2] and lipoproteins [3]), the synthesis of cholesterol and triglycerides [4], glycogen breakdown [5] and also play a key role in phospholipid metabolism [6].

It is therefore of obvious interest to determine whether the dynamic organization of lipids and proteins in this membrane is significantly different from other, less active, membranes and whether such differences could be attributed to functional requirements. Freeze etch studies of liver microsomes [7] reveal bilayer lipid structure in which proteins are embedded, which is typical of biological membranes in general. Also, differential scanning calorimetry studies [8] indicate that the lipids are in the fluid state at physiological temperatures, which is again typical of mammalian membranes.

In this work we examine the motional properties of phospholipids in liver microsomal membranes. ^{31}P NMR techniques are employed which, as detailed previously [9,10] employ the phospholipid phosphorus as a non-perturbing intrinsic probe of the local motion experienced by membrane phospholipids, and the macroscopic structures they adopt. It is shown that liver microsomal phospholipids experience more isotropic motion than is usual for phospholipids in a bilayer configuration, which is suggested to arise from transitory intramembrane non-bilayer lipid configurations.

Experimental

Preparation of microsomes

Microsomes were prepared from the livers of adult male Wistar rats or from fresh beef liver obtained from the local slaughterhouse. Livers were homogenized in 9 vols. of cold 0.25 M sucrose, 10 mM Tris-HCl (pH 7.2) and centrifuged for 15 min at 600 $\times g$. The supernatant was centrifuged for 20 min at 16 000 $\times g$. The resulting supernatant was carefully removed and centrifuged for 1 h at 100 000 $\times g$. The pellet was then resuspended in 100 mM NaCl, 0.2 mM EDTA, 25 mM Tris/acetic acid buffer, pH 7.0, containing 10% ^2H_2O, until a concentration of 50—75 mg microsomal protein per ml was achieved. Rough and smooth microsomes were prepared according to the method of Dallner or Rothschild as described by Gram [11]. Protein content was determined according to Lowry et al. [12] and phosphorus according to Chen et al. [13] after destruction of the sample as described by Ames and Dubin [14]. Unless specified, the term microsomes refers to the total mixture of rough and smooth microsomes.

Lipids and preparation of lipid dispersions

Egg phosphatidylcholine was isolated from hen eggs according to established procedures. Microsomal lipids were obtained from microsomes by extraction according to Bligh and Dyer [15] or according to Folch et al. [16]. A chloroform solution containing 50—100 μmol of microsomal lipid was dried down in a 10-mm NMR tube under N_2. After overnight storage under high vacuum the lipid film was dispersed in 100 mM NaCl, 0.2 mM EDTA, 25 mM Tris/acetic acid pH 7.0, containing 10% ^2H_2O by shaking on a Vortex mixer. Egg phosphatidylcholine vesicles were prepared by ultrasonication as described before [17]. Dibucaine was obtained from the Onderlinge Pharmaceutische Groothandel (Utrecht, Netherlands).

NMR

^{31}P NMR measurements were performed at 35 ± 1°C on a Bruker WH 90 spectrometer operating at 36.4 MHz. Spectra were recorded under conditions of 18-W broad-band proton decoupling using a sweep width of 20 KHz. 10 000—50 000 transients were accumulated using 45° radiofrequency pulses with an interpulse time of 0.17 s. The sample volume in general was 1.5 ml. To quantify the amount of signal the standard approach employing an external reference and recording the spectrum with gated decoupling and $5T_1$ waiting times between successive pulses could not be used because the very long accumulation times would certainly result in decomposition of the microsomal membrane. Therefore the following approach was chosen. First, the ^{31}P NMR spectrum of sonicated egg phosphatidylcholine and an external triphenylphosphine standard was recorded using gated decoupling with 90° radiofrequency pulses, and 15-s waiting times between subsequent pulses. By comparing the signal intensity per μmol phospholipid with the signal intensity of a standard phosphate solution it could be calculated that 95—100% of the phospholipid in the vesicles were observed in the spectrum. Then the ^{31}P NMR spectra of the egg phosphatidylcholine vesicles and the microsomes were recorded with an external trimethylphosphate standard using the fast pulse conditions described above. From the amount of phosphorus in the samples and the computer integration of the spectra the amount of signal observed in the case of the microsomes relative to the amount of signal observed for the egg phosphatidylcholine vesicles could be obtained. For smooth rat liver (Rothschild) microsomes this ratio was found to be 1.01. Since almost all phosphate is derived from the membrane phospholipids (see Results) and the T_1 values of different phospholipids are similar [18] this demonstrates that 95—100% of the phosphate present in the microsomal membrane is observed in the NMR spectrum.

Results

The ^{31}P NMR spectrum obtained from bovine liver microsomes at 35°C is illustrated in Fig. 1A. Very similar spectra were obtained for rat liver microsomes. This spectrum is remarkable in that it is relatively narrow and almost symmetrical. Such characteristics are in marked contrast to the ^{31}P NMR spectrum obtained from an aqueous dispersion of the extracted microsomal lipids (Fig. 1C) which is much broader and asymmetrical. The latter lineshape is characteristic of bilayer liquid crystalline phospholipids in large structures [19,20] and has been observed for aqueous dispersions of synthetic phospholipids [9,21] and several biological membranes, including the erythrocyte membrane [22], the vesicular stomatitis virus membrane [23], the chromaffin granule membrane [19] and the *Acholeplasma laidlawii* cell membrane [24]. The liver microsomal ^{31}P NMR spectrum is therefore quite anomalous.

Two possible explanations for this anomaly are that not all the phospholipids in the membrane are contributing to the observed ^{31}P NMR spectrum, and that the majority of the signal arises from other phosphate containing compounds, or that the constituent phospholipids experience more isotropic motion than is available to phospholipids in other biological membranes. We deal

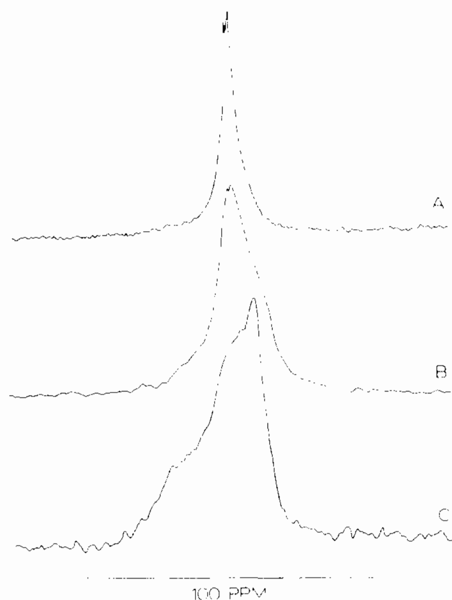

Fig. 1. 36.4 MHz ^{31}P NMR spectra of: A, bovine liver microsomes; B, freeze-dried bovine liver microsomes hydrated with 20% $^{2}H_2O$ (w/w); C, aqueous dispersion of extracted microsomal lipids. Since the microsomes used in this experiment consist of a mixture of rough and smooth microsomes part of the signal in A and B arises from non-phospholipid phosphorus (see text and Fig. 2).

with the former possibility first. Total liver microsomes contain non-phospholipid phosphorus, mainly in the form of RNA. Rough rat liver microsomes have been found to contain at least twice as much RNA phosphorus as phospholipid phosphorus [4]. In order to trace the influence of RNA on the ^{31}P NMR spectrum, rat liver microsomes were fractionated into smooth and rough microsomes employing the methods of Dallner and of Rothschild [11]. The ^{31}P NMR spectra of the various subfractions are illustrated in Fig. 2. The very narrow spectral component observed in the spectra of the rough microsomal fractions (Figs. 2B, 2D) may be tentatively attributed to non-phospholipid phosphorus compounds, as such a spectral feature is much less pronounced in, or absent from, the smooth microsome spectra. A phospholipid to total phosphorus ratio of 0.92 was obtained from smooth (Rothschild) microsomes, employing the lipid extraction procedure of Folch et al. [16]. The amount of phosphorus actually detected in the ^{31}P NMR spectra of smooth (Rothschild) microsomes was determined employing the technique described under Experimental to be 90—100% of the total phosphorus present. Thus in the case of the smooth microsomes it may be concluded that at least 87% of the phospholipid present contributes to the observed spectrum (Fig. 2C). This is in accord with results obtained from the erythrocyte ghost membrane where it was reported that virtually all (>97%) of the phospholipids are observed in the ^{31}P NMR spectra [22]. Since the spectrum of Fig. 2C is much narrower than the derived liposome spectrum, and yet at least 87% of the signal derives from membrane phospholipids, it must be concluded that microsomal phospholipids experience appreciably more isotropic motion.

We now consider possible sources of this additional motion. The most ob-

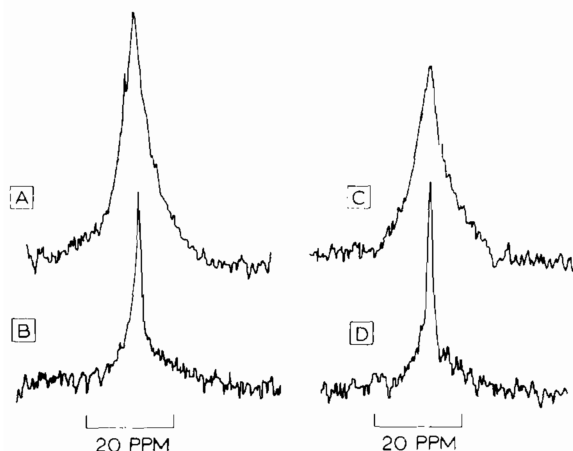

Fig. 2. 36.4 MHz ^{31}P NMR spectra of: A, smooth rat liver microsomes prepared according to the method of Dallner; B, rough rat liver microsomes prepared according to the method of Dallner; C, smooth rat liver microsomes prepared according to the method of Rothschild; D, rough rat liver microsomes prepared according to the method of Rothschild.

vious is that since microsomal vesicles are smaller than the derived liposomes tumbling of the entire vesicle provides an additional motional averaging mechanism, similar to the situation in sonicated vesicles [17]. The following considerations argue against this possibility. First, addition of 20% (w/w) sucrose to the microsomal suspension increases the medium viscosity by a factor of 2, and would reduce the tumbling rate accordingly, yet has no effect on the ^{31}P NMR spectrum. Secondly, freeze-dried microsomes hydrated with 20% (w/w) ^2H_2O evidenced ^{31}P NMR spectra which were still appreciably narrower than the spectra of the derived liposomes (Fig. 1B). This sample was effectively solid. The appearance of a lowfield shoulder does suggest that this treatment induces a fraction of phospholipid molecules with structural properties characteristic of phospholipids in a bilayer configuration. Thirdly, addition of 20 mM Ca^{2+} to the smooth microsomes resulted in precipitation of the vesicles, yet had no effect on the ^{31}P NMR spectrum.

Further, the ^{31}P NMR spectra arising from smooth (Rothschild) microsomes which had been incubated in the presence of the local anaesthetic dibucaine (0.5 mM) showed spectra (Fig. 3) which were appreciably broader than in the absence of dibucaine. The small narrow component observed is similar to a narrow spectral component observed in phosphatidylethanolamine-phosphatidylserine model membrane systems when the ratio of dibucaine to phosphatidylserine exceeds unity (Cullis, P.R., unpublished) and possibly reflects lytic effects obtained at high anaesthetic concentrations [25]. The important point is that the broadening of the bulk of the ^{31}P NMR spectrum in the presence of anaesthetic is most unlikely to result from larger structures, as local anaesthetics inhibit fusion in other biological systems [25]. This observation also argues against lateral diffusion around the vesicle entity providing a significant motional averaging mechanism, as local anaesthetics are thought to "fluidize" biological membranes [26] and thus would be expected to induce line-narrowing via an enhanced lateral diffusion rate. This observation is, how-

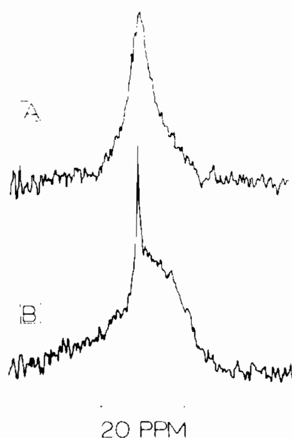

Fig. 3. 36.4 MHz ^{31}P NMR spectra of smooth rat liver microsomes (prepared according to the method of Rothschild) in thc absence (A) and presence (B) of 0.5 mM dibucaine.

ever, fully consistent with the ability of dibucaine to inhibit non-bilayer phases (such as the hexagonal H_{II}) in model systems [10,27] in which lipids may experience more isotropic motion than in the bilayer phase, as well as with the ability of related anaesthetics to induce more order in the polar headgroup region of dipalmitoylphosphatidylcholine [28].

A final argument against the possibility that tumbling or lateral diffusion provides effective isotropic averaging may be taken from the observation of Moore et al. [23] on the vesicular stromatitis virus. This virus has a size (600 × 1800 Å, [23]) which is similar to that of microsomal vesicles (500 × 3000 Å, [11]) but in this case the ^{31}P NMR spectrum of the virus is very similar to that of aqueous dispersions of the extracted lipids.

Discussion

The central result presented here is that a sizeable fraction of microsomal phospholipids experience nearly isotropic motion on the NMR timescale (10^{-5} s) whereas in model membranes composed of the extracted lipids spectra characteristic of liquid crystalline bilayer phospholipids are observed. Further, we have presented arguments which indicate that tumbling or lateral diffusion of lipids around the microsomal vesicle does not contribute significantly to the observed motional narrowing. Such behaviour is in strong contrast to behaviour reported for other biological membranes [19,22–24], for which typical "bilayer" spectra are observed for both the intact membrane and derived liposomes.

We consider two possible sources of additional phospholipid motion in the microsomal membrane to be most probable, both involving the protein component and both involving the presence of non-bilayer lipid phases. The first of these is that membrane protein tends to destabilize bilayer structure directly in local regions, inducing nearby lipids to adopt transitory non-bilayer configurations which allow appreciably more isotropic motion via lateral diffusion

processes [29]. That such effects are possible is indicated by the fact that the polypeptide gramicidin tends to favour formation of the hexagonal (H_{II}) phase over the bilayer phase in egg yolk phosphatidylethanolamine model membrane systems (de Kruijff, B. and Cullis, P.R., unpublished). Alternatively, the membrane proteins may induce heterogeneous lipid distributions in the plane of the membrane and/or across the membrane. As has been discussed elsewhere [10,29] many membrane lipid components do not naturally assume the bilayer phase and it would be expected that local high concentrations of "non-bilayer" lipids would destabilize bilayer structure. Such possibilities apply particularly to the endogenous phosphatidylethanolamine which accounts for 20—25% of membrane phospholipid [4,7,30]. This compound is relatively unsaturated [30], and would certainly be expected to assume the hexagonal (H_{II}) phase at physiological temperatures (in the absence of bilayer stabilizing effects) by analogy with results obtained for phosphatidylethanolamines from other natural sources [31]. Further, the presence of cholesterol in rat liver microsomes (the cholesterol to phospholipid molar ratio is 0.16, [4]) would also tend to favour formation of non-bilayer lipid phases in regions consisting of phosphatidylethanolamine and phosphatidylcholine [29,32]. Finally, the microsomal membrane also contains small amounts of phospholipid precursors such as fatty acids and diglycerides. These agents cannot form bilayers independently, and tend to induce alternative configurations, as was demonstrated recently by introducing oleic acid into the erythrocyte membrane [33]. In the case of the aqueous dispersion of the microsomal lipids the various components would be mixed randomly such that local concentrations of non-bilayer lipids would not exceed a critical value and therefore the normal bilayer type of spectrum is observed.

A question which then arises is how such transitory non-bilayer lipid configurations may occur without irreversibly destroying cellular integrity. We suggest that transitory inverted micellar or hexagonal (H_{II}) phases may occur in the bilayer as indicated in Fig. 6c of ref. 29. We further suggest that the bulk (bilayer) phospholipid experiences rapid exchange with these inverted configurations, leading to the observed motional averaging of the bulk phospholipid. Evidence which supports, but does not prove, such possibilities may be obtained from three observations. First, the reversion to a more usual "bilayer" type spectrum for the smooth microsomes in the presence of dibucaine is consistent with the ability of dibucaine to inhibit formation of hexagonal H_{II} phases in particular in selected model membrane systems (refs. 10 and 27 and Cullis, P.R., unpublished). In the liver microsomal membrane the inhibition of these intramembrane inverted phases would then reduce or eliminate sources of isotropic motional averaging. A second point concerns the observation of Davis and Inesi [34] who concluded from ^{1}H NMR studies on the related sarcoplasmic reticulum membrane that some 20% of the lipid experiences nearly isotropic motion. Finally, one of the main consequences of an intramembrane phase in equilibrium with the bilayer phase would be a fast transbilayer exchange of lipid molecules (flip-flop) as described in Fig. 6 of ref. 29. As the biosynthesis of microsomal phosphatidylcholine and phosphatidylethanolamine is reported to occur only on the cytoplasmic side of the membrane [35,36], transbilayer movements of these phospholipids must occur.

Indeed, evidence for a rapid transbilayer exchange of at least part of the phospholipid components of liver microsomal membranes is now accumulating [37,38].

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