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Calcium-Induced Phase Separation Phenomena in Multicomponent Unsaturated Lipid Mixtures†

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ABSTRACT: The ability of calcium to induce phase separation in multicomponent lipid mixtures containing various unsaturated species of acidic and neutral phospholipids has been investigated by ^{31}P NMR, ^{2}H NMR, and small-angle X-ray diffraction techniques. It is shown that, in unsaturated (dioleoyl-) phosphatidylglycerol (PG)/phosphatidylethanolamine (PE) (1:1) and phosphatidic acid (PA)/phosphatidylcholine (PC) (1:1) mixtures, calcium is unable to induce lateral phase separation of the acidic and neutral lipids and that all the lipids adopt a hexagonal (H_{II}) phase in the presence of calcium. In multicomponent mixtures containing one or more acidic species the presence of cholesterol either facilitates calcium-induced lamellar to hexagonal (H_{II}) transitions for all the lipid components or, in systems already in a hexagonal (H_{II}) phase, mitigates against calcium-induced lateral phase separations. Further, cholesterol is shown to exhibit no preferential interaction on the NMR time scale with either PC, PE, or phosphatidylserine (PS) when the lipids are in the liquid-crystal state. The ability of cholesterol to directly induce H_{II} phase formation in PC/PE mixtures is also shown to be common to various other sterols including ergosterol, stigmasterol, coprostanol, epicoprostanol, and androstanol.

The addition of calcium to liposomal systems composed of acidic (negatively charged) phospholipids mixed with net neutral lipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) can have two major effects on membrane morphology. The first of these, commonly called "lateral phase separation", refers to the ability of calcium to sequester the acidic species into separate bilayer domains which usually exhibit gel-state characteristics. Such effects have been observed in many binary mixtures, including PC/phosphatidylserine (PS) systems (Ohnishi & Ito, 1974; Johnson & Papahadjopoulos, 1975; van Dijck et al., 1978) and mixtures of PC and phosphatidic acid (PA) (Ohnishi & Ito, 1974; Galla & Sackmann, 1975), among others. It has been proposed that

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such lateral phase separations may play a role in membrane fusion processes (Düzgünes & Papahajopoulos, 1983). However, the occurrence of lateral phase separations in complex mixtures of unsaturated lipids such as occur in biological membranes is not established.

The second type of structural rearrangement that calcium can initiate in mixtures of acidic and neutral lipids is the bilayer to hexagonal (H_{II}) polymorphic phase transition. Examples include mixtures of unsaturated PE and PS (Cullis & Verkleij, 1979; Tilcock & Cullis, 1981; Bally et al., 1983; Tilcock et al., 1984), as well as mixtures of unsaturated PE with phosphatidylglycerol (PG) (Farren & Cullis, 1980) and cardiolipin (Cullis et al., 1980). In some cases these polymorphic phase transitions are accompanied by Ca^{2+}-induced lateral phase separations. For dioleoyl-PE (DOPE)/dioleoyl-PS (DOPS) (1:1) systems, for example, Ca^{2+} induces a lateral phase separation of the DOPS into crystalline domains, allowing the DOPE to adopt the H_{II} phase it prefers in isolation. This does not appear to be a general occurrence, however, as calcium does not induce lateral phase separation in PE/PS mixtures containing more unsaturated varieties of PS or in DOPE/DOPS/cholesterol (1:1:1) systems (Tilcock et al., 1984). In the latter system, calcium effects a bilayer to H_{II} polymorphic phase transition where both the DOPE and DOPS adopt the H_{II} phase.

It is clearly important to establish the conditions under which calcium-induced bilayer to H_{II} polymorphic phase transitions are accompanied by lateral phase separations of acidic or neutral lipids. In this work we investigate the influence of divalent cations on the behavior of mixtures of DOPE with dioleoyl varieties of PS, PG, and phosphatidic acid (PA), paying particular attention to the influence of cholesterol. It is shown that in DOPG- and DOPA-containing systems Ca^{2+} is unable to induce lateral phase separation of the acidic species and all component lipids adopt the hexagonal H_{II} phase on addition of Ca^{2+}. The presence of cholesterol facilitates this Ca^{2+}-induced transition and, in multicomponent systems containing dioleoyl species of PC, PE, and PS, can promote Ca^{2+}-induced bilayer to H_{II} transitions where all component lipids adopt hexagonal H_{II} structures.

Materials and Methods

Lipid Synthesis. 11,11-Dideuteriooleic acid ([11,11-$^2H_2$-oleic acid) was synthesized according to Farren et al. (1984). Dioleoylphosphatidylcholine (DOPC) and [11,11-$^2H_2$]DOPC (deuterium label on both acyl chains) were synthesized and purified as previously described (Tilcock et al., 1982). Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylglycerol (DOPG), and dioleoylphosphatidic acid (DOPA), both deuteriated and nondeuteriated species, were derived from their respective phosphatidylcholine employing the base exchange capacity of phospholipase D (Comfurius & Zwaal, 1977). DOPE was purified as previously described (Tilcock et al., 1984). DOPG, DOPS, and DOPA were purified by using (carboxymethyl)cellulose column chromatography and converted to their sodium salts (Hope & Cullis, 1980).

Nuclear Magnetic Resonance. ^2H and ^{31}P NMR spectra were obtained by using a Bruker WP-200 spectrometer operating at 30.7 MHz for ^2H and 81 MHz for ^{31}P. Nondeuteriated phospholipids (30–50 μmol) were dispersed by vortex mixing in 0.8–1 mL of buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 100 mM NaCl, pH 7] to which was added the calcium ionophore A23187 (10 μL of a 2 mg/mL solution in Me_2SO) to facilitate equilibration of added calcium. Deuteriated lipids were dispersed as above except that buffer was prepared with deuterium-depleted water. Calcium was added as aliquots from a 100 mM stock solution of the chloride salt. Where signal intensities were measured, triphenyl phosphate (10% v/v in $CHCl_3$) was used as an external standard, located in a central insert in the NMR tube. For ^{31}P NMR, spectra were accumulated for up to 1000 transients with a 15-μs 90° radio frequency pulse, 20-kHz sweep width, and 1-s interpulse delay in the presence of broad-band proton decoupling. For ^2H NMR, spectra were accumulated for up to 50 000 transients with a phase-cycled quadrupolar echo sequence with a 15-μs 90° pulse, 100-kHz sweep width, and 200-μs echo delay.

X-ray Diffraction. Nickel-filtered Cu Kα (λ = 1.54 Å) X-rays were generated on a Rigaku RU-200 microfocus rotating anode generator. X-rays were collimated and focused by using either single or double Franks mirror optics and slits. Diffraction intensities were recorded on either of two image-intensified X-ray detectors (Reynolds et al., 1978; Gruner et al., 1982a,b). Since only lattice information was required for this study, an X-ray sensitivity correction parameter was not applied; this accounts for the asymmetry in peak intensities seen to either side of the beam stop in some of the diffraction data. Details of reduction and analysis of the data are described elsewhere (Gruner et al., 1982a,b; Tilcock et al., 1984).

Lipids for X-ray were dispersed in buffer to approximately 50% w/w (employing the same buffer as used for NMR) in acid-cleaned, 1.5 mm diameter glass X-ray capillaries which were then sealed with epoxy plugs. Sample temperature was under computer control to ±1 °C in a thermoelectrically regulated copper sample holder. Diffraction patterns were typically acquired in less than 2 min of X-ray exposure.

Results

The effect of calcium upon the phase behavior of DOPE/[11,11-$^2H_2$]DOPG (1:1) mixtures is illustrated in Figure 1. In the absence of calcium, ^{31}P NMR indicates a predominantly extended lamellar structure for the PE/[11,11-$^2H_2$]DOPG mixtures with some evidence of an H_{II} phase component (Figure 1D). This is also indicated by the corresponding ^2H NMR spectrum (Figure 1E) which indicates the presence of environments giving rise to at least two distinct averaging processes. The majority of the ^2H NMR signal arises from a component with a quadrupole splitting (ΔQ) of 8.3 kHz, from [11,11-$^2H_2$]DOPG in a lamellar phase. There is a less intense component with a ΔQ of 2.2 kHz due to [11,11-$^2H_2$]DOPG in the hexagonal H_{II} phase. We cannot exclude the possibility that a small component corresponding to lipid experiencing isotropic motion on the ^2H NMR time scale is superimposed upon the weak, central signal from residual deuterium in the deuterium-depleted water used for buffer preparation. Addition of calcium to a Ca^{2+}/DOPG molar ratio of 0.5 gives rise to a ^{31}P NMR spectrum (Figure 1B) that is consistent with a hexagonal H_{II} phase for the mixture as a whole. Unlike Ca^{2+}/PS complexes which exhibit rigid-lattice ^{31}P NMR spectra (Hope & Cullis, 1980), addition of calcium to DOPG liposomes gives rise to a ^{31}P NMR spectrum consistent with lipid in a lamellar, gel-state environment (spectrum not shown). Such a spectral component was never observed in the DOPE/DOPG systems, suggesting that no Ca^{2+}-induced phase separation occurred. Direct evidence for the incorporation of DOPG into the hexagonal H_{II} phase is given by ^2H NMR (Figure 1C) which shows that only averaged motion characterized by one quadrupole splitting of 2.2 kHz was observed from [11,11-$^2H_2$]DOPG in the presence of calcium. Small-angle X-ray diffraction of the mixture in the presence of calcium (Figure 1A) indicated only one hex-

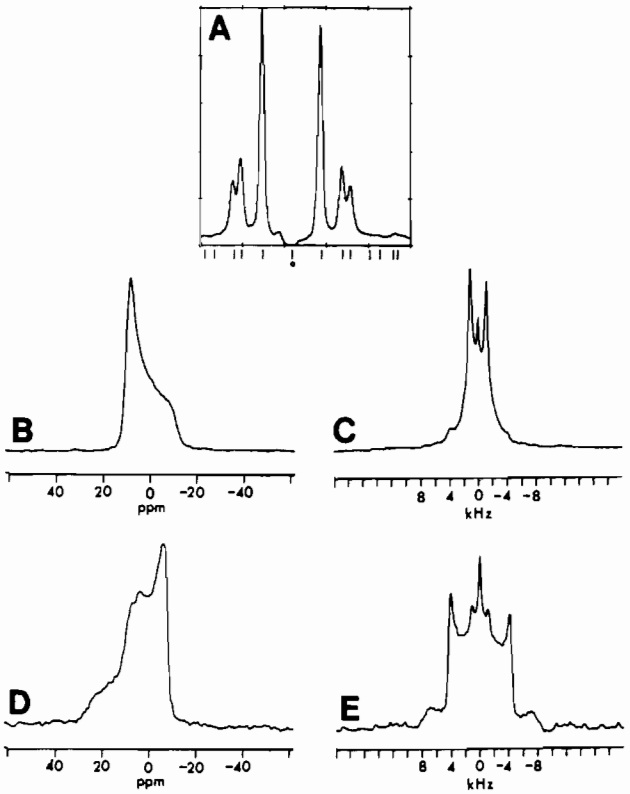

FIGURE 1: (A) Small-angle X-ray diffraction from a DOPE/DOPG/calcium (1:1:0.5) mixture at 30 °C. The diffraction is consistent with a hexagonal lattice of basis 68 ± 1 Å. For this figure and Figures 2–4, the diffracted X-ray intensity (in arbitrary units) is shown versus scattering angle. Zero scattering angle is indicated by a zero beneath the central dip due to the beam stop shadow. Tick marks, where shown, indicate the expected positions of peaks from a hexagonal lattice least-squares fit to the major visible peaks. (B) 81-MHz ^{31}P NMR spectrum and (C) 30.7-MHz ^{2}H NMR spectrum from a DOPE/[^{2}H]DOPG/calcium (1:1:0.5) mixture at 30 °C. (D) 81-MHz ^{31}P NMR spectrum and (E) 30.7-MHz ^{2}H NMR spectrum from a DOPE/[^{2}H]DOPG (1:1) mixture at 30 °C.

agonal phase with basis 68 ± 1 Å with no evidence of a coexisting lamellar lattice. This result supports previous findings for soya PE/egg PG mixtures in the presence of calcium (Farren & Cullis, 1980), which also indicated direct incorporation of the PG into the hexagonal (H_{II}) phase. This first example shows unequivocally that, even in binary systems without cholesterol, the mechanism of lamellar–H_{II} transitions does not necessarily involve phase separation phenomena as detectable on the NMR time scale or longer.

The ability of calcium to induce phase separation in mixed lipid systems is markedly dependent upon the acyl chain unsaturation of the constituent lipids. Previous studies of calcium-induced phase separations in PC/PA mixtures have made use of saturated species such as dimyristoyl-PC/dimyristoyl-PA (van Dijck et al., 1978), dipalmitoyl-PC/dipalmitoyl-PA (Jacobson & Papahadjopoulos, 1974; Galla & Sackmann, 1975), or spin-labeled stearoyl-PA (Ito & Ohnishi, 1974). In Figure 2, we present results upon unsaturated DOPC/DOPA (1:1) and also DOPC/DOPA/cholesterol (1:1:1) mixtures in the absence and presence of calcium. In the absence of calcium, the DOPC/DOPA (1:1) sample gave rise to a small-angle diffraction pattern (Figure 2a), to which neither a single hexagonal nor a single lamellar lattice could be well fitted. The corresponding ^{31}P NMR spectrum indicates motion for the ensemble consistent with an extended lamellar assembly, in agreement with previous results (Farren et al., 1983). In the presence of calcium (Figure 2b), small-angle X-ray diffraction data are consistent with a single hexagonal lattice with basis 70 ± 2 Å. There was no evidence of a coexisting lamellar lattice, indicating that both the DOPC and DOPA adopt the hexagonal phase; i.e., there is no phase separation. The corresponding ^{31}P NMR spectrum also indicates motional properties for the ensemble consistent with a hexagonal H_{II} phase with no evidence of phase separation. For similar systems containing cholesterol (DOPC/DOPA/cholesterol 1:1:1), in the absence of calcium, the small-angle X-ray diffraction indicates a single hexagonal (H_{II}) phase with basis 80 ± 0.5 Å (Figure 2c), a result also indicated by the accompanying ^{31}P NMR data. In the presence of calcium, the small-angle X-ray diffraction is consistent with a poorly formed hexagonal lattice with a 90 ± 2 Å basis. The corresponding ^{31}P NMR spectrum also indicates motion with a hexagonal H_{II} ensemble with no evidence of an extended lamellar component. Thus for this system with cholesterol, calcium is unable to induce a lateral phase separation on time periods longer than 10^{-5} s.

As previously noted (Cullis & de Kruijff, 1982; Tilcock et al., 1984), cholesterol can destabilize lamellar structure in mixed (unsaturated) PE/PC and PE/PS systems, either inducing the hexagonal (H_{II}) phase directly or else facilitating calcium-induced lamellar to hexagonal (H_{II}) transitions. In the following studies we demonstrate the generality of this effect for various multicomponent unsaturated lipid mixtures. We present first detailed studies upon the effects of calcium on a DOPE/DOPC/DOPS/cholesterol (1:1:1:3) mixture. Mixtures containing either deuteriated PC, deuteriated PE,

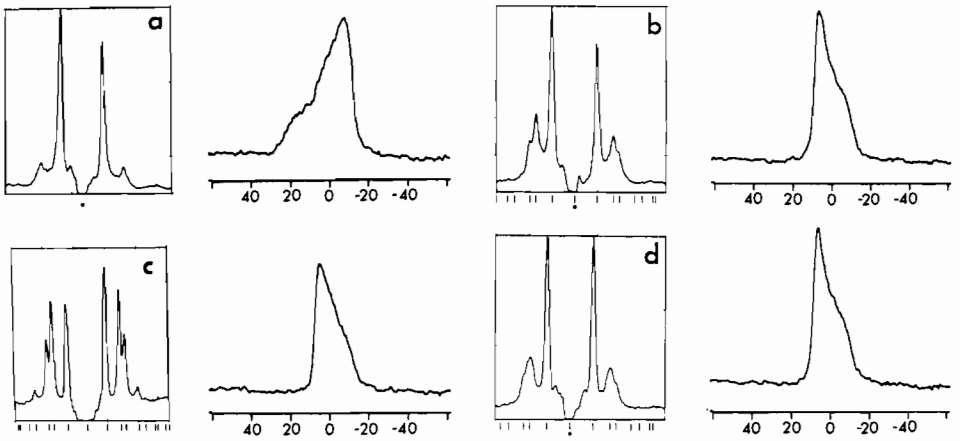

FIGURE 2: Small-angle X-ray diffraction and corresponding 81-MHz ^{31}P NMR spectra from (a) DOPC/DOPA (1:1), (b) DOPC/DOPA/calcium (1:1:0.5), (c) DOPC/DOPA/cholesterol (1:1:1), and (d) DOPC/DOPA/cholesterol/calcium (1:1:1:0.5). All spectra and diffraction patterns were measured at 30 °C.

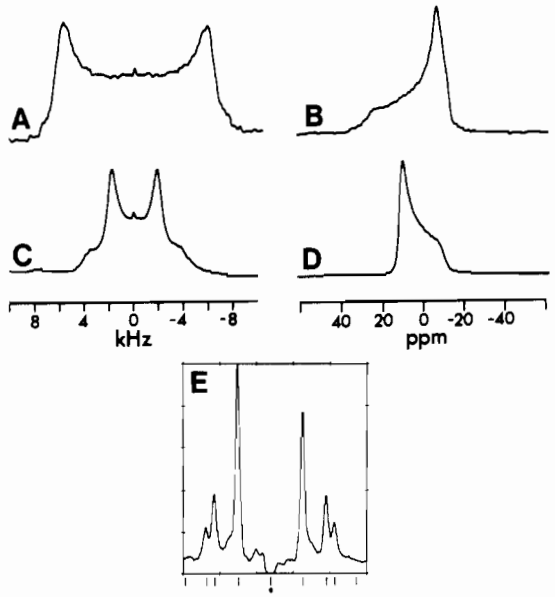

FIGURE 3: (A) 30.7-MHz ^2H NMR spectrum of a DOPE/DOPC/[^2H]DOPS/cholesterol (1:1:1:3) mixture at 30 °C; (B) corresponding 81-MHz ^{31}P NMR spectrum; (C) 30.7-MHz ^2H NMR spectrum of a DOPE/DOPC/[^2H]DOPS/cholesterol/calcium (1:1:1:3:0.5) mixture at 30 °C; (D) corresponding 81-MHz ^{31}P NMR spectrum; and (E) corresponding small-angle X-ray diffraction.

or deuteriated PS were prepared which were then examined by ^2H NMR in order to be able to monitor the phase behavior of the individual components. The results for a mixture containing ^2H-labeled DOPS are shown in Figure 3. In the absence of calcium, ^2H NMR (Figure 3A) indicated a quarupole splitting (ΔQ) of 10.8 kHz for the lamellar PS component, whereas ^{31}P NMR (Figure 3B) indicated motional characteristics consistent with an extended lamellar phase. In the presence of calcium, ^2H NMR (Figure 3C) indicated a decrease in ΔQ from 10.8 (for [^2H]DOPS) to 3.6 kHz, consistent with incorporation of the [^2H]DOPS into the hexagonal H_{II} phase. Note that the deuterium label is in the acyl chain, not in the head group; therefore, these spectral changes cannot be explained in terms of changes in the head group conformation. The corresponding ^{31}P NMR spectra (Figure 3D) indicated motional characteristics consistent with a hexagonal H_{II} phase. It is important to note that, for equivalent systems containing either [11,11-$^2H_2$]DOPE or [11,11-$^2H_2$]DOPC (spectra not shown), ^2H NMR indicated a reduced ΔQ, also to a value of 3.6–3.7 kHz for both systems, again indicating that both the DOPE and DOPC are directly incorporated into the hexagonal H_{II} phase. The equivalence of the observed quadrupole splittings also indicates that the systems remain well mixed and that there is no preferential association of cholesterol with any of the phospholipids in the liquid-crystal state, consistent with previous observations on PE/PC/cholesterol mixtures (Tilcock et al., 1982). The NMR results were confirmed by small-angle X-ray diffraction (Figure 3E) which demonstrated unequivocally that only a single hexagonal lattice with basis 72 ± 1 Å was observed in the presence of calcium, with no evidence for a coexisting lamellar lattice. These results clearly demonstrate that calcium does not induce lateral phase separations in these systems. It is of interest that two of the three phospholipids (DOPC and DOPS) incorporated in this mixture do not spontaneously adopt a hexagonal H_{II} phase either alone or in the presence of calcium, yet the distributed presence of 16 mol % PE in the DOPC/DOPE/DOPS/cholesterol mixture enables all of the lipids to enter the hexagonal H_{II} phase in the presence of calcium.

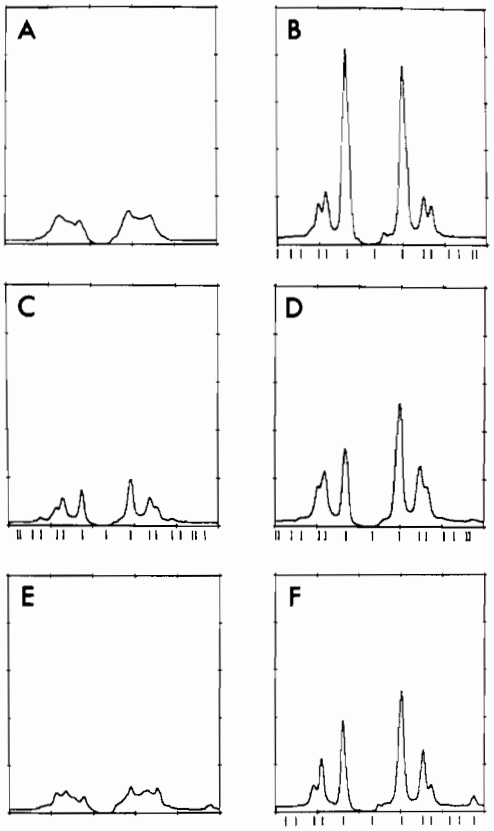

FIGURE 4: Small-angle X-ray diffraction patterns from (A) DOPG/DOPA/cholesterol (1:1:1); (B) DOPG/DOPA/cholesterol/calcium (1:1:1:0.5); (C) DOPC/DOPE/DOPA/cholesterol (1:1:1:3); (D) DOPC/DOPE/DOPA/cholesterol/calcium (1:1:1:3:0.5); (E) DOPE/DOPS/DOPG/cholesterol (1:1:1:3); and (F) DOPE/DOPS/DOPG/cholesterol/calcium (1:1:1:3:0.5). All patterns were recorded at 40 °C. Tick marks indicate the least-squares best fit to the expected peak positions of a hexagonal lattice. The absence of tick marks indicates no unambiguous fit to a lattice was possible.

The generality of the effect of cholesterol upon calcium-induced phase separations is illustrated in Figure 4 for a variety of mixed lipid systems. Figure 4A,B shows the effect of added calcium on a DOPG/DOPA/cholesterol (1:1:1) mixture. In the absence of calcium (Figure 4A), small-angle X-ray yields no sampled diffraction, indicative of the absence of a lattice structure, although ^{31}P NMR indicates an extended lamellar phase (spectrum not shown), which may arise from lamellae which are not organized in a lattice. In the presence of calcium (Figure 4B), only a hexagonal lattice with basis 72 ± 2 Å was observed. Figure 4C–F illustrates similar effects in mixtures containing four lipids including cholesterol. Figure 4C,D shows the effect of calcium on a DOPC/DOPE/DOPA/cholesterol (1:1:1:3) mixture. Without calcium (Figure 4C), the small-angle X-ray diffraction indicates a poor fit to a hexagonal lattice with basis 80 ± 2 Å. In the presence of calcium (Figure 4D), only a single hexagonal lattice with basis 73 ± 2 Å was observed. There is no calcium-induced phase separation in this system, analogous to the results presented in Figure 2. Figure 4E,F illustrates the effects of calcium on a multicomponent system that contains two charged lipid species, DOPE/DOPS/DOPG/cholesterol (1:1:1:3). In the absence of calcium (Figure 4E), small-angle X-ray diffraction suggests the presence of multiple lamellar lattices. This may represent a spontaneously phase separated system. However, addition of calcium (Figure 4F) gives rise to a diffraction pattern indicative of only a single hexagonal lattice with basis 68 ± 2 Å.

The mechanism by which cholesterol either directly induces

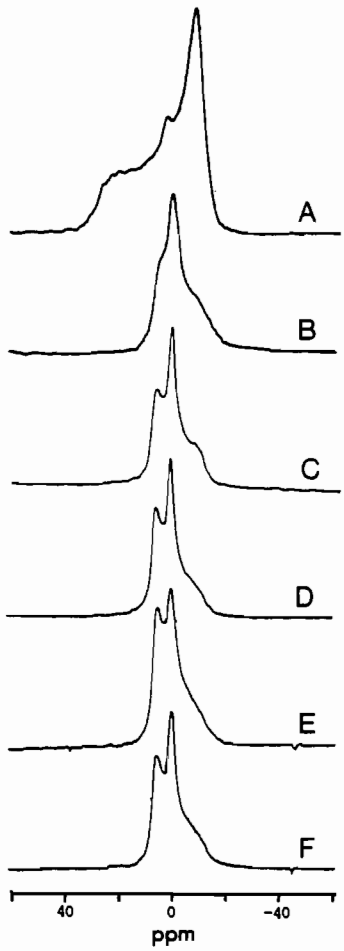

FIGURE 5: 81-MHz ^{31}P NMR spectra of DOPE/DOPC (4:1) mixtures at 40 °C: (A) in the absence of added sterol; in the presence of 4 mol % of either (B) ergosterol, (C) stigmasterol, (D) coprostanol, (E) epicoprostanol, or (F) androstanol.

H_{II} phase formation or else facilitates lamellar to hexagonal H_{II} transitions by exogenous calcium remains unknown. However, it may be noted from Figure 5 that the destabilizing influence of cholesterol in mixed DOPC/DOPE (1:4) systems (Tilcock et al., 1982) is also shared by a variety of other sterols. It is clear that all the sterols examined can induce a phase transformation from lamellar to hexagonal H_{II} and, to a lesser extent, a structure whose lipids undergo isotropic motion on the NMR time scale (Seelig, 1978). It is also remarkable that as little as 4 mol % sterol can induce the phase transformation.

Discussion

On the basis of observations that sonicated unilamellar vesicles composed of saturated PC's fused when incubated near their gel–liquid-crystal transition temperature (Papahadjoupoulos et al., 1974) or that the ion specificity for the fusion of PS vesicles is correlated with the relative effectiveness of different divalent cations to affect the thermotropic properties of PS (Jacobson & Papahadjopoulos 1974), it was suggested that ionotropic-, pH-, or protein-induced lateral phase separations may be of relevance to fusion processes. In this hypothesis, the phase discontinuities between a crystalline, phase-separated, e.g., Ca^{2+}/PS, complex and the surrounding fluid lipid serve as the initiation site for the fusion event (Düzgünes & Papahadjopoulos, 1983).

Many of the model membrane systems used to investigate calcium-induced phase separation phenomena have been composed of disaturated lipids such as dipalmitoyl or dimyristoyl species. In eukaryotic membranes such saturated species represent a small fraction of the lipid chains; hence, it is legitimate to question whether such prior studies have provided relevant insight into processes in eukaryotic membranes.

We have shown that calcium is unable to induce a phase separation in either DOPE/DOPG (1:1) or DOPC/DOPA (1:1) systems, whereas calcium can induce a phase separation in dimyristoyl-PC/dimyristoyl-PA (van Dijck et al., 1978) or dipalmitoyl-PC/dipalmitoyl-PA mixtures (Jacobson & Papahadjopoulos, 1974). In the case of DOPE/DOPG, addition of Ca^{2+} results in a lamellar to hexagonal H_{II} phase transformation where both phospholipid species enter the hexagonal H_{II} phase. This may be contrasted with the known ability of calcium to cause a lateral phase separation of the PS component into an anhydrous complex in DOPE/DOPS mixtures (Tilcock et al., 1984), although it should be noted that calcium is unable to induce a similar effect in DOPE/dilinoleoyl-PS mixtures (Tilcock et al., 1984). This, together with the DOPC/DOPA results presented herein, indicates that increased acyl chain unsaturation mitigates against calcium-induced lateral phase separations. We have also shown for various systems containing dioleoyl phospholipids and cholesterol and either one or two charged lipid species that calcium is similarly unable to induce phase separations for time periods of the order of or longer than the NMR time scale (10^{-5} s). We cannot discount the possibility of microdomain formation on time periods less than $\simeq 10^{-5}$ s.

In addition, we have shown conclusively for a DOPE/DOPC/DOPS/cholesterol (1:1:1:3) mixture that, in the presence of calcium sufficient to bind all the PS, the entire lipid mixture undergoes a lamellar–H_{II} transition. This is a remarkable result considering that neither DOPC nor DOPS, either in the absence or in the presence of calcium, at the experimental temperature or pH, will adopt the hexagonal H_{II} phase. It would also appear that cholesterol does not exhibit any preferential association with any of the phospholipids on the NMR time scale when the lipids are in the liquid-crystal state, consistent with previous findings for a PC/PE/cholesterol mixture (Tilcock et al., 1982). The observation that a variety of sterols, despite wide variation in structure, can all destabilize the lamellar phase in mixed PC/PE systems demonstrates that neither the conformation of the hydroxyl moiety at the 3-position, the presence of a coplanar ring system, nor even the presence of a side chain is of importance with regard to the destabilization. This finding is of interest given the observation that there is a direct relationship between the decrease in the temperature of the lamellar to H_{II} transition for PE and the mean molecular areas occupied by the sterols (Gally & de Kruijff, 1982) and would suggest that the effects of cholesterol (and other sterols) may best be rationalized in terms of generalized spacing effects and the influence of the sterols upon parameters such as the intrinsic radius of curvature or the packing of the hydrocarbon chains of the lipid mixtures (Gruner, 1985).

Registry No. DOPG, 62700-69-0; DOPA, 14268-17-8; DOPC, 10015-85-7; DOPE, 2462-63-7; DOPS, 6811-55-8; Ca, 7440-70-2; cholesterol, 57-88-5; ergosterol, 57-87-4; stigmasterol, 83-48-7; coprostanol, 360-68-9; epicoprostanol, 516-92-7; androstanol, 58855-92-8.

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A 13-Kilodalton Protein Purified from Milk Fat Globule Membranes Is Closely Related to a Mammary-Derived Growth Inhibitor

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ABSTRACT: With the use of specific antibodies against a previously purified [Boehmer, F.-D., Lehmann, W., Schmidt, H., Langen, P., & Grosse, R. (1984) *Exp. Cell Res. 150*, 466–477] and sequenced mammary-derived growth inhibitor (MDGI) [Boehmer, F.-D., Kraft, R., Otto, A., Wernstedt, C., Hellmann, U., Kurtz, A., Mueller, T., Rohde, K., Etzold, G., Lehmann, W., Langen, P., Heldin, C.-H., & Grosse, R. (1987) *J. Biol. Chem. 262*, 15137–15143], the localization and relative amount of immunoreactive 13-kilodalton (kDa) antigen in different fractions of bovine milk were determined. The highest amount of antigen was found to be associated with the milk fat globule membranes (MFGM). As revealed by a dot immunobinding assay, the amount of immunoreactive bovine and human MFGM-associated antigen increased dramatically with the onset of lactation after delivery. This finding corresponds to earlier data obtained for MDGI and indicates a relationship between the proliferative state of mammary epithelial cells and the amount of immunoreactive antigen. The 13-kDa antigen has been purified from MFGM to homogeneity by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroelution. The MFGM-derived 13-kDa polypeptide was found to be almost identical with MDGI as demonstrated by tryptic digestion and partial amino acid sequence analysis of tryptic fragments of both proteins. The results clearly show the presence of a membrane-bound MDGI-related 13-kDa protein, thus supporting the possible involvement of membrane-associated growth inhibitors in growth regulation of mammary epithelial cells.

Polypeptide growth factors have acquired an established place among growth modulators of the mammary gland during the last decade. For example, epidermal growth factor (EGF)[1] receptor level and transforming growth factor (TGF) action seem to be correlated with growth of breast cancer cells (Fitzpatrick et al., 1984; Sporn et al., 1986; Spitzer et al., 1987). Demonstration of numerous growth stimulatory factors in milk (Shing & Klagsbrun, 1984; Bano et al., 1985) also points to the mammary gland as an important source of growth factors. Polypeptide growth inhibitors (Wang & Hsu, 1986) are possibly of similar importance for the regulation of mammary epithelial cell proliferation (Dickson & Lippman, 1987). We have described previously a 13-kDa growth inhibitor

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[1] Abbreviations: DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; TGF, transforming growth factor; MDGI, mammary-derived growth inhibitor; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TBS/Tween, Tris-buffered saline, pH 7.4, containing 0.2% Tween 20; MFGM, milk fat globule membrane(s); Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); HPLC, high-performance liquid chromatography; RP, reverse phase; TFA, trifluoroacetic acid.

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