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THE EFFECT OF CALCIUM ON THE BILAYER STABILITY OF LIPIDS FROM BOVINE ROD OUTER SEGMENT DISK MEMBRANES

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The phase behavior of bovine rod outer segment disk lipids has been investigated using freeze-fracture and ^{31}P nuclear magnetic resonance (NMR) techniques. ^{31}P-NMR spectra of isolated disk membranes were taken as a function of temperature between 25°C and 45°C. The ^{31}P-NMR spectrum characteristic of phospholipid bilayers was observed at all temperatures both in the absence of Ca^{2+} and in the presence of 10 mM and 50 mM Ca^{2+}. A similar study was performed on lipids isolated from the disk membranes. In the absence of Ca^{2+} only lamellar phase behavior was observed. In the presence of less than 10 mM Ca^{2+}, however, there was a change in morphology to non-lamellar structures. Removal of the Ca^{2+} caused the system to reassume the lamellar form.

Introduction

The behavior of lipids and rhodopsin in the retinal rod outer segment dish membrane has been the subject of considerable study. Studies focused on the composition of the phospholipids show the disk membrane lipids to consist largely of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. The fatty acyl side chains have been found to be highly unsaturated [1–7]. Rhodopsin-lipid interactions in the intact disk membrane have been investigated and there is evidence indicating that rhodopsin influences some motional properties of some of the surrounding lipids [8–12].

It has been demonstrated that lipids extracted from biological membranes can exist in conformations other than bilayer [12–15]. The stability of these lipids in the bilayer phase relative to the non-bilayer conformation, has been shown to be dependent on headgroup, degree of unsaturation, pH, temperature and the presence of cations [15]. Calcium has been widely regarded as important in visual function. It has been suggested that Ca^{2+} is the principle messenger between the light-sensitive rhodopsin in the disk membrane and the rod outer segment plasma membrane [16]. In this paper we demonstrate that rod outer segment disk lipid structure is sensitive to Ca^{2+} concentrations. These lipids can exist in the bilayer conformation at low Ca^{2+} concentrations (less than 4 mM) but at higher

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Ca^{2+} concentrations exhibit non-bilayer characteristics. This bilayer destabilization by Ca^{2+} is not observed in the intact membrane by the ^{31}P-NMR technique used here.

Methods

Bovine retinas were purchased from American Stores (Nebraska). Osmotically intact disks were prepared as described by Smith et al. [17]. Disks typically had a 280/500 spectral ratio of 2.2–2.3. Disk lipids were obtained by solubilizing the disks in octylglucoside and eluting them over a concanavalin A-Sepharose 4B column in the same manner as used to purify rhodopsin [18]. Fractions which did not bind to the column contained the disk lipids. These were pooled and concentrated using an Amicon ultra filtration device. The lipids were dialyzed against 2 liters of 10 mM Hepes/1 mM EDTA, pH 7 to remove the detergent and then lyophilized. Finally the lipid was suspended in 2 ml of 10 mM Hepes, pH 7 and dialyzed against 2 liters of 10 mM Hepes, pH 7. Fatty acid determinations were carried out after formation of the methyl esters by gas chromatography in a Varian 2100 gas chromatograph using a 15% HI-EF-IBP (diethylamine succinate) column at an operation temperature of 160°C. The fatty acid composition agreed with previously published results [4–6]. Notably there was 40% 22 : 6.

All buffers were perfused with argon before use and samples containing lipid were always layered with argon to prevent oxidation.

Phospholipid determinations were made using the method of Bartlett [19] with modifications as described by Litman [20].

^{31}P-NMR spectra were obtained at 109 MHz on a JEOL FX 270 Fourier transform NMR spectrometer at stated temperatures in 10 mm tubes. Free induction decays were collected using a 32 pulse fully phase cycled CSA echo sequence with a 20 μs echo. No first-order phase corrections were used in transforming the data. 50 kHz sweep widths with 1 second delays between sets of pulses were employed. (The CSA echo sequence was kindly provided by Drs. M. Rance, A. Byrd and I.C.P. Smith.) Generally 2000 scans were accumulated for each spectrum. Spectra for rod outer segment disks were obtained in the dark. Chemical shifts are in ppm from external phosphoric acid.

Samples for freeze-fracture electron microscopy were frozen using an ultrarapid freezing technique [21]. The lipid dispersions were sandwiched between two thin copper strips and frozen by plunging rapidly in liquid propane. Freeze-fracture replicas were prepared in a Polaron E7500 unit and the replicas examined in a Siemen 101 electron microscope.

Results

Effect of Ca^{2+} and temperature on disk lipids

The concentration of Ca^{2+} in the disk lipid dispersion was varied from 0 to 10 mM and the temperature from 25 to 45°C. Fig. 1 shows a composite of ^{31}P-NMR spectra of the disk lipids in 10 mM Hepes (no calcium) at different temperatures. No detectable change in the lineshape is found in any of the spectra which are characteristic of phospholipids in lamellar phase. Upon ad-

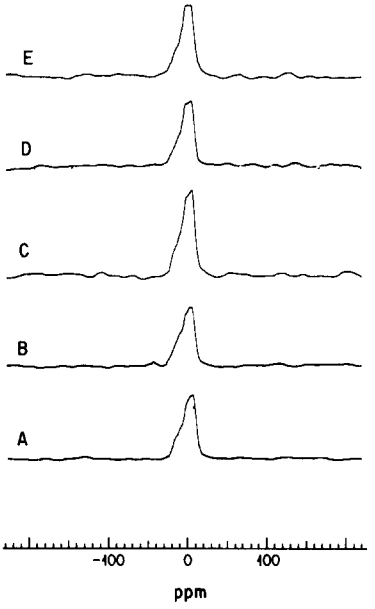

Fig. 1. 109 MHz ^{31}P-NMR spectra, taken as described in Methods, of a dispersion of lipids, obtained as described in Methods, from bovine rod outer segment disc membranes, in 1 ml of 10 mM Hepes, pH 7. No Ca^{2+} was added. Samples were incubated at the indicated temperatures for 10 min before obtaining a measurement. Spectra were obtained at 25, 30, 35, 40 and 45°C (A, B, C, D, and E, respectively). 2000 scans were obtained for each measurement.

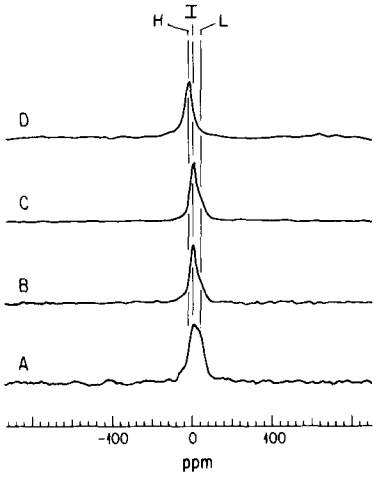

Fig. 2. Effect of calcium on the ^{31}P-NMR spectra of lipid dispersions obtained from disc membranes. The spectra were obtained at 25°C. Spectra were measured in the presence of 4, 4 (after heating to 45°C and cooling to 25°C), 6, and 10 mM Ca^{2+} (A, B, C, and D). The positions for the maxima expected for resonances due to hexagonal (H), isotropic (I) and lamellar (L) phases are indicated.

ding 2 mM Ca^{2+} (final concentration) to the medium no change is observed in the ^{31}P-NMR lineshape. The spectra remain that of lamellar phospholipids between 20 and 45°C (data not shown).

A rather dramatic change occurs on increasing the Ca^{2+} concentration to 4 mM. The spectra changes from that characteristic of lamellar to a non-lamellar phase (Fig. 2). Though still largely lamellar, some isotropic phase is apparent. Addition of more Ca^{2+} (Fig. 2C) produces a three phase system, containing lamellar, isotropic and hexagonal II structure. On addition of more Ca^{2+} (Fig. 2D) the spectra assumes an hexagonal II line shape which is not completely reversed on cooling back to 25°C (Fig. 2B). Higher concentrations of Ca^{2+} (up to 10 mM) cause only modest additional changes in the lineshape of the ^{31}P-NMR spectra. Higher temperatures (upto 45°C) did not cause any further change in the spectra (data not shown).

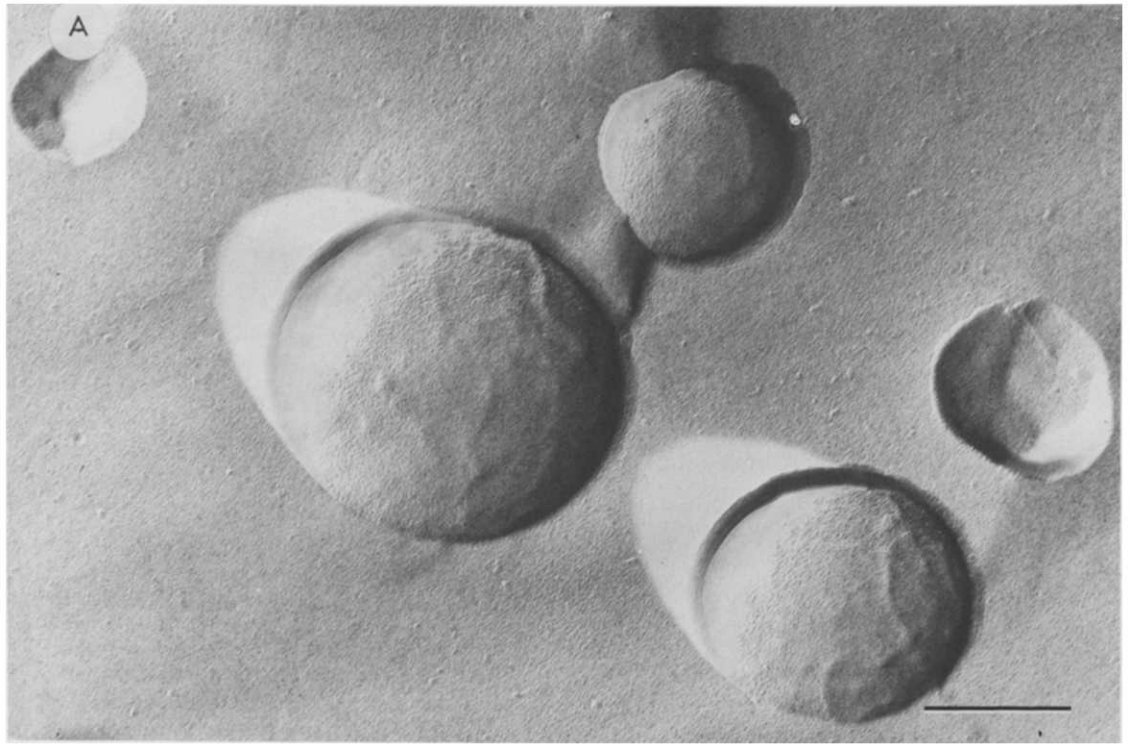

Fig. 3A.

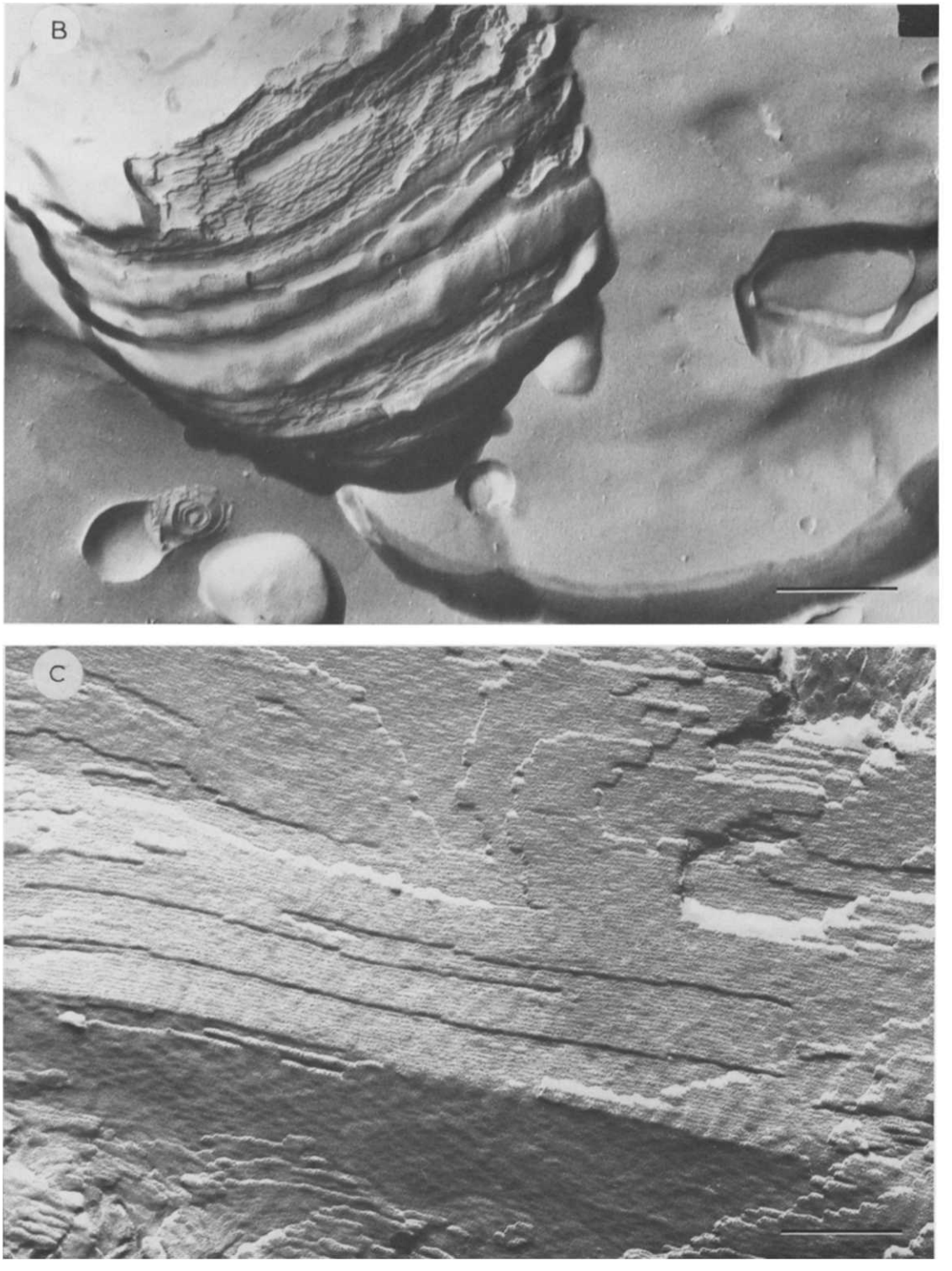

Fig. 3. Freeze-fracture electron micrographs of lipids extracted from bovine rod outer segment disc membranes, dispersed in 10 mM Hepes, pH 7. (A) no added Ca^{2+}; (B) with 4 mM Ca^{2+}; (C) with 8 mM Ca^{2+}. The scale bar represents 250 nm.

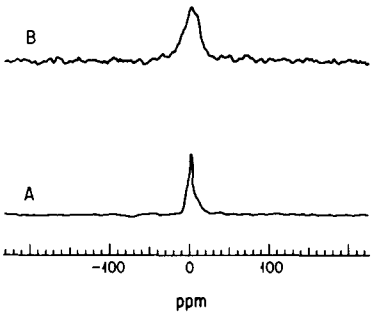

Fig. 4. (A) ^{31}P-NMR spectrum of the extracted disc lipids in the presence of 8 mM Ca^{2+}; (B) ^{31}P-NMR spectrum of the same sample in A after extensive dialysis to remove the Ca^{2+}, as described in the text.

Since phase structures cannot be unambiguously assigned based on ^{31}P-NMR spectra alone, we studied the structures formed by the disk lipid dispersions at different Ca^{2+} concentrations using freeze-fracture electron microscopy. Fig. 3 shows typical areas from freeze fracture replicas of disk lipid dispersions with 0, 4, and 10 mm Ca^{2+}. In the absence of Ca^{2+} (Fig. 3A) only smooth lipid vesicles are observed in the replica. At 4 mM Ca^{2+}, while vesicles are still found in the freeze-fracture replica, new structures appear (Fig. 3B). These structures morphologically look like intermediate structures between lamellar and hexagonal II phases [22]. The appearance of these structures corresponds with the appearance of the isotropic lineshape in the ^{31}P-NMR spectra coexisting with the lamellar lineshape (Fig. 2). On further increasing the concentration of Ca^{2+} to 10 mM only ordered tube-like structures are found in the freeze-fracture replicas (Fig. 3C). These structures are morphologically identical to hexagonal II structure reported for phosphatidylethanolamine [23] and other lipids [24]. This is consistent with the ^{31}P- NMR spectra (Fig. 4) which show largely a hexagonal resonance.

To further assess whether Ca^{2+} is responsible for the bilayer to hexagonal II phase transition, the disk lipid dispersion containing 10 mM Ca^{2+} was dialyzed against a buffer containing EGTA. (The dialysis was performed at pH 4 as it was not possible to remove the Ca^{2+} from the lipids at pH 7. The pH was brought back to 7 before recording the ^{31}P-NMR spectra.) The ^{31}P-NMR spectrum taken after this dialysis is shown in Fig. 4B and is characteristic of lamellar phospholipid.

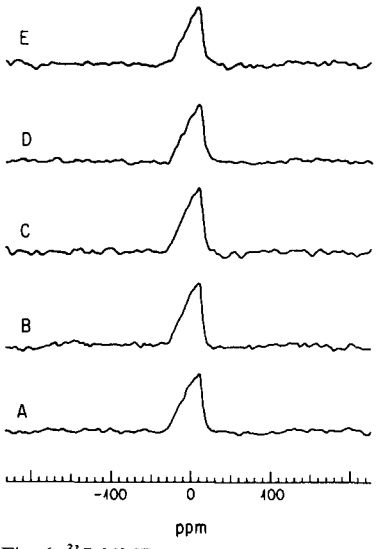

Fig. 6. ^{31}P-NMR spectra of unbleached rod outer segment disk membranes, as in Fig. 5, without (A) and with (B) 10 mM Ca^{2+}, at 30°C. 8000 transients were accumulated.

Effect of Ca^{2+} and temperature on disk membranes

To test whether Ca^{2+} can produce a similar phase change in disk membranes, ^{31}P-NMR spectra were recorded for disk membranes suspended in 10 mM Hepes with different concentrations of added Ca^{2+} from 0 to 50 mM and the temperature was varied from 25 to 45°C. Only lamellar lineshapes are observed at Ca^{2+} concentrations upto 50 mM and at temperatures up to 45°C (Fig. 5).

Fig. 6 shows the comparison between disk membrane with and without 10 mM Ca^{2+} at an increased number of scans. As shown elsewhere

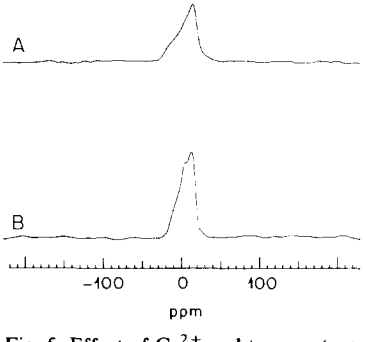

Fig. 5. Effect of Ca^{2+} and temperature on ^{31}P-NMR spectra of osmotically intact, unbleached bovine rod outer segment disk membranes from 50 retinas in 1 ml of 10 mM Hepes, pH 7. 10 mM Ca^{2+} was added. Spectra were obtained at 25, 30, 35, 40, and 45°C (A, B, C, D, and E, respectively).

[25] this lineshape can be satisfactorily computed simulated, using the short T_2 measured for the system, using only a lamellar component.

Discussion

The release of Ca^{2+} upon illumination from rod outer segment disk membranes has been regarded as important in the function of the rod outer segment in visual transduction. The molecular interactions of Ca^{2+} with components of the rod outer segment, however are not known. One possible site of interaction of Ca^{2+} with a membrane is at the negatively charged headgroup of phosphatidylserine. Approximately 11–16% of the disk phospholipids are phosphatidylserine [1,6]. Recently some evidence was presented that Ca^{2+} tends to exclude phosphatidylserine from the environment around rhodopsin in disk membranes [26]. In this paper we have demonstrated that millimolar concentrations of Ca^{2+} destabilize the bilayer structure of extracted disk membrane lipids. This destabilization can be reversed by the removal of Ca^{2+}.

The presence of the negatively charged phosphatidylserine tends to stabilize the bilayer structure. The divalent cations can neutralize the charge on the lipid and thereby destabilize the charge on the bilayer. Gounaris et al. [15] in their recent report have studied the effect of cations in such mixed lipid systems and have shown that neutralization of the negative charges either by the addition of cations or lowering of pH is the first step in the destabilization of the bilayer phase. These effects must be considered when using the extracted lipids for model studies.

These studies conclusively show that Ca^{2+} can destabilize the bilayer structure of isolated disk lipids. However, the intact disk remains bilayer even in the presence of high concentrations of Ca^{2+}. This indicates that rhodopsin may play a role in bilayer stabilization as suggested by De Grip et al. [27]. While this conclusion is reasonable, the effect of the asymmetric arrangement of the rhodopsin and the phospholipids in the disk membrane must also be evaluated. There is a preliminary study which deals with this issue [28]. In this study the ^{31}P-NMR spectra of disks which were solubilized in octylglucoside then reconstituted by dialysis were compared to Folch-extracted disk lipids which were then solubilized and dialyzed in the same manner. This procedure should largely eliminate the asymmetry of the system. The lipids produced a non-bilayer, isotropic spectra while the recombined total disk produced a bilayer type spectrum.

There is at present an apparent conflict in the literature regarding the phase behavior of lipids isolated from rod outer segment disk membranes. De Grip et al. [27] reported that lipids isolated from these membranes can form non-bilayer structures when suspended in buffer. Another group [29,30] reported that only bilayer structures were found in phospholipid preparations from disk membranes. A closer examination of these reports in light of the data presented here reveals that the conflicting conclusions are due to the differences in the buffer systems used by the groups. The buffer system used by De Grip et al. had 2 mM Ca^{2+} and 3 mM Mg^{2+} while Deese et al. reported no divalent cations in their buffer system. The significance of this difference was not appreciated by the latter investigators. We have demonstrated that 4 mM Ca^{2+} is sufficient to trigger bilayer to non-bilayer transition. It is expected that another divalent cation (Mg^{2+}) can enhance to some degree the effect of Ca^{2+}.

In conclusion, it appears that rhodopsin and Ca^{2+} interact with the lipids in an antagonistic manner. Rhodopsin stabilizes the bilayer structure and Ca^{2+} destabilizes it. While we observed no gross changes in intact disk membrane structure in the presence of Ca^{2+}, it may have a physiological role in destabilizing microdomains of the bilayer at some point during the visual process. The small (less than 1% of the total lipid) population of lipids in an isotropic environment in the disk membrane with calcium (Fig. 6) may be indicative of such an effect.

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