^{31}P NUCLEAR MAGNETIC RESONANCE STUDIES OF CELL MEMBRANES LABELLED WITH PHOSPHONIUM PHOSPHATIDYLCHOLINE

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1. Introduction

It has been demonstrated that phosphonium choline (fig.1) is incorporated into phospholipid as the phosphonium analogue of phosphatidylcholine in cultured cells [1] and in rat tissues [2]. Phosphonium phosphatidylcholine does not inhibit the growth of cultured cells and is turned over at a similar rate to phosphatidylcholine [1].

Phosphonium phosphatidylcholine has also been shown to have similar packing properties to phosphatidylcholine in artificial membrane systems [3]. Therefore the structure of natural membranes containing phosphonium phosphatidylcholine is unlikely to be significantly perturbed.

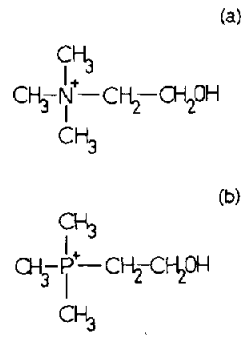

Fig.1. Structural formulae of (a) choline and (b) the phosphonium analogue.

The ^{31}P NMR signal due to the phosphonium group of phosphonium phosphatidylcholine is distinct from the phosphate resonance [2,3] and is thus a useful probe of the cationic region of the headgroup of phosphatidylcholine in natural membranes. In addition, it has been demonstrated that the linewidth of the phosphonium ^{31}P NMR signal, like that of the phosphate resonance, is dependent on the physical state of phospholipids, as the linewidth increases below the phase transition temperature [3]. Since phosphatidylcholine and the phosphonium analogue have similar phase transition temperatures for a defined fatty acid composition, it is valid to use phosphonium labelled natural membranes for investigations of possible phase transitions in the phosphatidylcholine portion of membrane lipids.

In this study, natural membranes, labelled with phosphonium phosphatidylcholine, have been used to investigate possible phase transitions in membrane phospholipids. Further, the effects of protein–lipid interactions on the motion of the polar head group have been studied. Finally, the application of ^{31}P NMR of whole cultured cells containing the phosphonium analogue is illustrated and discussed.

2. Materials and methods

2.1. *Rat feeding*

Freshly weaned male Wistar rats were fed on a defined diet [2] containing exclusively choline (N fed) or the phosphonium analogue (P fed) to a final concentration 6 mmol/kg diet as choline source.

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2.2. *Erythrocyte ghost preparation*

After 4–6 weeks on the defined diets, rats were anaesthetised with ether and blood was collected into acid citrated dextrose solution by cardiac puncture. Erythrocyte ghosts were prepared as described by Steck [4]. In order to concentrate the pellet for obtaining NMR spectra, ghosts were frozen, thawed and sedimented by centrifugation (50 000 × *g*, 10 min).

2.3. *Pronase digestion*

Freeze-thawed ghosts (29.4 mg protein) were incubated (37°C, 15 min) with pronase (0.35 mg) in 10 mM Tris-buffered saline, pH 7.4, made 5 mM with respect to $CaCl_2$ [5]. The mixture was chilled in ice and ghost fragments were isolated and washed once. Approx. 40% of the membrane protein, as measured by the increase in phospholipid:protein ratio, was digested by this treatment.

Protein content was determined by the method of Lowry et al. [6] and phospholipid phosphorus was measured as previously described [1].

2.4. *Liver sub-cellular fractions*

Livers were removed from N fed or P fed rats immediately post mortem and mitochondrial and microsomal fractions were prepared as described by Wirtz and Zilversmit [7].

2.5. *Cell culture*

Nil 8 cells, cultured in Dulbecco's medium, with choline replaced by the phosphonium analogue where indicated (P Dulbecco's) were harvested at confluency as described previously [1]. Cells (approx. 5×10^8) were washed twice with 10 mM Tris-buffered saline, pH 7.4, and the resulting cell pellet was used directly for obtaining ^{31}P NMR spectra.

2.6. *Lipids*

Total lipids were extracted as described by Folch et al. [8] and resuspended in Tris-buffered saline, to a final concentration of approx. 1 mg phospholipid phosphorus/ml for NMR studies.

Phosphonium phosphatidylcholine accounted for 30% and 45% of the total phosphatidylcholine content of erythrocyte ghosts prepared from rats fed on the phosphonium choline diet for 4 weeks and 6 weeks, respectively.

2.7. *NMR spectroscopy*

^{31}P NMR spectra were recorded using a 129 MHz Fourier transform spectrometer which was built in this department [9] and was interfaced with a Nicolet B-NC-12 computer and equipped with temperature control and field stabilisation via a deuterium lock. Spectra were obtained at 30°C unless otherwise stated. All spectra are illustrated with field strength increasing to the right.

2.8. *Materials*

Phosphonium choline was synthesized as described by Edwards and Hands [2]. Cell culture media and sera were obtained from Flow Laboratories Ltd, Irvine, Scotland. All other reagents were purchased from Sigma Chem. Co., Surrey, or Fisons, Loughborough, England.

3. Results and discussion

3.1. *^{31}P NMR spectra of membranes*

^{31}P NMR spectra recorded from normal rat erythrocyte ghosts and phosphonium labelled erythrocyte ghosts have a broad asymmetric phosphate resonance peak (fig.2) which contains contributions from all phospholipid species. Such 'solid state' ^{31}P NMR spectra are characteristic of phospholipids in a bilayer above the phase transition and have been observed for model membrane systems [10] and also from natural membranes [11,12].

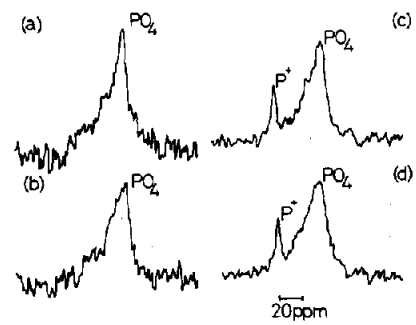

Fig.2. ^{31}P NMR spectra were recorded from freeze-thawed rat erythrocyte ghosts (a,b) and phosphonium labelled ghosts (c,d) at 1°C (a,c) and 40°C (b,d). 30% of total phosphatidylcholine was labelled with the phosphonium analogue, where indicated. P^+ denotes the phosphonium resonance. The phosphate resonance is denoted by PO_4.

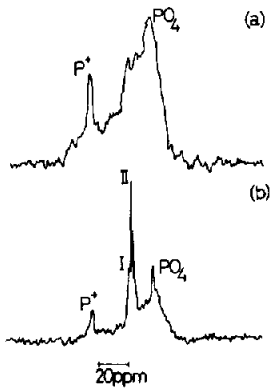

Fig.3. Sub-cellular fractions were prepared from the livers of rats fed on the phosphonium choline diet for 6 weeks. ^{31}P NMR spectra of the microsomal fraction (a) and the mitochondrial fraction (b) were recorded at 1°C. Peaks I and II are identified as water soluble phosphate esters and inorganic phosphate, respectively. Line-broadening, as a result of exponential multiplication of the free induction decay to improve signal-to-noise, is 80 Hz for the mitochondrial spectrum and 160 Hz for the microsomal spectrum.

^{31}P NMR spectra from phosphonium labelled ghosts have, in addition, a sharp peak attributable to the phosphonium moiety of phosphatidylcholine (fig.2c,d). A similar spectrum has previously been obtained for aqueous dispersions of phosphonium phosphatidylcholine above the phase transition temperature [3].

Phosphonium labelled microsomes show similar ^{31}P NMR spectra to ghost fragments (fig.3a). The spectrum of a phosphonium-labelled mitochondrial fraction also shows the sharp phosphonium peak, whilst the asymmetric peak from the phospholipid phosphate has narrow components superimposed on the downfield shoulder (denoted by I and II in fig.3b). These phosphate resonances are due to water-soluble phosphate-esters (I) and inorganic phosphate (II) within the mitochondrial particles [13] and are absent from the spectrum of the microsomal fraction since hypotonic washes are used in the latter preparation [7].

3.2. *Effect of temperature*

Over the temperature range investigated (1–40°C) the linewidth of the phosphonium peak, which can be determined more accurately than the linewidth of the asymmetric phosphate peak, does not vary for rat erythrocyte ghosts (fig.2c cf. d). This is also true for spectra of extracted lipids (fig.5) indicating that the majority of membrane phosphatidylcholine does not participate in a gel–liquid crystalline phase transition over the physiological temperature range.

Ladbrooke and Chapman [14] have likewise inferred from differential scanning calorimetry studies, that there is no observable thermal phase transition in erythrocyte ghosts between 1°C and 40°C. X-ray data [15] are also in accordance with the results presented here. On the other hand, viscosity measurements [16] have indicated that a phase transition occurs in erythrocyte membranes and extracted lipids at approximately 20°C. Other investigators [17] employing Raman spectroscopy, have determined that the major phase transition in erythrocyte membranes occurs below 0°C but that there is an additional transition around 17°C. It is therefore possible that a minor portion of erythrocyte membrane phosphatidylcholine, undetectable by ^{31}P NMR measurements, undergoes a phase transition between 0°C and 40°C. In this regard, it has been suggested that only a small portion of phospholipids in cholesterol-poor regions of the membrane participates in a phase transition [12].

As with the erythrocyte membrane fragments, no change in the linewidth of the phosphonium signal from microsomes has been observed between 1°C and 30°C. Therefore, in agreement with differential scanning calorimetry studies [18] the phospholipids

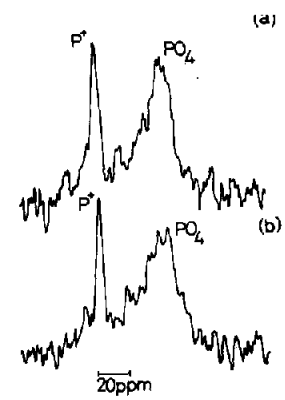

Fig.4. Freeze-thawed ghosts were pepared from erythrocytes of rats fed on the phosphonium choline diet for 6 weeks. ^{31}P NMR spectra were recorded at 25°C before (a) and after (b) proteolytic digestion with pronase, as described in the text. Line-broadening (see legend to fig.3) is 160 Hz.

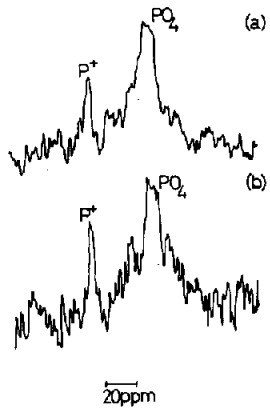

Fig.5. Total lipids were extracted from phosphonium labelled erythrocyte ghosts, with 45% of phosphatidylcholine replaced by the phosphonium analogue, and resuspended in Tris-buffered saline. ^{31}P NMR spectra were recorded at 1°C (a) and 30°C (b). Line-broadening (see legend to fig.3) is 160 Hz.

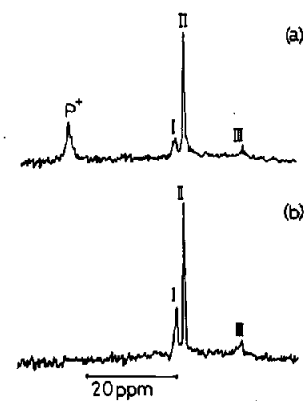

Fig.6. Nil cells (5×10^8) were grown in P-Dulbecco's (a) or Dulbecco's (b) medium. ^{31}P NMR spectra were recorded at 20°C. Phosphate peaks I, II and III are tentatively identified as sugar phosphate, inorganic phosphate and the β-phosphate of nucleoside triphosphate, by comparison with published spectra [13]. P^+ indicates the phosphonium resonance.

of subcellular membrane fractions, and in particular phosphatidylcholine, do not participate in a concerted phase transition and are predominantly fluid at temperatures as low as 0°C. However, the change of a small portion of microsomal phosphatidylcholine to a different physical state [19] is not excluded.

3.3. *Effect of protein removal*

Pronase digestion of erythrocyte membrane proteins does not affect the ^{31}P NMR spectrum of phosphonium labelled ghost fragments (fig.4). Membrane lipids themselves give rise to ^{31}P NMR spectra similar to those of intact membranes (fig.5) and the linewidth of the phosphonium peak is similar to that in spectra of membranes. These results indicate that lipid–protein interactions in the polar headgroup region of phosphatidylcholine do not cause extensive immobilization thus suggesting that lipid–protein interactions are predominantly apolar in nature, as indicated by other investigators [12,20].

3.4. *Phosphonium labelled cultured cells*

The ^{31}P NMR spectrum of Nil cells grown in the presence of phosphonium choline is shown in fig.6. The phosphate region of the spectrum is complicated, containing contributions from water-soluble phosphate esters superimposed on a broad resonance derived from all phospholipid phosphate groups. The phosphonium signal, in contrast, is a single resonance peak which may be attributed to the phosphatidylcholine analogue, since spectra of a total lipid extract and of the phosphonium phosphatidylcholine fraction of Nil cells also show this peak. Moreover, the concentration of water-soluble choline derivatives is 100-fold less than the concentration of cellular phosphatidylcholine [21].

Therefore it is possible to use whole cells with phosphatidylcholine specifically labelled with the phosphonium group for ^{31}P NMR studies. Such an approach should prove useful in determining the effect of fusion-promoting agents [22] on membrane phospholipids. It also has the potential for determining the distribution of phosphatidylcholine across the plasma membrane of mammalian cells since the phosphonium ^{31}P NMR signal is sensitive to paramagnetic ions [3].

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