Strength of Ca^{2+} Binding to Retinal Lipid Membranes: Consequences for Lipid Organization

Daniel Huster,*,† Klaus Arnold,† and Klaus Gawrisch*

*Laboratory of Membrane Biochemistry and Biophysics, NIAAA, National Institutes of Health, Rockville, Maryland 20852 USA, and †Institute of Medical Physics and Biophysics, University of Leipzig, 04103 Leipzig, Germany

ABSTRACT There is evidence that membranes of rod outer segment (ROS) disks are a high-affinity Ca^{2+} binding site. We were interested to see if the high occurrence of sixfold unsaturated docosahexaenoic acid in ROS lipids influences Ca^{2+}-membrane interaction. Ca^{2+} binding to polyunsaturated model membranes that mimic the lipid composition of ROS was studied by microelectrophoresis and ^2H NMR. Ca^{2+} association constants of polyunsaturated membranes were found to be a factor of ~2 smaller than constants of monounsaturated membranes. Furthermore, strength of Ca^{2+} binding to monounsaturated membranes increased with the addition of cholesterol, while binding to polyunsaturated lipids was unaffected. The data suggest that the lipid phosphate groups of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) in PC/PE/PS (4:4:1, mol/mol) are primary targets for Ca^{2+}. Negatively charged serine in PS controls Ca^{2+} binding by lowering the electric surface potential and elevating cation concentration at the membrane/water interface. The influence of hydrocarbon chain unsaturation on Ca^{2+} binding is secondary compared to membrane PS content. Order parameter analysis of individual lipids in the mixture revealed that Ca^{2+} ions did not trigger lateral phase separation of lipid species as long as all lipids remained liquid-crystalline. However, depending on temperature and hydrocarbon chain unsaturation, the lipid with the highest chain melting temperature converted to the gel state, as observed for the monounsaturated phosphatidylethanolamine (PE) in PC/PE/PS (4:4:1, mol/mol) at 25°C.

INTRODUCTION

Ca^{2+} ions act as intracellular messengers that relay information within cells to regulate their activity (Berridge et al., 1998). This applies in particular to the regulation of the photoresponse of retinal rod outer segments (ROSs). Activity of several enzymes in the signal transduction pathway of the visual system is regulated by internal Ca^{2+} concentration (Kawamura, 1992; Hsu and Molday, 1993; Ichikawa, 1994; Koch and Stryer, 1988). Internal Ca^{2+} is tightly controlled by cation channels and Na^+-Ca^{2+}-K^+ exchangers in the ROS plasma membrane (Yau and Nakatani, 1985). The Ca^{2+} concentration in cells is buffered by numerous binding sites. It has been suggested that negative electric charges at the membrane-water interface of ROS discs bind most of the intracellular Ca^{2+} ions (Schnetkamp, 1985). When Ca^{2+} channels open, waves of high Ca^{2+} concentration propagate away from the channel's mouth (Berridge et al., 1998). Most likely, the buffer capacity of ROS membranes for Ca^{2+} is a major factor that determines the velocity of propagation of such Ca^{2+} gradients (Ichikawa, 1996; McLaughlin and Brown, 1981).

Ca^{2+} binding to acidic and zwitterionic phospholipid membranes has been intensively studied (McLaughlin et al., 1981; Ohki et al., 1982; Altenbach and Seelig, 1984; Seelig, 1990). It was established that acidic phospholipid membranes bind Ca^{2+} with high affinity, while Ca^{2+} binding to zwitterionic membranes appears to be weak. An important contributing factor in binding to negatively charged membranes is the increased cation concentration near negatively charged surfaces that can be explained by the Gouy-Chapman theory of the electric double layer (McLaughlin et al., 1981; McLaughlin, 1977). Ca^{2+} binding to zwitterionic lipids is weak because the positive charge of bound Ca^{2+} ions reduces unbound Ca^{2+} concentration near the lipid-water interface. After correction for differences in electric surface potentials, the intrinsic Ca^{2+} binding constant of PC is similar to those of the acidic PS (Seelig, 1990; Altenbach and Seelig, 1984).

ROS disk membranes deviate significantly from other cell membranes in their fatty acid composition. From 20 to 50% of all fatty acids in the phospholipids are docosahexaenoic acid (22:6) (DHA), a long-chain, highly unsaturated ω3 fatty acid that is essential for neural development and function (Fliesler and Anderson, 1983; Salem and Ward, 1993; Salem and Niebylski, 1995). Phospholipid membranes that contain large amounts of polyunsaturated lipids have unique biophysical properties that may enhance or reduce the capability of these lipids to act as binding sites for Ca^{2+}. In particular, polyunsaturated lipids have a larger area per molecule (Holte et al., 1995; Separovic and Gawrisch, 1996; Koenig et al., 1997) and a very different response to the addition of cholesterol (Huster et al., 1998). Furthermore, Ca^{2+} may have different effects on area per molecule in mono- and polyunsaturated lipids. We have reported earlier that the addition of Ca^{2+} ions increases hydrocarbon chain order, equivalent to a reduction in lipid area per

Received for publication 1 October 1999 and in final form 28 February 2000.

Address reprint requests to Dr. Klaus Gawrisch, Laboratory of Membrane Biochemistry and Biophysics, NIAAA, National Institutes of Health, 12420 Parklawn Dr., Rm. 158, Rockville, MD 20852. Tel.: 301-594-3750; Fax: 301-594-0035; E-mail: gawrisch@helix.nih.gov.

0006-3495/00/06/3011/08 $2.00

molecule (Huster et al., 1997). It was suggested that the affinity of divalent cations for PS decreases with increasing lipid chain unsaturation because unsaturation increases area per lipid molecule and thus decreases the surface charge density of the liposomes (Casal et al., 1989).

The three major phospholipids in ROS membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) at a molar ratio of ~4:4:1. The average cholesterol content is ~10 mol% (Fliesler and Anderson, 1983; Albert et al., 1996). In a recent study, we characterized the lateral organization of mono- and polyunsaturated PC/PE/PS (4:4:1, mol/mol) membranes in the presence and absence of cholesterol by NMR. We reported evidence for the existence of short-lived cholesterol/PC microdomains in polyunsaturated PC/PE/PS/cholesterol membranes (Huster et al., 1998). Considering the importance of Ca^{2+} binding to these membranes, it was questioned whether the lateral lipid organization in these membranes is also influenced by Ca^{2+}-lipid interaction. Numerous studies on Ca^{2+}-induced demixing of acidic and zwitterionic phospholipids have been carried out (Hui et al., 1983; Tokutomi et al., 1981; Feigenson, 1989; Coorssen and Rand, 1995; van Dijck et al., 1978; Silvius and Gagne, 1984a,b; Tilcock et al., 1984). It was reported that Ca^{2+} binding segregates the negatively charged PS into gel-phase domains that are depleted of zwitterionic lipids (Coorssen and Rand, 1995; Feigenson, 1989; Silvius and Gagne, 1984a,b; Hauser and Shipley, 1984; Casal et al., 1987). Very little is known about the influence of Ca^{2+} on lateral lipid organization of polyunsaturated membranes. Tilcock et al. suggested that a segregation of PS may not be possible for more unsaturated PS molecules (Tilcock et al., 1984).

In this study, we investigated Ca^{2+} binding to complex mixtures of monounsaturated 18:0–18:1 PC/PE/PS (4:4:1, mol/mol) and polyunsaturated 18:0–22:6 PC/PE/PS (4:4:1) mixtures in the presence and absence of 10 mol% cholesterol. Association constants of Ca^{2+} binding were derived from electric surface potential measurements. The influence of Ca^{2+} binding on lipid packing was investigated by ^2H NMR order parameter measurements. The order parameters of deuterated stearic acid chains in the *sn*-1 position of PC, PE, and PS were determined individually. Changes in chain order were recorded as a function of Ca^{2+} concentration and cholesterol content. Order parameters of phospholipids in the mixture were analyzed for signal superposition and order differences between lipid species. Ca^{2+} binding raises lipid order parameters. The selective increase in chain order for one of the lipids in the mixture would be a reflection of a preferential Ca^{2+} interaction with this lipid and, most likely, demixing of lipid species.

MATERIALS AND METHODS

Materials

The phospholipids 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18:0–18:1 PC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (18:0–18:1 PE), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (18:0–18:1 PS), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0–22:6 PC), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (18:0–22:6 PE), and 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoserine (18:0–22:6 PS) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The subscript d35 denotes a perdeuterated stearic acid chain in the *sn*-1 position. Lipid purity was checked by high-performance liquid chromatography before and after the experiments and was judged to be near 98%. The phospholipids containing DHA were stored in organic solvent at −60°C with butylated hydroxytoluene (BHT) added at a lipid/BHT molar ratio of 250:1 to prevent DHA oxidation.

Sample preparation

DHA-lipid sample preparation was carried out in a Plexiglas glove box under an argon atmosphere. Known quantities of each lipid dissolved in organic solvent were mixed in a glass tube. The organic solvent was removed under a stream of argon. The lipid films were redissolved in ~500 μl cyclohexane (depleted in oxygen by aeration with argon), and the solution was frozen in liquid nitrogen. Samples were freeze dried at a pressure of 50 μm Hg for at least 4 h, resulting in a fluffy lipid powder. Small aliquots of lipid (1 wt%) were dispersed in a buffer solution containing 100 mM NaCl and 10 mM HEPES buffer, adjusted to pH 7.4, and the appropriate amount of Ca^{2+}. To ensure equilibration of calcium concentration inside and outside the multilamellar liposomes, the buffer contained the calcium ionophore A23187 (10 μl of a 2 mg/ml dimethyl sulfoxide solution per ml buffer; see Pressman, 1976; Tilcock et al., 1984, 1988). Samples were equilibrated in 10 freeze-thaw cycles. Suspensions were centrifuged at 27,000 × *g*, and the lipid pellets were transferred to 5-mm glass sample tubes that were sealed with a ground glass joint and cap. We coated the ground glass joint with a thin film of Teflon grease to prevent oxygen from entering the sample, and we secured the cap with a layer of Parafilm.

Microelectrophoresis

Surface potential measurements were performed on a Mark II apparatus from Rank Brothers (Bottisham, UK), using a cylindrical cell. Multilamellar liposome dispersions prepared as described above were diluted to a concentration of 0.01 wt%, using the same buffer. Before the measurements, the cell was flushed thoroughly with the appropriate Ca^{2+}-containing buffer solution to eliminate an influence from Ca^{2+} binding to glass surfaces. Measurements were conducted from low to high Ca^{2+} concentration. The electric field strength along the capillary was measured with a high input resistance electrometer (Keithley, Cleveland, OH). Data points represent the average of backward and forward velocities of 10 particles. The ζ-potentials were calculated using the Helmholtz-Smoluchowski equation (Aveyard and Haydon, 1973):

$$\zeta = \frac{u\eta}{\varepsilon\varepsilon_0}, \tag{1}$$

where u is the electrophoretic mobility, η is the water viscosity, ϵ is the relative permittivity of water, and ϵ_0 is the permittivity of vacuum. All measurements were carried out at 25°C.

^2H NMR spectroscopy

The ^2H NMR spectra were acquired on a Bruker DMX300 spectrometer (Billerica, MA) at a frequency of 46.06 MHz and a spectral width of 200 kHz, using a high-power probe with a 5-mm solenoid coil. A phase-cycled quadrupolar echo pulse sequence was used (Davis et al., 1976), with 2.1-μs

90° pulses, a 50-μs delay between pulses, and a relaxation delay of 0.5 s. DePaked spectra (Sternin et al., 1983) were calculated using the algorithm of McCabe and Wassall (1995). Chain segment order parameters were determined from the relation

$$S(n) = \frac{\Delta\upsilon_Q}{\frac{3}{4}\frac{e^2qQ}{h}}, \quad (2)$$

where e^2qQ/h is the quadrupolar coupling constant (167 kHz for deuterons in the C-^{2}H bond). Smoothed order parameter profiles were computed according to the method of Lafleur et al. (1989). Average order parameters were calculated by adding all order parameters and dividing them by the number of deuterated carbons in the chain. Unless stated otherwise, the NMR measurements were carried out at a temperature of 25°C. All data processing starting from the manipulation of the free induction decay was done with a program written for Mathcad (Mathsoft, Cambridge, MA).

Calculation of area per molecule

The average chain length, $\langle L\rangle$, defined as the projection of the chain on the bilayer normal, is calculated from the average order parameter according to

$$\langle L\rangle = l(0.5 + \langle S\rangle). \quad (3)$$

In Eq. 3, l is the length of an all-*trans* chain calculated from $l = m^*$ 1.27 Å, where m is the number of carbons in the chain and 1.27 Å is the distance between neighboring carbons (Bunn, 1939). The area per lipid is determined from

$$A = \frac{V}{\langle L\rangle} = \frac{V}{l(0.5 + \langle S\rangle)}, \quad (4)$$

where V is the lipid chain volume. The lipid chain volume, V, of mono- and polyunsaturated mixed chain lipids was calculated from data obtained in a combined analysis of x-ray and ^{2}H NMR experiments on pure 18:0–18:1 PC and 18:0–22:6 PC (Koenig et al., 1997). The area per lipid for 18:0–18:1 PC is A = 61.4 Å^2 at $\langle S\rangle$ = 0.158, and for 18:0–22:6 PC it is A = 68.0 Å^2 at $\langle S\rangle$ = 0.149. We assumed that the chain volume, V, is independent of lipid mixing and the presence Ca^{2+} ions and that changes in order parameters of individual lipids reflect changes in area per molecule of these lipids that can be calculated with Eq. 4.

THEORY

Analysis of Ca^{2+} binding to membrane surfaces

The surface charge density of liposomes in the presence of Ca^{2+} was calculated from the measured ζ-potential by the Graham equation:

$$\sigma = \frac{\varepsilon\varepsilon_0\zeta}{\lambda_D}, \quad (5)$$

where λ_D is the Debye length. The surface concentrations of sodium, $[Na]_S$, and calcium, $[Ca]_S$, were calculated according to the Boltzmann distribution law,

$$[Na]_S = [Na]_0\exp(-F\zeta/RT) \quad (6a)$$

$$[Ca]_S = [Ca]_0\exp(-2F\zeta/RT), \quad (6b)$$

where $[Na]_0$ and $[Ca]_0$ are the bulk Na^+ and Ca^{2+} concentrations, F is Faraday's constant, R is the gas constant, and T is the absolute temperature.

For the Na^+ and Ca^{2+} binding analysis, it was assumed that PC, PE, and PS have identical intrinsic association constants for the cations. It was further assumed that Na^+ forms a 1:1 complex with the phospholipid. Na^+ binding reduces the surface concentration of free phospholipid, $\{PL\}_{free}$, that is calculated by the mass action law according to

$$\{PL\}_{free} = \frac{\{PL\}_{total}}{1 + k_{Na}[Na]_S}. \quad (7)$$

Concentrations that are reported in moles per unit of surface are given in curled braces, and concentrations that are reported in moles per liter are given in square brackets. The total area concentration of phospholipid in the membrane, $\{PL\}_{total}$, was calculated from the area per phospholipid molecule according to $\{PL\}_{total} = 1/9(4A_{PC} + 4A_{PE} + A_{PS})$. The calculation of lipid area per molecule, $A_{PC/PE/PS}$, has been described above. For cholesterol-containing samples, an area per cholesterol molecule of 37.7 Å^2 was assumed (Smaby et al., 1997). The Na^+ binding constant, k_{Na}, was determined from ζ-potential measurements in the absence of Ca^{2+}.

For the analysis of divalent Ca^{2+} binding a 1:2 stoichiometry of Ca^{2+}-lipid complexes was assumed. The net surface charge of the lipid bilayer is the sum of the surface charges of free phospholipids, $\{PL\}_{free}$, phospholipid/Na complexes, {PLNa}, and phospholipid/Ca^{2+} complexes $\{(PL)_2Ca\}$ according to

$$\frac{\sigma}{e} = -\frac{\{PL\}_{free}}{9} + \frac{8}{9}\{PLNa\} + \frac{8}{9}\{(PL)_2Ca\}. \quad (8)$$

For simplicity, we assumed that the effective charge of every lipid in the PC/PE/PS (4:4:1, mol/mol) mixture is $-e/9$, where e is the elementary charge. The concentration of Ca^{2+}-lipid complexes is related to the Ca^{2+} association constant according to

$$k_{Ca} = k^*_{Ca}\{PL\}_{total} = \frac{\{(PL)_2Ca\}\{PL\}_{total}}{\{PL\}^2_{free}[Ca]_S}. \quad (9)$$

The total lipid surface concentration is

$$\{PL\}_{total} = \{PL\}_{free} + \{PLNa\} + 2\{(PL)_2Ca\}. \quad (10)$$

The system of Eqs. 5–10 allows the calculation of the Ca^{2+} association constant, k_{Ca}. Calculations were conducted with Mathcad (Mathsoft, Cambridge, MA).

RESULTS

Ca^{2+} binding to PC/PE/PS mixed bilayers

In Fig. 1, the influence of Ca^{2+} binding on the ζ-potential of mixed phospholipid membranes with and without added

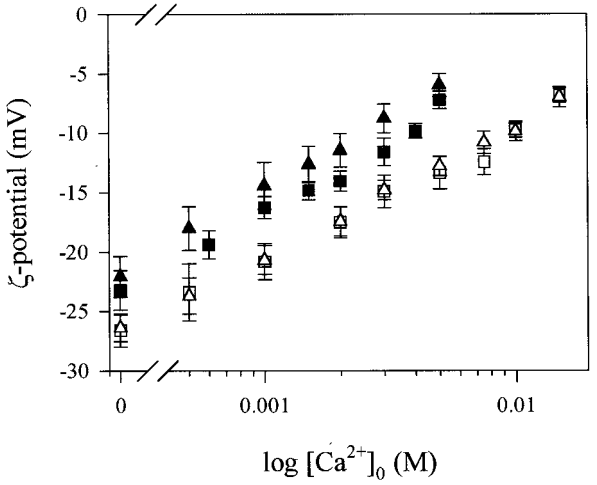

FIGURE 1 Increase in ζ-potentials due to Ca^{2+} binding to 18:0–18:1 PC/PE/PC membranes (4:4:1, mol/mol) (▲, ■) and 18:0–22:6 PC/PE/PS membranes (4:4:1) (△, □) in the presence (▲, △) and absence (■, □) of 10 mol% cholesterol at 25°C.

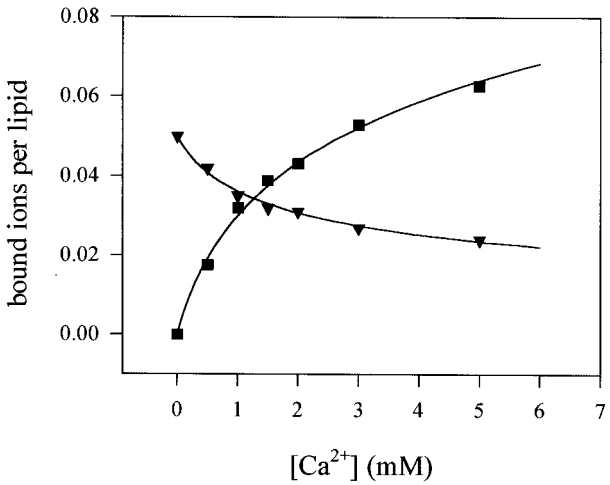

FIGURE 2 Number of bound Na^+ (▼) and Ca^{2+} (■) ions per lipid for monounsaturated 18:0–18:1 PC/PE/PS/cholesterol (4:4:1:1, mol/mol) membranes as a function of bulk Ca^{2+} concentration. Because of the larger Ca^{2+} binding constant and the larger relative increase in Ca^{2+} concentration near the negatively charged surface, the Na^+ ions are forced from the membrane by competing Ca^{2+} ions. Binding isotherms were derived assuming that Na^+ ions form 1:1 phospholipid/Na^+ complexes and Ca^{2+} ions form 2:1 phospholipid/Ca^{2+} complexes.

cholesterol is shown. With increasing Ca^{2+} concentration, the ζ-potential of all membranes becomes less negative. The ζ-potentials of monounsaturated mixtures are lower than those of polyunsaturated mixtures. The addition of 10 mol% cholesterol to the phospholipid mixtures lowered the ζ-potential of monounsaturated lipids but had no measurable influence on the ζ-potential of polyunsaturated lipids.

Data were analyzed in terms of a Na^+ and Ca^{2+} binding constant as explained above. The values are reported in Table 1. Polyunsaturated membranes bind cations with about twofold lower binding constants compared to monounsaturated membranes. The addition of cholesterol to monounsaturated membranes increases Ca^{2+} binding constants but has no measurable effect on binding in the equivalent polyunsaturated mixture. In Fig. 2, isotherms for binding of Na^+ and Ca^{2+} to the monounsaturated lipid mixture in the presence of cholesterol are shown. Increasing Ca^{2+} bulk concentration leads to an increase in the number of bound Ca^{2+} ions, while displacing bound Na^+ ions from the membrane surface. The results for the other lipid mixtures are qualitatively similar.

^{2}H NMR order parameters

We studied lipid chain order of all three phospholipid species in the membrane mixture by individual ^{2}H-labeling of the stearic acid chain in PC, PE, or PS. Fig. 3 shows the ^{2}H NMR powder spectra of PE in mono- and polyunsaturated PC/PE/PS mixtures in the presence and absence of 5 mM Ca^{2+}. ^{2}H NMR spectra of monounsaturated phospholipids show larger quadrupolar splittings than polyunsaturated mixtures, indicating higher lipid chain order.

TABLE 1 Na^+ and Ca^{2+} binding constants at 25°C of mono- and polyunsaturated PC/PE/PS mixtures (4:4:1, mol/mol) in the presence and absence of 10 mol% cholesterol

	k_{Na} (M^{-1})		k_{Ca} (M^{-1})	
Mixture	—	10 mol%	—	10 mol%
18:0–18:1	0.22 ± 0.03	0.22 ± 0.02	8.05 ± 1.04	11.52 ± 0.88
18:0–22:6	0.11 ± 0.01	0.09 ± 0.01	4.26 ± 0.75	4.27 ± 0.70

The addition of Ca^{2+} ions to the polyunsaturated mixture resulted in very small changes in *sn*-1 chain quadrupole splittings (spectra C and D). In contrast, the addition of 5 mM Ca^{2+} to monounsaturated mixtures partly converts the PE into the gel phase at 25°C, as seen from the superposition of the broad gel phase pattern in spectrum B. No such conversion was observed for PC and PS (spectra not shown). Raising the temperature to 30°C converted the gel-phase PE back into the liquid-crystalline state.

^{2}H NMR powder spectra were dePaked (McCabe and Wassall, 1995), and smoothed order parameters for the stearic acid chain were calculated (Lafleur et al., 1989). Fig. 4 shows typical order parameter profiles for mono- and polyunsaturated PC/PE/PS/cholesterol (4:4:1:1) mixtures in the absence (Fig. 4 *A*) and presence (Fig. 4 *B*) of 5 mM calcium. Small order parameter differences between lipid species are observed for monounsaturated mixtures in the presence and absence of Ca^{2+}. The addition of either Ca^{2+} ions or cholesterol raises the hydrocarbon chain order of all three phospholipids.

In polyunsaturated lipid mixtures, order differences are small in the absence of cholesterol. The addition of 10 mol% cholesterol raises the order of 18:0–22:6 PC much more than it does the order of other lipid species (Fig. 4 *A*).

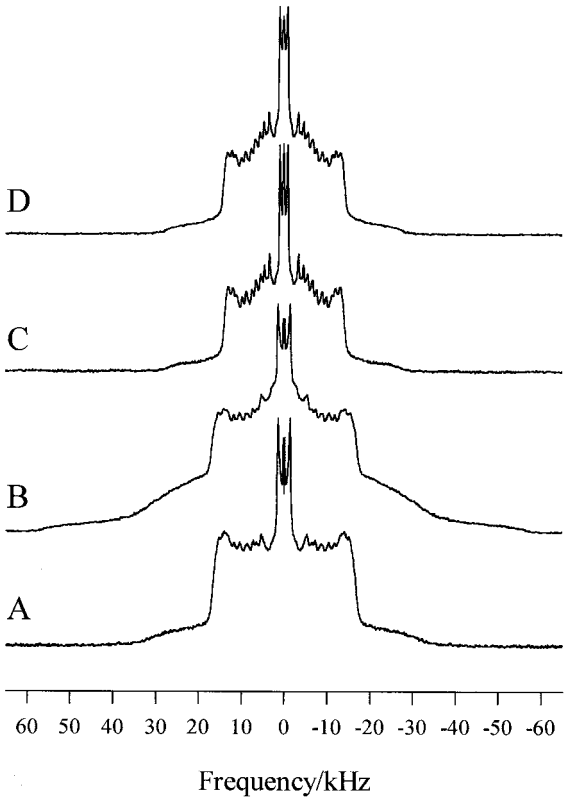

FIGURE 3 ^2H NMR powder spectra of *sn*-1 chain perdeuterated PE_{d35} in mono- and polyunsaturated membrane mixtures of PC/PE_{d35}/PS (4:4:1, mol/mol) at 25°C. (*A*) Monounsaturated mixtures in the absence of Ca^{2+}. (*B*) The addition of 5 mM Ca^{2+} partly converts the monounsaturated PE into the gel phase. (*C*) Polyunsaturated mixtures in the absence of Ca^{2+}. (*D*) Polyunsaturated mixtures in the presence of 5 mM Ca^{2+}.

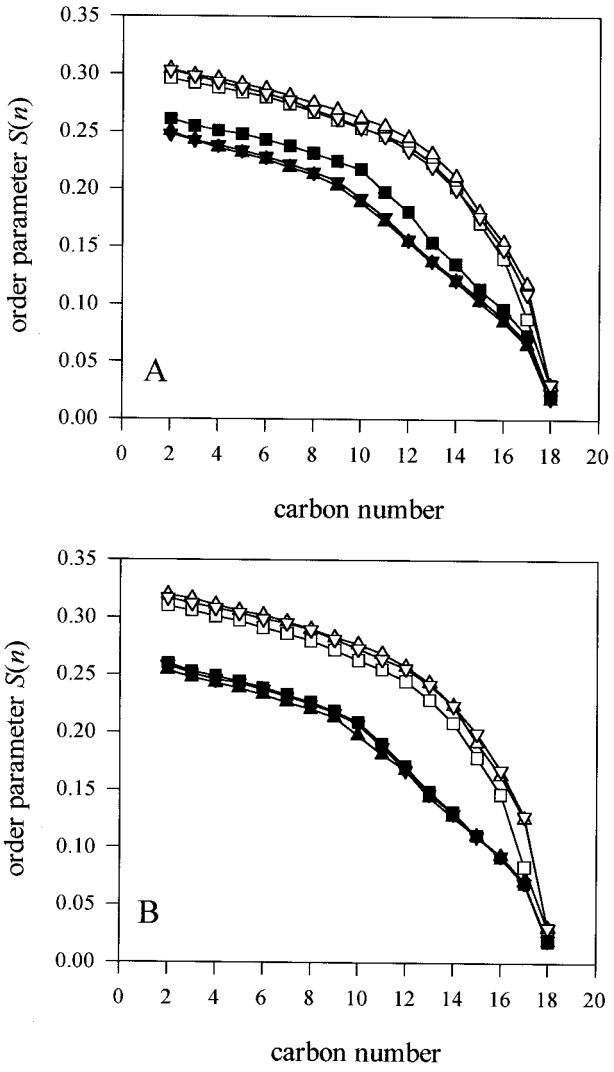

FIGURE 4 Stearic acid ^2H NMR order parameter profiles of 18:0–18:1 PC/PE/PS/cholesterol mixtures (4:4:1:1, mol/mol) (△, ▽, □) and 18:0–22:6 PC/PE/PS/cholesterol mixtures (4:4:1:1) (▲, ▼, ■) in the absence (*A*) and presence (*B*) of 5 mM Ca^{2+}. Order parameter profiles are measured for PC (■, □), PE (▲, △), and PS (▼, ▽) individually. Order parameter differences between lipids in the polyunsaturated mixture are the result of a preferential interaction between cholesterol and PC (Huster et al., 1998). The addition of 5 mM Ca^{2+} ions abolishes this preferential interaction.

After the addition of 5 mM Ca^{2+}, these differences disappear (Fig. 4 *B*). Results of the order parameter investigations on all phospholipid mixtures are summarized in Table 2.

Ca^{2+}- and cholesterol-induced changes in area per lipid molecule

From the average order parameters, the cross-sectional area per lipid molecule in the mixture was calculated according to the procedure described in Materials and Methods. Data are given in Table 3. The area per lipid molecule in the membrane is significantly larger for polyunsaturated phospholipids than for monounsaturated phospholipids. Furthermore, the addition of cholesterol to monounsaturated lipids results in larger area condensation. In monounsaturated lipids, the order increase after the addition of 5 mM Ca^{2+} ions is equivalent to a decrease in area per molecule of ~1 $Å^2$. In contrast, the packing of polyunsaturated mixtures without cholesterol is hardly influenced by Ca^{2+} interaction. In the presence of cholesterol, the addition of Ca^{2+} ions increases the area per molecule of PC by 0.4 $Å^2$ and reduces the area per molecule of PE and PS by 0.5 $Å^2$ and 0.3 $Å^2$, respectively.

DISCUSSION

Calcium binding to mono- and polyunsaturated phospholipids

Ca^{2+} ion binding to membranes is determined primarily by the electrical surface potential and secondarily by the binding affinity of lipids. The negatively charged surface poten-

TABLE 2 Average order parameter* of perdeuterated stearic acid in mono- and polyunsaturated PC/PE/PS mixtures (4:4:1, mol/mol) and PC/PE/PS/cholesterol mixtures (4:4:1:1, mol/mol) in the presence and absence of 5 mM Ca^{2+} at 25°C

Cholesterol	S_{av} —	S_{av} —		S_{av} 10 mol%	S_{av} 10 mol%	
Ca^{2+}	—	5 mM	ΔS_{av}	—	5 mM	ΔS_{av}
18:0–18:1						
PC_{d35}/PE/PS	0.196	0.206	+0.010	0.226	0.234	+0.008
PC/PE_{d35}/PS	0.201/0.182†	gel/0.192†	+0.010†	0.238	0.251	+0.013
PC/PE/PS_{d35}	0.204	0.216	+0.012	0.231	0.242	+0.011
18:0–22:6						
PC_{d35}/PE/PS	0.162	0.163	+0.001	0.186	0.182	−0.004
PC/PE_{d35}/PS	0.159	0.160	+0.001	0.175	0.180	+0.005
PC/PE/PS_{d35}	0.169	0.172	+0.003	0.178	0.181	+0.003

*The experimental error is ±0.002.
†Measurement at 30°C.

tial raises the Ca^{2+} ion concentration near the surface. As a result, Ca^{2+} binding to all lipids in the membrane increases. On the basis of previously published results (Altenbach and Seelig, 1984; Seelig, 1990), we assumed that one Ca^{2+} ion binds two phospholipids, and that binding affinities of zwitterionic and negatively charged phospholipids are identical. The latter assumption is strongly supported by our ^2H NMR order parameter measurements in the presence of Ca^{2+} ions, which indicate an order increase of all phospholipids in the mixture. We propose that Ca^{2+} ions bind primarily to the negatively charged lipid phosphate groups of all phospholipids, independent of their headgroup charge.

Binding constants of cations binding to polyunsaturated phospholipids are a factor of ~2 lower than binding constants of cations binding to monounsaturated phospholipids. Furthermore, the addition of 10 mol% cholesterol to the membranes increases binding constants of cations binding to monounsaturated lipids but has no measurable effect on cation binding to polyunsaturated lipids. These changes in cation binding parallel the influence of polyunsaturation and cholesterol on the area per lipid molecule. The smaller area per phospholipid in monounsaturated mixtures appears to favor Ca^{2+} ion binding, and further reduction of phospholipid area due to the addition of cholesterol increases the binding constant. In contrast, polyunsaturated membranes show much less area reduction after the addition of cholesterol and no measurable change in Ca^{2+} binding.

Binding constants of the monovalent Na^+ ions are more than one order of magnitude smaller than binding constants of the divalent Ca^{2+} ions. Furthermore, because of their larger positive charge, the relative increase in Ca^{2+} ion concentrations near the surface is higher than the corresponding increase in Na^+ concentration. As a consequence, the divalent cations are more successful in binding to the bilayer surface compared to monovalent cations, despite their much lower concentration in the electrolyte solution (see Fig. 2).

Overall, differences in cation binding to mono- and polyunsaturated membranes are much smaller than the differences in binding constants would suggest. The differences are very much attenuated by changes in the electrical surface potential due to cation binding. Stronger binding to phospholipids reduces the negative potential. Consequently, the cation concentration near the membrane decreases, and fewer cations are bound to the membrane surfaces. Therefore, it is fair to state that the content of negatively charged lipids is more important for cation binding than are the calculated differences in cation binding constants.

Influence of Ca^{2+} binding on lateral lipid organization and lipid order

Lateral separation of lipids into gel-phase domains or liquid-crystalline clusters was studied by chain order parame-

TABLE 3 Area per lipid molecule (given in Å²) in mixtures of PC/PE/PS in the presence and absence of 10 mol% cholesterol at 25°C

	A_{PC}	A_{PE}	A_{PS}	A_{PC}	A_{PE}	A_{PS}
Cholesterol	—	—	—	10 mol%	10 mol%	10 mol%
18:0–18:1, no Ca^{2+}	58.0	59.2*	57.4	55.7	54.7	55.3
18:0–18:1, 5 mM Ca^{2+}	57.2	58.4	56.4	55.0	53.8	54.4
18:0–22:6, no Ca^{2+}	67.8	68.2	67.1	65.5	66.5	66.2
18:0–22:6, 5 mM Ca^{2+}	67.7	68.0	66.8	65.9	66.0	65.9

*At 30°C.
The lipid area per molecule was calculated from order parameter changes compared to pure 18:0–18:1 PC (A = 61.4 Å²) and 18:0–22:6 PC (A = 68.0 Å²), as determined from a combined ^2H NMR and x-ray approach (Koenig et al., 1997) (see Materials and Methods for details).

ter analysis of the lipids in the mixture. Formation of gel-phase domains or large liquid-crystalline clusters with diameters much bigger than 50 nm results in signal superposition from the lipids in different environments that is easily detected. When clusters are less than 50 nm in diameter, lipid molecules exchange rapidly between different environments on the NMR time scale, resulting in a single, well-resolved chain order profile for every lipid in the mixture. Formation of significant quantities of smaller clusters is detected when absolute values of chain order parameters between the lipids in the mixture are compared. Lipid order parameters depend heavily on the statistics of next-neighbor interaction between lipid species. The average order parameters of pure lipids with identical hydrocarbon chains but different headgroups deviate to a significant extent (Huster et al., 1998). Typically, membranes that are enriched in PE have the highest lipid order (Separovic and Gawrisch, 1996), with a lower order for PS and an even lower order for PC-enriched membranes (Huster et al., 1998). In homogenous lipid mixtures, lipid hydrocarbon chain orders of all lipid species approach identical values. In clusters the lipids interact preferentially with a limited spectrum of other lipids. As a consequence, the differences in average order parameters between lipids remain large (Huster et al., 1998). Independent confirmation for the link between lipid chain order and lateral organization was obtained by nuclear Overhauser enhancement spectroscopy (Huster et al., 1998).

After the addition of 5 mM Ca^{2+} ions to the monounsaturated PC/PE/PS mixture, part of the zwitterionic PE formed gel-phase domains, while the acidic PS and the zwitterionic PC remained liquid-crystalline. An increase in temperature of just 5°C was sufficient to return the PE to the liquid-crystalline state. In previous investigations, a segregation of the charged phospholipid into gel-phase domains was typically related to the lipid headgroup charge (Hui et al., 1983; Tokutomi et al., 1981; Feigenson, 1989; Coorssen and Rand, 1995; van Dijck et al., 1978; Silvius and Gagne, 1984a,b; Tilcock et al., 1984). In contrast, we propose that the conversion of lipids to the gel state is directly linked to their individual phase transition temperatures. The 18:0–18:1 PE is the phospholipid with the highest phase transition temperature. Ca^{2+} ion binding further increases phase transition temperatures (Chapman et al., 1977) and drives this lipid into gel-phase domains. The observation that Ca^{2+} triggered conversion of PE to the gel phase is further confirmation that Ca^{2+} ions bind to both negatively charged and zwitterionic lipids.

Provided that all lipids stay in the liquid-crystalline phase, there is no indication that the addition of Ca^{2+} ions triggers the formation of lipid clusters. In both mono- and polyunsaturated PC/PE/PS lipid mixtures, differences in hydrocarbon chain order after the addition of Ca^{2+} ions remained very small. In a previous study (Huster et al., 1998), we reported that cholesterol preferentially associates with PC in the polyunsaturated mixture, as seen by nuclear Overhauser enhancement spectroscopy and the preferential increase in PC chain order (see Table 2). The addition of 5 mM Ca^{2+} ions to this mixture effectively removed all differences in lipid chain order, suggesting that Ca^{2+} ion binding eliminates the PC/cholesterol-enriched clusters.

CONCLUSIONS

We found about a twofold weaker binding of Ca^{2+} ions to polyunsaturated phospholipids compared to monounsaturated phospholipids. Furthermore, the addition of 10 mol% cholesterol to monounsaturated lipids increased Ca^{2+} binding constants, while no effect from cholesterol addition was observed in polyunsaturated lipids. The ^2H NMR order parameter studies on PC, PE, and PS in the mixture support a model with the lipid phosphate group as the primary site of attachment for Ca^{2+}. The negative charge of the PS headgroup had no effect on the specific binding of Ca^{2+} ions to PS but was essential for recruiting cations to the lipid-water interface. Overall, cation binding is strongly influenced by the magnitude of the membrane electric surface potential. Differences in Ca^{2+} association constants result in small but significant changes in the amount of bound Ca^{2+}. As long as all lipids remained liquid-crystalline, we did not observe Ca^{2+}-induced lateral demixing of phospholipids. However, the addition of Ca^{2+} ions to the monounsaturated PC/PE/PS mixtures resulted in the partial conversion of PE to the gel state that disappeared after the sample temperature was raised slightly. We conclude that the binding and buffering capacity of ROS membranes is slightly lower than the binding capacity of less unsaturated membranes with a similar composition of phospholipids. However, in practical terms, the differences in the amount of bound Ca^{2+} are primarily determined by the amount of negatively charged lipid in the membrane and not by the differences in binding constants.

DH and KA acknowledge support by the Deutsche Forschungsgesellschaft (SFB 197, A10), and DH is grateful for a grant from the Studienstiftung des deutschen Volkes.

REFERENCES

Albert, A. D., J. E. Young, and P. L. Yeagle. 1996. Rhodopsin-cholesterol interactions in bovine rod outer segment disk membranes. *Biochim. Biophys. Acta.* 1285:47–55.

Altenbach, C., and J. Seelig. 1984. Ca^{2+} binding to phosphatidylcholine bilayers as studied by deuterium magnetic resonance. Evidence for the formation of a Ca^{2+} complex with two phospholipid molecules. *Biochemistry.* 23:3913–3920.

Aveyard, R., and D. A. Haydon. 1973. An Introduction to the Principles of Surface Chemistry. Cambridge University Press, London.

Berridge, M. J., M. D. Bootman, and P. Lipp. 1998. Calcium—a life and death signal. *Nature.* 395:645–648.

Bunn, C. W. 1939. The crystal structure of long-chain paraffin hydrocarbons. *Trans. Faraday Soc.* 35:482–491.

Casal, H. L., H. H. Mantsch, and H. Hauser. 1987. Infrared studies of fully hydrated saturated phosphatidylserine bilayers. Effect of Li^+ and Ca^{2+}. *Biochemistry.* 26:4408–4416.

Casal, H. L., H. H. Mantsch, and H. Hauser. 1989. Infrared and ^{31}P-NMR studies of the interaction of Mg^{2+} with phosphatidylserines: effect of hydrocarbon chain unsaturation. *Biochim. Biophys. Acta.* 982:228–236.

Chapman, D., W. E. Peel, B. Kingston, and T. H. Lilley. 1977. Lipid phase transitions in model biomembranes. The effect of ions on phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 464:260–275.

Coorssen, J. R., and R. P. Rand. 1995. Structural effects of neutral lipids and divalent cation-induced interactions for phosphatidylserine-containing bilayers. *Biophys. J.* 68:1009–1018.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Feigenson, G. W. 1989. Calcium-ion binding between lipid bilayers—the four-component system of phosphatidylserine, phosphatidylcholine, calcium chloride and water. *Biochemistry.* 28:1270–1278.

Fliesler, S. J., and R. E. Anderson. 1983. Chemistry and metabolism of lipids in the vertebrate retina. *Prog. Lipid Res.* 22:79–131.

Hauser, H., and G. G. Shipley. 1984. Interactions of divalent cations with phosphatidylserine bilayer membranes. *Biochemistry.* 23:34–41.

Holte, L. L., S. A. Peter, T. M. Sinnwell, and K. Gawrisch. 1995. ^2H NMR order parameter profiles suggest a change of molecular shape for phosphatidylcholines containing a polyunsaturated acyl chain. *Biophys. J.* 68:2396–2403.

Hsu, Y. T., and R. S. Molday. 1993. Modulation of the CGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature.* 361:76–79.

Hui, S. W., L. T. Boni, T. P. Stewart, and T. Isac. 1983. Identification of phosphatidylserine and phosphatidylcholine in calcium-induced phase separated domains. *Biochemistry.* 22:3511–3516.

Huster, D., K. Arnold, and K. Gawrisch. 1998. Influence of docosahexaenoic acid and cholesterol on lateral lipid organization in phospholipid mixtures. *Biochemistry.* 37:17299–17308.

Huster, D., A. J. Jin, K. Arnold, and K. Gawrisch. 1997. Water permeability of polyunsaturated lipid membranes measured by O-17 NMR. *Biophys. J.* 73:855–864.

Ichikawa, K. 1994. Critical processes which characterize the photocurrent of retinal rod outer segments to flash stimuli. *Neurosci. Res.* 19: 201–212.

Ichikawa, K. 1996. Modeling and analysis of spatio-temporal change in $[Ca^{2+}](i)$ in a retinal rod outer segment. *Neurosci. Res.* 25:137–144.

Kawamura, S. 1992. Light-sensitivity modulating protein in frog rods. *Photochem. Photobiol.* 56:1173–1180.

Koch, K. W., and L. Stryer. 1988. Highly cooperative feedback-control of retinal rod guanylate-cyclase by calcium-ions. *Nature.* 334:64–66.

Koenig, B. W., H. H. Strey, and K. Gawrisch. 1997. Membrane lateral compressibility determined by NMR and x-ray diffraction: effect of acyl chain polyunsaturation. *Biophys. J.* 73:1954–1966.

Lafleur, M., B. Fine, E. Sternin, P. R. Cullis, and M. Bloom. 1989. Smoothed orientational order profile of lipid bilayers by ^2H NMR. *Biophys. J.* 56:1037–1041.

McCabe, M. A., and S. R. Wassall. 1995. Fast-Fourier-transform dePaking. *J. Magn. Reson. B.* 106:80–82.

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.

McLaughlin, S., and J. Brown. 1981. Diffusion of calcium ions in retinal rods. A theoretical calculation. *J. Gen. Physiol.* 77:475–487.

McLaughlin, S., N. Mulrine, T. Gresalfi, G. Vaio, and A. McLaughlin. 1981. Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* 77:445–473.

Ohki, S., N. Duzgunes, and K. Leonards. 1982. Phospholipid vesicle aggregation: effect of monovalent and divalent ions. *Biochemistry.* 21: 2127–2133.

Pressman, B. C. 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* 45:501–530.

Salem, N., Jr., and C. D. Niebylski. 1995. The nervous-system has an absolute molecular species requirement of proper function. *Mol. Membr. Biol.* 12:131–134.

Salem, N., Jr., and G. R. Ward. 1993. Are omega-3 fatty acids essential nutrients for mammals? *World Rev. Nutr. Diet.* 72:128–147.

Schnetkamp, P. P. 1985. Ca^{2+} buffer sites in intact bovine rod outer segments: introduction to a novel optical probe to measure ionic permeabilities in suspensions of small particles. *J. Membr. Biol.* 88: 249–262.

Seelig, J. 1990. Interaction of phospholipids with Ca^{2+} ions—on the role of the phospholipid head groups. *Cell Biol. Int. Rep.* 14:353–360.

Separovic, F., and K. Gawrisch. 1996. Effect on unsaturation on the chain order of phosphatidylcholines in a dioleoylphosphatidylethanolamine matrix. *Biophys. J.* 71:274–282.

Silvius, J. R., and J. Gagne. 1984a. Calcium-induced fusion and lateral phase separations in phosphatidylcholine-phosphatidylserine vesicles. Correlation by calorimetric and fusion measurements. *Biochemistry.* 23:3241–3247.

Silvius, J. R., and J. Gagne. 1984b. Lipid phase behavior and calcium-induced fusion of phosphatidylethanolamine-phosphatidylserine vesicles. Calorimetric fusion studies. *Biochemistry.* 23:3232–3240.

Smaby, J. M., M. M. Momsen, H. L. Brockman, and R. E. Brown. 1997. Phosphatidylcholine acyl unsaturation modulates the decrease in interfacial elasticity induced by cholesterol. *Biophys. J.* 73:1492–1505.

Sternin, E., M. Bloom, and A. L. MacKay. 1983. De-Pake-ing of NMR spectra. *J. Magn. Reson.* 55:274–282.

Tilcock, C. P. S., M. B. Bally, S. B. Farren, P. R. Cullis, and S. M. Gruner. 1984. Cation-dependent segregation phenomena and phase behavior in model membrane systems containing phosphatidylserine: influence of cholesterol and acyl chain composition. *Biochemistry.* 23:2696–2703.

Tilcock, C. P. S., P. R. Cullis, and S. M. Gruner. 1988. Calcium-induced phase separation phenomena in multicomponent unsaturated lipid mixtures. *Biochemistry.* 27:1415–1420.

Tokutomi, S., R. Lew, and S. Ohnishi. 1981. Ca^{2+}-induced phase separation in phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine mixed membranes. *Biochim. Biophys. Acta.* 643:276–282.

van Dijck, P. W., B. de Kruijff, A. J. Verkleij, L. L. van Deenen, and J. de Gier. 1978. Comparative studies on the effects of pH and Ca^{2+} on bilayers of various negatively charged phospholipids and their mixtures with phosphatidylcholine. *Biochim. Biophys. Acta.* 512:84–96.

Yau, K. W., and K. Nakatani. 1985. Light-induced reduction of cytoplasmic free calcium in retinal rod outer segment. *Nature.* 313:579–582.