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MODULATION OF MEMBRANE STRUCTURE BY Ca^{2+} AND DIBUCAINE AS DETECTED BY ^{31}P NMR

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Summary

The polymorphic phase behaviour of model membrane systems consisting of 20 mol% bovine brain phosphatidylserine and 80 mol% egg yolk phosphatidylethanolamine has been examined employing ^{31}P NMR techniques. It is shown that the addition of Ca^{2+} to such systems can trigger isothermal bilayer to hexagonal (H_{II}) phase transitions, and that such effects can be reversed by the subsequent incorporation of the local anaesthetic dibucaine. These results are discussed in terms of a recent model for membrane fusion (Cullis, P.R. and Hope, M.J. (1978) Nature 271, 672—674) and mechanisms of anaesthesia.

It has recently been proposed that lipids in local regions of biological membranes may assume transitory non-bilayer configurations [1], and that formation of these alternative structures may be vital to many aspects of membrane function, including 'flip-flop' [1], membrane fusion [2] exo- and endocytosis [3] as well as facilitated transport [3]. It is therefore of interest to demonstrate that agents which modulate such processes may also modulate membrane lipid structure in a compatible manner. Two such agents are Ca^{2+} and local anaesthetics which stimulate [4] and inhibit [5] membrane fusion phenomena respectively. In this work we have therefore investigated the effects of Ca^{2+} and the local anaesthetic dibucaine on the polymorphic phase behaviour of mixed phosphatidylethanolamine-phosphatidylserine model membrane systems. We show that Ca^{2+} can trigger

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formation of hexagonal (H_{II}) phase structure, whereas the subsequent addition of dibucaine causes a reversion to the bilayer phase. These observations are discussed in terms of a previous model of membrane fusion [2] and possible molecular mechanisms of anaesthetics.

The polymorphic phase behaviour of mixed aqueous dispersions of egg yolk phosphatidylethanolamine and 0—50 mol% beef brain phosphatidylserine were investigated employing ^{31}P NMR techniques, which, as indicated elsewhere [1,6] provide a convenient method of phase identification. Mixtures containing 20 mol% phosphatidylserine were selected for detailed study, as effects observed for this system provided the clearest indication of the effects we wish to emphasize here. As indicated in Fig. 1(a), such dispersions at 37°C exhibit broad asymmetric ^{31}P NMR spectra characteristic of bilayer liquid crystalline phospholipids [1,6—8], with a narrow component indicating a small fraction ($\leqslant 5\%$) of the lipid in structures allowing effectively isotropic motion. The subsequent addition of Ca^{2+} to a Ca^{2+}/phosphatidylethanolamine ratio of 0.5 (mol/mol) then results in the appearance of a narrower spectrum (Fig. 1(b)), with reversed asymmetry compared to bilayer spectra, which has been previously identified as characteristic of phospholipids in the hexagonal (H_{II}) phase [1,6,8,9]. Additional Ca^{2+} produced no further effects. Finally, the addition of dibucaine to a dibucaine/phosphatidylserine ratio of 1 (mol/mol) to this system caused a total reversion to the bilayer phase as indicated by the lineshape of Fig. 1(c).

In order to interpret these effects some knowledge of the behaviour of the individual lipid species and the effects of Ca^{2+} and dibucaine is required. It has been shown elsewhere [8] that pure egg yolk phosphatidylethanolamine assumes the hexagonal (H_{II}) phase at 37°C, and thus the observation of bilayer structure in the presence of 20 mol% phosphatidylserine implies a bilayer stabilizing role of phosphatidylserine. This is consistent with the observation that pure phosphatidylserine preferentially adopts the bilayer phase, as indicated by the characteristic ^{31}P NMR lineshape (see Fig. 2 (a)) displayed by the bulk of the phospholipids. The narrow spectral component has been attributed by other workers [10] to small bilayer structures for which tumbling or lateral diffusion [11] become effective motional averaging mechanisms.

The triggering of hexagonal (H_{II}) phase structures on addition of Ca^{2+} may be attributed to a diminished bilayer stabilizing capacity of the phosphatidylserine in the presence of Ca^{2+}, allowing the preference of the phosphatidylethanolamine component for the hexagonal (H_{II}) phase to predominate. This is consistent with the observation that the presence of Ca^{2+} at the concentrations employed here ($\approx$ 15 mM) had no effect on the phase behaviour of egg yolk phosphatidylethanolamine, in agreement with previous studies [8]. The addition of equimolar Ca^{2+} to pure beef brain phosphatidylserine on the other hand had dramatic effects, precipitating the lipid with concommitant total loss of the ^{31}P NMR signal. Such effects correspond to formation of the 'cochleate' structures described elsewhere [12] and indicate that the phosphate group motion in such structures is severely restricted, resulting in very broad ^{31}P NMR spectra which we are not able to detect on the apparatus employed here. Similar, but less ex-

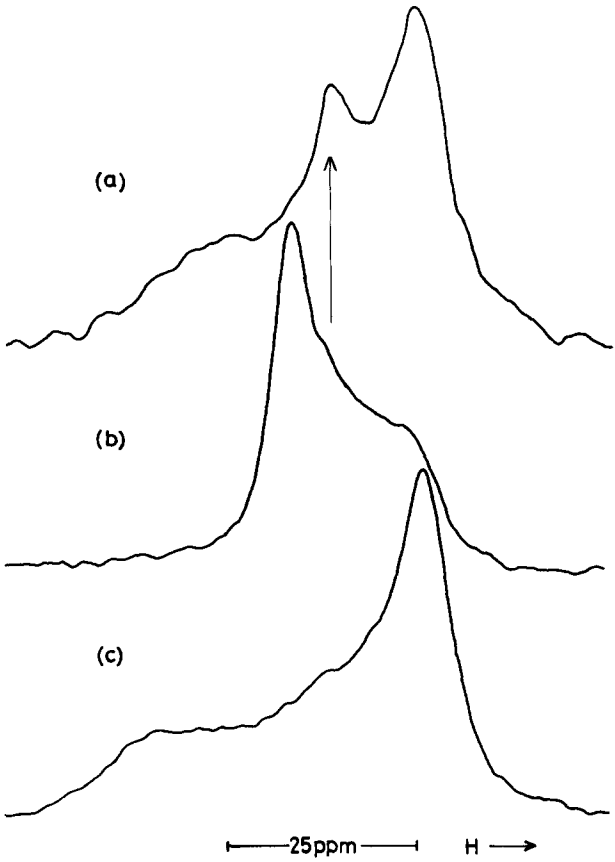

Fig.1. 36.4 MHz ^{31}P NMR spectra of an aqueous dispersion of 20 mol% bovine brain posphatidylserine and 80 mol% egg yolk phosphatidylethanolamine at 37°C: (a) In the absence of Ca^{2+} or dibucaine; (b) In the presence of Ca^{2+} (Ca^{2+} to phosphatidylserine ratio of 0.5 (mol/mol)); (c) as (b) plus dibucaine (dibucaine to phosphatidylserine ratio 1.0 (mol/mol)). The lipid dispersion was prepared from a chloroform solution containing appropriate amounts of the two lipid species (100 mg total phospholipid) where the chloroform was evaporated under N_2 and subsequent high vacuum. The lipid was then hydrated in 1 ml ^2H_2O containing 50 mM Tris/acetic acid (p^2H 7.2) and 300 mM NaCl by vortexing. Ca^{2+} was added from a stock solution of 100 mM $CaCl_2$. Dibucaine hydrochloride (Cinchocaine, Ciba) was added from a freshly prepared solution of 100 mM dibucaine where the pH was raised to 6.0 by addition of NaOH. The p^2H of the lipid dispersion was checked at each stage and raised back to 7.0 if required by addition of aliquots of 100 mM Tris. After adding Ca^{2+} or dibucaine the lipid dispersion was freeze-thawed (solid CO_2 /acetone) to ensure equilibrium partitioning of these agents. Egg yolk phosphatidylethanolamine was isolated as indicated elsewhere [8], and phosphatidylserine was isolated from the total lipids of bovine brain according to well established procedures [19] and was subsequently converted to the sodium salt by the method of Papahadjopoulos and Miller [20]. Both lipid species were at least 99% pure as indicated by thin layer chromatography. The arrow in (a) indicates the resonance position of spectra arising from phospholipids experiencing isotropic motion (e.g. sonicated vesicles).

treme broadening of the ^{31}P NMR resonance of less pure phosphatidylserine dispersions in the presence of Ca^{2+} has been reported elsewhere [10].

In the case of the mixed lipid dispersions it did not appear that the component phosphatidylserine formed separate cochleate structures in the presence of Ca^{2+} as appreciable loss of signal intensity was not observed even in systems containing 50 mol% phosphatidylserine. We therefore attribute the formation of H_{II} structures on addition of Ca^{2+} to either of two sources. First, Ca^{2+} may induce segregation of the phosphatidylserine

enabling the phosphatidylethanolamine to revert to the hexagonal phase. Alternatively, small Ca^{2+}-phosphatidylserine complexes may be formed which alter the dynamic shape of these molecules, thus affecting their ability to stabilize bilayer structure (for an extended discussion of these molecular shape concepts and their relation to lipid polymorphism, see Ref. 3). Whatever the detailed mechanism, however, the Ca^{2+} induced phase change appears to result from relatively specific Ca^{2+}-phosphatidylserine interactions, and not from non-specific charge neutralization effects, as Mg^{2+} was unable to induce H_{II} phase formation in similar systems even at Mg^{2+} concentrations corresponding to a Mg^{2+}/phosphatidylserine ratio of 3.0.

The ability of dibucaine to cause a reversion to bilayer structure may be partly attributed to its ability to displace Ca^{2+} from the lipid-water interface [13—15] thus causing dispersal of the phosphatidylserine and subsequent restabilization of the bilayer phase. In addition, it would be expected that the positively charged dibucaine would interact preferentially with the negatively charged phosphatidylserine, and it is therefore of interest to establish that the possible dibucaine-phosphatidylserine complexes formed preferentially assume the bilayer phase and may also act to stabilize bilayer structure. That this is the case is illustrated in Fig. 2 (b) for phosphatidylserine in the presence of equimolar dibucaine, for which a ^{31}P NMR spectrum characteristic of bilayer liquid crystalline phospholipids is observed. This contrasts with effects observed for pure egg phosphatidylethanolamine

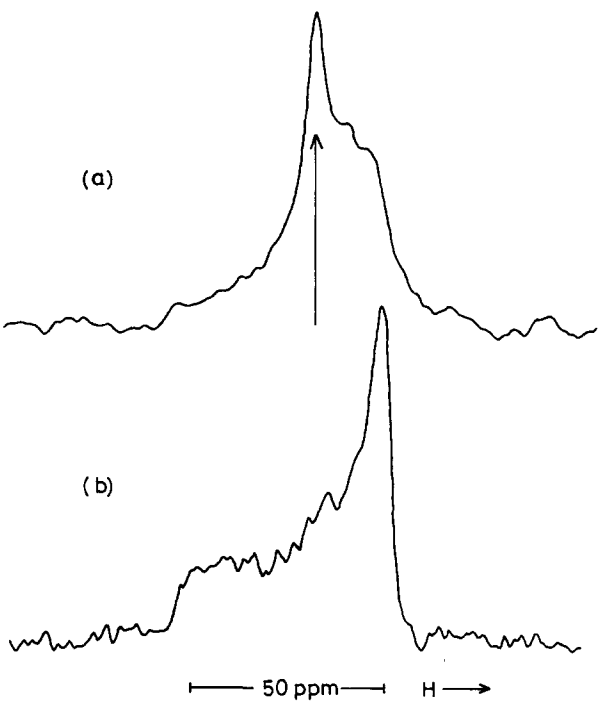

Fig.2. 36.4 MHz ^{31}P NMR spectra of an aqueous dispersion of bovine brain phosphatidylserine at 37°C: (a) In the absence of Ca^{2+} or dibucaine; (b) In the presence of equimolar dibucaine. All other conditions were as described for Fig.1. The arrow in (a) has a similar meaning as in Fig.1.

where increasing concentrations of dibucaine resulted in an increasing proportion of the lipid adopting structures or phases characterized by the possibility of isotropic motional averaging with an associated narrow spectral component.

One aspect of the spectrum of Fig. 2 (b) which requires further attention is the large negative value of the effective chemical shift anisotropy [1] ($\Delta\sigma_{CSA}^{EFF} = -53$ ppm) observed. Such effects suggest an ability of the local anaesthetic to induce appreciably more order in the phosphate region, which is consistent with recent observations for phosphatidylcholine-local anaesthetic dispersions [16]. A final technical point concerns the need for a freeze-thaw procedure in order to observe these spectral changes. Addition of dibucaine to phosphatidylserine dispersions without freeze-thawing did not produce any detectable effect on the lipid dispersion or the ^{31}P NMR spectra obtained. After freeze-thawing, however, the lipid precipitated and the resulting spectra were then of the form of Fig. 2 (b). Such effects are tentatively attributed to an inability of the anaesthetic to traverse the outer membrane of the liposomal structures unless the membrane is made leaky by the freeze thaw procedure. It was as a result of this observation that the freeze-thaw process was included as part of the experimental protocol in all the experiments reported here.

It is of interest to note that the bilayer stabilizing capacity of dibucaine in the mixed phosphatidylethanolamine-phosphatidylserine-Ca^{2+} dispersions was apparent at pharmacological concentrations (< 0.5 mM) of dibucaine if the lipid was incubated in a large volume of the anaesthetic containing buffer (Cullis, P.R., unpublished observation). This is consistent with the results described here, due to the very high partition coefficients of dibucaine in lipid systems containing phosphatidylserine [17].

In an earlier work, it has been proposed that membrane fusion in vivo proceeds via formation of hexagonal (H_{II}) phase -or inverted micellar lipid structure as an intermediate event [2]. It has also been extensively documented that Ca^{2+} is required for fusion processes [4]. The results of the present investigation thus may therefore be relevant to fusion events between membranes if the apposed monolayers contain predominantly unsaturated phosphatidylethanolamine and phosphatidylserine. In particular, the requirement of Ca^{2+} for fusion may then arise due to its ability to engender the non-bilayer intermediary structures. Conversely, the inhibitory effects of local anaesthetics [5] for fusion events would correspond to an inhibition of such non-bilayer lipid configurations.

An additional point concerns the molecular mode of action of local anaesthetics. Previous investigators have largely postulated that anaesthetics modulate the fluidity of the bilayer matrix, thus gating hypothetical membrane bound ion channels [14,18]. The results presented here suggest an alternative possibility, in that local anaesthetics can modulate or inhibit formation of non-bilayer lipid structures. As indicated elsewhere [3] it is conceivable that biological ionophores may rely on the ability of membrane lipids to adopt transitory non-bilayer configurations.

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