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Biochimica et Biophysica Acta 1414 (1998) 140–154

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

^{113}Cd-, ^{31}P-NMR and fluorescence polarization studies of cadmium(II) interactions with phospholipids in model membranes

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Received 11 March 1998; received in revised form 19 August 1998; accepted 19 August 1998

Abstract

Cadmium(II) interactions with multilamellar vesicles of dimyristoyl (DM)- and dipalmitoyl (DP)-phosphatidylcholine (PC), -phosphatidylserine (PS), -phosphatidic acid (PA), -phosphatidylglycerol (PG) and -phosphatidylethanolamine (PE) have been investigated both from the metal and the membrane viewpoints, respectively, by solution ^{113}Cd-NMR and diphenylhexatriene fluorescence polarization coupled with solid-state ^{31}P-NMR. Results can be summarized as follows. (1) Strong cadmium binding to membrane phospholipids results in a decrease of the free Cd(II) ^{113}Cd-NMR isotropic signal and because of slow exchange, in the NMR time scale, between free and bound cadmium pools, the lipid/water partition coefficients, K_{lw}, of the Cd(II) species can be determined in the lamellar gel (fluid) phase. It is found $K_{lw}(\text{DMPC}) \approx K_{lw}(\text{EggPE}) \approx 2 \pm 2$ (2 ± 2); $K_{lw}(\text{DMPA}) = 392 \pm 20$ (505 ± 25); $K_{lw}(\text{DMPG}) = 428 \pm 21$ (352 ± 17); $K_{lw}(\text{DMPS}) = 544 \pm 27$ (672 ± 34). Cadmium interactions with membrane phospholipids are therefore electrostatic in nature and the phosphate moiety is proposed as a potential binding site. (2) The presence of Cd(II) stabilizes the gel phases of PG, PA and PS lipids and leads to suppression of the main phase transition for PA and PS. These effects are reduced upon increasing salinity to 0.5 M Cl^- and abolished at 1.8 M Cl^-, Cd(II) being removed from the membranes due to formation of soluble $CdCl_n$ species. Moving the pH from 7 to 6 also decreases Cd(II) binding to PA, because of surface charge reduction. (3) Cadmium promotes the formation of isotropic ^{31}P-NMR lines with PG systems and of a hexagonal phase on egg PE bilayers at 24°C, suggesting dramatic membrane reorganization. Properties of cadmium and calcium interacting with phospholipid model membranes are compared, and the potential roles of these interactions in the molecular mechanisms of cadmium uptake and toxicity in cells are discussed.

Keywords: Cadmium; Toxicity; Phospholipid membrane; Nuclear magnetic resonance; Fluorescence polarization; Partition coefficient

Abbreviations: DM, dimyristoyl-; DP, dipalmitoyl-; Cd(II), inorganic cadmium; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DPH, diphenylhexatriene; P, fluorescence polarization of DPH; T_m, lamellar gel-to-lamellar fluid phase transition temperature; R_i, lipid-to-cadmium molar ratio; pCl, $-\log[Cl^-]$; MLV, multilamellar vesicles; δ, isotropic NMR chemical shift; $\Delta\sigma$, NMR chemical shielding anisotropy; K_{lw}, lipid/water partition coefficient; MES, morpholino-ethane sulfonic acid; MOPS, morpholino-propane sulfonic acid; BBPS, bovine brain PS

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00162-X

1. Introduction

Cadmium is a nutritionally non-essential metal of increasing importance in environmental monitoring. Its toxicity to humans is subject to controversies and open outbreaks remain limited to the Itaï-Itaï disease [1]. However, there is no doubt that cadmium is amongst the most toxic of metals to aquatic biota and is a serious hazard to environmental health [2].

Cadmium has high affinity for proteinic thiol groups but also induces membrane damage (leakage, stiffening) that probably results from strong interactions with lipids [3–5]. Besides, the mechanism of cadmium uptake is often considered to involve electrostatic adsorption of ionic Cd^{2+} at the membrane surface, followed by protein-mediated internalization [6]. Acidic phospholipids have thus been proposed as Cd^{2+} binding sites [7]. Although Cd(II) interactions with membrane lipids have never been systematically investigated, binding to PS-containing artificial bilayers was reported [8,9] and low-affinity Cd–DPPC complexation has been demonstrated [10]. Using ^{31}P-NMR on vesicles or micelles of phospholipids extracted from intestinal brush border membrane, Tacnet et al. observed specific Cd(II) binding to anionic PI and PS, but not to PC, PE or sphingomyelin [11].

To gain knowledge in cadmium–membrane interactions, we have probed by ^{113}Cd-NMR the binding of Cd(II) to phospholipids in model membranes (MLV), depending on the phospholipid headgroup and on lipid-to-metal molar ratio. Fluorescence polarization using the standard DPH probe and ^{31}P-NMR were performed simultaneously, to investigate Cd(II)-induced membrane structural changes both at the core and headgroup levels, respectively. The effects of temperature, pH and salinity on these interactions were also explored, since these factors may greatly affect cadmium chemical speciation in the external medium, bioavailability to biological barriers, uptake and toxicity effects in the environment.

2. Materials and methods

2.1. Materials

Cadmium nitrate $Cd(NO_3)_2$, morpholino-ethane sulfonic acid (MES) and morpholino-propane sulfonic acid (MOPS) were obtained from Sigma (St. Louis, USA). DMPC, DMPS, DMPG, DPPS and DPPG were from Avanti Polar Lipids (Alabaster, USA), egg PE (EPE) and bovine brain PS (BBPS) from Lipid Products (South Nutfield, UK), DPPA and DMPA from Bachem (Bubendorf, Switzerland) and DMPE from Fluka (Buchs, Switzerland). Egg yolk PC was prepared according to the method of Singleton et al. [12]. Diphenylhexatriene (DPH) was obtained from Aldrich (Milwaukee, USA). Deionized water was used for buffer preparation. All other compounds were high purity reagents from Prolabo (France). Possible phospholipid degradation was checked by thin-layer chromatography (TLC) after completion of experiments. No significant hydrolysis was detected (data not shown).

2.2. Sample preparation

To obtain multilamellar vesicles, phospholipids were dispersed (50 mM) in MES (50 mM, pH 6.0) or MOPS (50 mM, pH 7.0) by several freeze–thaw–heating cycles and vortex stirring. For NMR experiments, pH values were chosen so that each anionic phospholipid bears only one negative charge (i.e., DMPA, DMPG: pH 6.0; DMPS: pH 7.0). Note that bilayer structure is not significantly modified by the one-unit pH difference [13]. PE and PC are zwitterionic at both pHs. MES and MOPS buffers have a similar structure, only differing by one additional $-CH_2-$unit in the MOPS side chain that results in pK_a of 6.0 and 7.0, respectively. We selected these buffers because they do not form complexes with Cd(II), as shown by preliminary ^{113}Cd-NMR experiments (see Section 3).

To form (1:1) DMPE/EPC MLV, DMPE and egg PC were first mixed together in $CHCl_3$ and stirred, then solvent was evaporated under gentle N_2 flow and the phospholipids were dispersed in MES as above.

For NMR experiments, variable amounts of MLV solutions (0 to 1500 μl) were added to 37.5 or 75 μl of $Cd(NO_3)_2$ from a stock aqueous solution (1 M), then samples were completed to 1.5 ml with appropriate buffer and stirred ([Cd(II)] = 25 or 50 mM, molar ratio R_i = [lipid]/[Cd] ranging from 0 to 2). An external cadmium reference (sealed glass tube, see NMR data) was added to samples for ^{113}Cd-

NMR only. Samples pH was checked before NMR acquisition and after 4 h delay.

Assuming that nitrate ions, hydroxide ions and buffers are the only Cd(II) ligands to be considered in solution, chemical speciation models based on available thermodynamic data predict that the Cd^{2+} species accounts for at least 90% of total Cd(II) under our experimental conditions (pH = 6.0 to 7.0). The remaining 10% correspond to the $CdNO_3^+$ species. Upon chloride addition, a mixture of $CdCl_n$ complexes (with $n = 1$–4) is formed. In the absence of phospholipids, the following distributions can be calculated using the MINEQL+2.23 chemical speciation program [14,15]:

- KCl = 0.5 M: Cd^{2+} 3.3%, $CdCl^+$ 35.6%, $CdCl_2$ 35.3%, $CdCl_3^-$ 11.1%, $CdCl_4^{2-}$ 14.6%
- NaCl = 0.8 M: Cd^{2+} 1.6%, $CdCl^+$ 24.6%, $CdCl_2$ 33.5%, $CdCl_3^-$ 14.4%, $CdCl_4^{2-}$ 25.7%
- NaCl = 1.8 M: $CdCl^+$ 4.7%, $CdCl_2$ 17.2%, $CdCl_3^-$ 17.8%, $CdCl_4^{2-}$ 60.3%

Note that KCl (0.5 M) instead of NaCl was employed in fluorescence polarization studies because high Na^+ concentrations induced large perturbations of the thermotropic behavior of phospholipid multilamellar vesicles in dilute solutions (0.2 mM, see below).

2.3. Fluorescence polarization

Stock dispersions of phospholipid multilamellar vesicles were prepared in adequate buffer by vortexing the lipid (6 mM) and the fluorescent probe DPH (1% vol. from a stock solution of 6 mM in tetrahydrofuran), to a final DPH/lipid molar ratio of 0.01. These dispersions were let equilibrate to allow THF evaporation from the solution. Aliquots were then diluted in buffer ([PL] = 0.2 mM) and enclosed in 10-mm wide quartz cells. DPH fluorescence polarization (P) was measured as previously described [16], as a function of sample temperature (±0.1°C), in the absence and presence of Cd(II) (R_i = [lipid]/[Cd] = 2 or 0.5). A 4-h delay before data acquisition was kept in the presence of cadmium, to allow system equilibration. Sample cooling was operated manually, and cooling rate was about 10°C/h. Each data point was the average of three separate measurements. Note that DPH steady-state fluorescence is unaffected by Cd(II) [7]. To study the influence of salinity on Cd–lipid interactions, samples and controls were also recorded in MES or MOPS buffers (0.5 M KCl).

2.4. NMR

^{113}Cd-NMR and ^{31}P-NMR spectra were recorded using 10-mm diameter NMR tubes on a Bruker ARX 300 spectrometer operating in unlocked mode. ^{31}P-NMR was performed at 121.5 MHz with the Hahn-echo sequence [17] under ^{1}H spin–lock decoupling conditions [18]. Experimental parameters were: 50 kHz spectral window, 1000–2800 scans, 9 μs $\pi/2$ pulses, 6 s recycle time and 30 μs delay between pulses to form the echo. A Lorentzian line broadening of 100 Hz was applied before Fourier transformation. ^{31}P-NMR chemical shifts are expressed relative to 85% H_3PO_4, and chemical shift anisotropies ($\Delta\sigma$) are given with a 3 ppm accuracy. ^{113}Cd-NMR spectra were acquired at 66.6 MHz using the single pulse acquisition sequence with gated broadband proton decoupling, in the presence of an external reference ($Cd(NO_3)_2$ 1 M in water, 3 mm diameter, vol. 100 μl, $\delta = -11.5$ ppm) for calibration. A T_1 value of 7.8 s was determined for 50 mM $Cd(NO_3)_2$ in water using an impulsion–recovery sequence (data not shown). Typical experimental parameters were: 20 kHz window, 100 or 360 scans (depending on Cd concentration), 15 μs $\pi/2$ pulses and 40 s recycle time. A 25 Hz line broadening was applied before Fourier transformation. ^{113}Cd-NMR chemical shifts are expressed relative to 0.1 M $Cd(ClO_4)_2$, with 1 ppm accuracy. Peak area integration was performed using Bruker standard software. Reference peak area was used to calculate sample mole number and concentration, with 10% accuracy.

To explore the consequences of the membrane physical state (gel or fluid) on lipid–cadmium interactions, NMR experiments were performed at two temperatures: 24°C and 5°C above the main transition temperature of the Cd(II)–lipid system, as determined by fluorescence polarization. Salinity effects were also explored: NaCl (0.8 M and 1.8 M) was added to Cd–DMPG, Cd–DMPA and Cd–DMPS samples at various R_i, and ^{113}Cd-NMR spectra were recorded at 24°C.

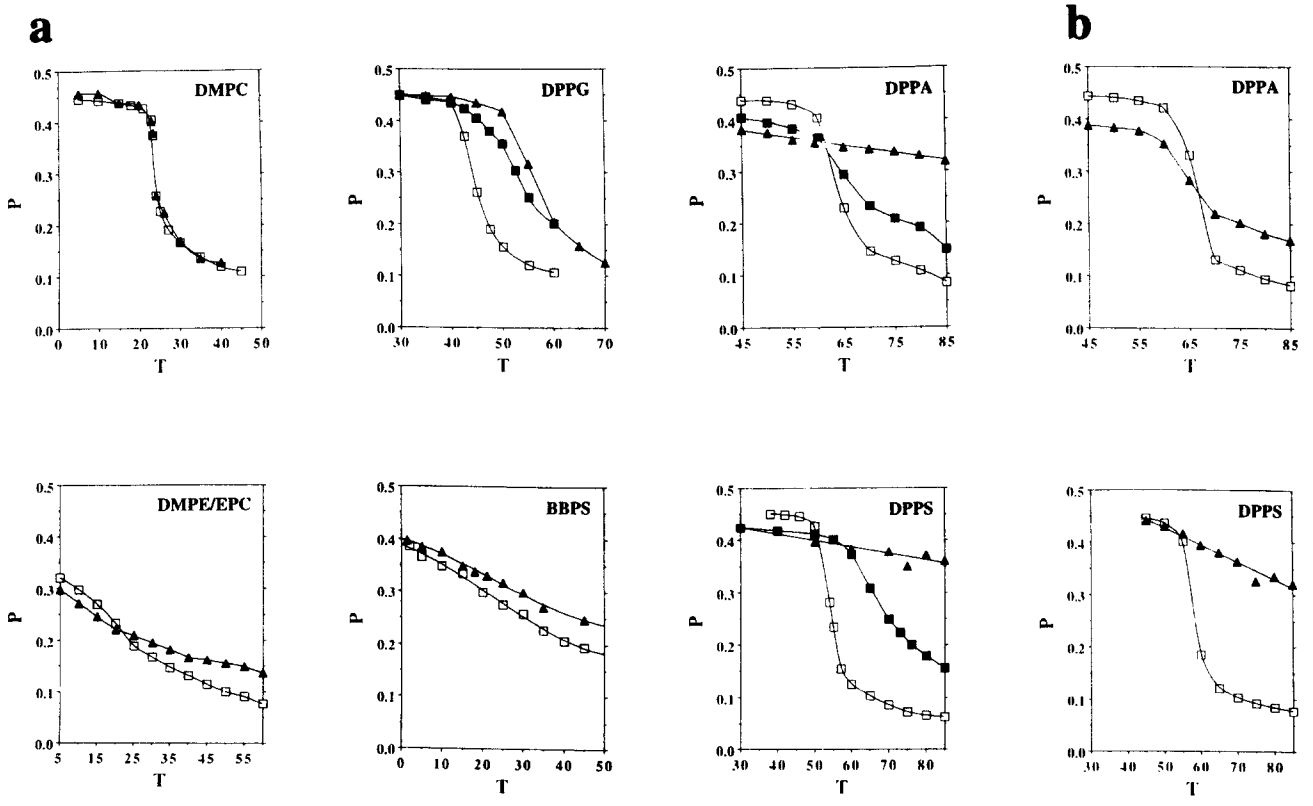

Fig. 1. Fluorescence polarization (P) of DPH (60 μM) embedded in multilamellar vesicles of phospholipids (6 mM in MES or MOPS buffers, pH 6 or 7, [KCl] = 0) as a function of temperature (T in °C), in the absence (□) and presence of Cd(II), at $R_i = 0.5$ (▲) or 2 (■). Experimental conditions: (a) pH 7.0, (b) pH 6.0. Nature of phospholipid is indicated on graph. Solid lines are drawn to help reading the figure. $\lambda_{excitation} = 360$ nm, $\lambda_{emission} = 445$ nm.

3. Results

3.1. Fluorescence polarization

Fluorescence polarization of DPH embedded in phospholipid MLV was recorded as a function of temperature and in the absence and presence of Cd(II) ($R_i = 2$ or 0.5), Fig. 1. The polarization, P, of DPH embedded in a lipid bilayer is related to the membrane physical state: P values are high in the gel-crystalline phase and low in the fluid phase [19]. Thermotropic variations therefore allow determination of the gel-to-fluid phase transition temperature (T_m) of the membrane, depending on composition, experimental conditions and the presence of Cd(II).

Table 1
Main phase transition temperatures (T_m) of phospholipid multilamellar vesicles in the absence and presence of cadmium

	DMPC	DMPE/EPC	DPPG	DPPA	DPPS	BBPS
Controls	23.5	22.0	44.0	63.0	54.0	18.0
Controls+Cd ($R_i = 2$)	23.5	–	52.5	64.0	66.0	–
Controls+Cd ($R_i = 0.5$)	23.5	>	57.5	>	>	25.0
Controls+KCl	–	22.0	43.0	65.5	58.0	19.0
Controls+Cd ($R_i = 0.5$)+KCl	–	23.0	45.5	66.0	>	19.0

T_m values are given in degrees Celsius, with a 1°C accuracy. They are estimated from the fluorescence polarization measurements of 60 μM DPH embedded in 6 mM MLV, in the absence and presence of Cd(II). KCl is added at 0.5 M total concentration.
–, No data; >, phase transition no longer detectable.

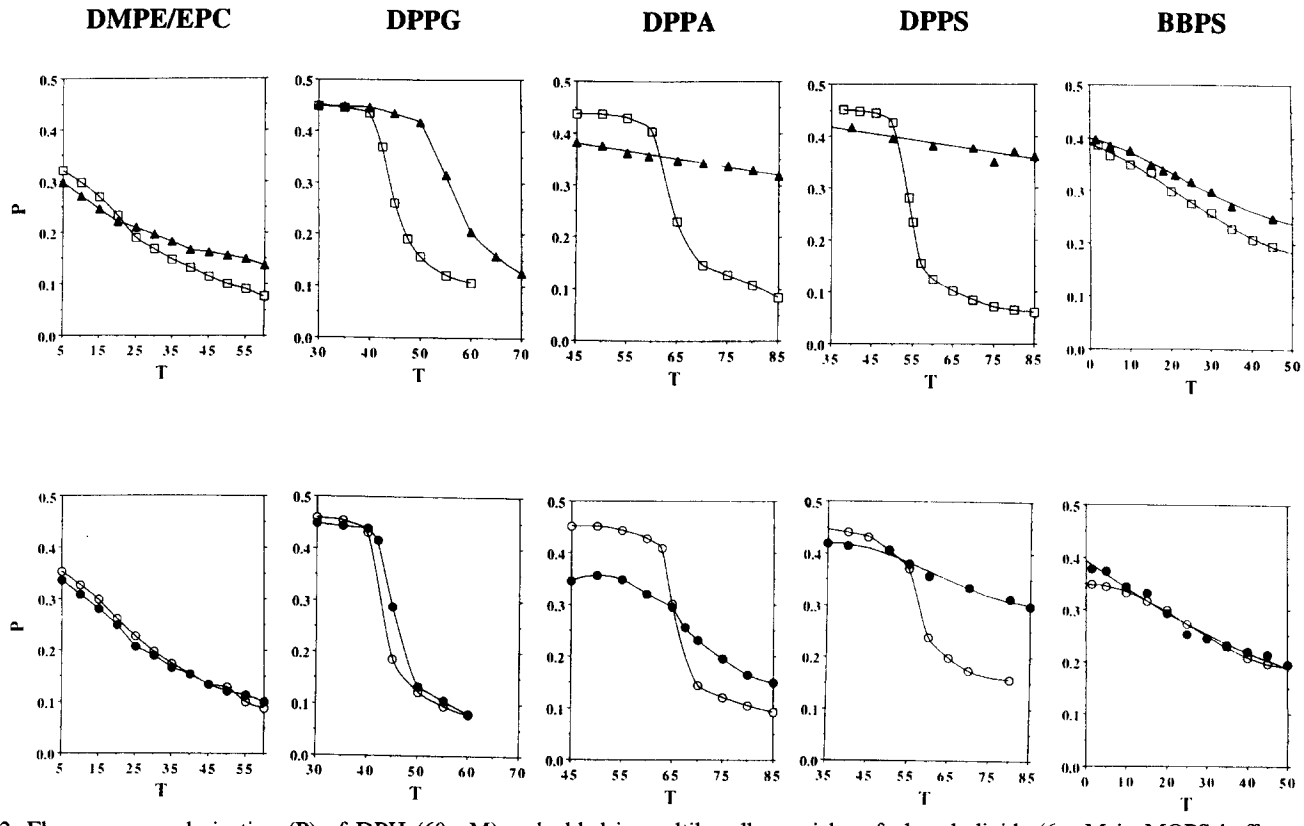

Fig. 2. Fluorescence polarization (P) of DPH (60 μM) embedded in multilamellar vesicles of phospholipids (6 mM in MOPS buffers, pH 7) as a function of temperature (T in °C), in the absence (□, ○) and presence of Cd(II) at $R_i = 2$ (▲, ●). Experimental conditions: (a) [KCl] = 0, (b) [KCl] = 0.5 M. Nature of phospholipid is indicated on graph. Solid lines are drawn to help reading the figure. $\lambda_{excitation} = 360$ nm, $\lambda_{emission} = 445$ nm.

Cd^{2+} addition has no significant effect on DMPC P values, whatever the R_i. However, at pH 7.0 and $R_i = 0.5$, it increases the polarization of DPH for all other phospholipids in their fluid phase in the order: DMPE/EPC ≈ BBPS < DPPS (Fig. 1a). With DMPE/EPC, DPPA and DPPS, a slight decrease of P in the gel phase is also observed. Decreasing R_i from 2 to 0.5 does not change the P increase of DPPG bilayers, but results in very high P values, up to 85°C, for DPPA and DPPS. Main phase transition temperatures T_m as observed by DPH fluorescence polarization are reported in Table 1. Values for pure systems are in good agreement with literature data [20]. It is remarkable that cadmium induces a T_m increase for DPPG, DPPS and BBPS vesicles. No significant T_m changes are detected for DMPC and DPPA. However, excess Cd^{2+} ($R_i = 0.5$) completely suppresses the gel-to-fluid phase transition of DPPA, DPPS and DMPE/EPC MLV at pH 7.0 (Fig. 1a). Cadmium effects on DPH fluorescence in DPPA bilayers are markedly reduced upon decreasing the pH to 6.0 (Fig. 1b). The gel-to-fluid phase transition remains detectable and P values are close to those obtained at $R_i = 2$, pH 7.0. Interactions of Cd(II) with DPPG (not shown) and DPPS are barely affected by a pH decrease to 6.0.

The influence of salinity on the cadmium–lipid interactions is shown in Fig. 2. Upon addition of 0.5 M KCl (Fig. 2b), a reduction of Cd(II) effects is observed on DPH fluorescence polarization. P and T_m values are the same for controls and in the presence of Cd^{2+} ($R_i = 0.5$) for DMPE/EPC, BBPS and DPPG bilayers, except for a slight T_m increase with DPPG (Fig. 2b and Table 1). The gel-to-fluid phase transition of DPPA is now observable and P values are close to those obtained at $R_i = 2$ in the absence of KCl. Cd(II) effects on DPPS are nearly unaffected by the presence of KCl and the gel-to-fluid phase transition is not recovered, despite a slight decrease in P values in the fluid phase compared to Fig. 2a.

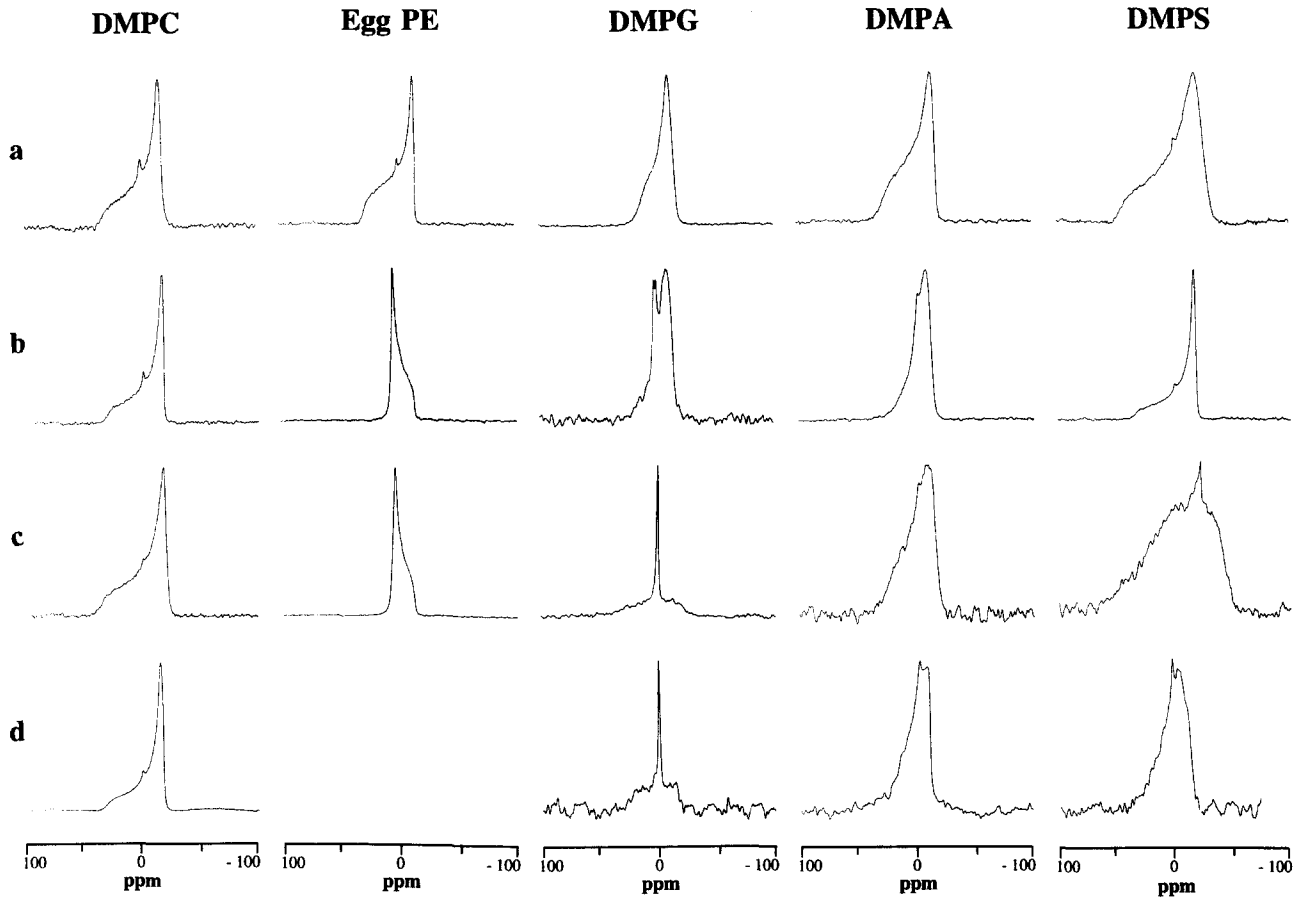

Fig. 3. Solid state ^{31}P-NMR spectra of phospholipid multilamellar vesicles, in the absence and presence of Cd(II) ($R_i = 2$). The two upper rows spectra are controls: (a) $T = 24$°C, (b) +5°C above T_m of the cadmium–lipid systems, i.e., 35°C for DMPC, 60°C for DMPG and 70°C for DMPA and DMPS. For egg PE, temperature at line b is 40°C, i.e., such that the system is in the hexagonal H_{II} (the lamellar-to-hexagonal phase transition occurs at 35°C). The two lower rows display spectra after Cd(II) addition and equilibration: (c) $T = 24$°C, (d) +5°C above T_m. Chemical shifts are expressed relative to 85% H_3PO_4.

3.2. ^{31}P-NMR

^{31}P-NMR powder spectra of MLV in the absence and presence of Cd(II) ($R_i = 2$) are given in Fig. 3. Spectra for controls at 24°C show well-defined, axially symmetric powder pattern lineshapes characteristic of the lamellar fluid (DMPC, EPE) or gel (DMPG, DMPA, DMPS) phases (Fig. 3a). Controls at high temperatures all are in the lamellar fluid phase, except for EPE which forms a hexagonal H_{II} phase above 35°C (Fig. 3b). Almost all control spectra show some percents of an isotropic component, probably corresponding to the formation of micelles or small unilamellar vesicles [21]. For DMPG another minor spectral component appears, possibly related to H_{II} phase formation [22].

At 24°C, lineshape changes occur within one hour following Cd^{2+} addition, except for DMPA where one must wait a few hours to record reproducible ^{31}P-NMR spectra (Fig. 3c). No lineshape changes were detected for DMPC in the presence of cadmium, up to $R_i = 2$. Cd^{2+} addition to EPE MLV induces a transition to the H_{II} phase. A spectral broadening is observed for DMPG, DMPA and especially DMPS. An isotropic line accounts for more than 50% of the DMPG spectra, revealing extensive lipid phase restructuration. A minor isotropic component is also observed with DMPS. DMPG and DMPS samples immediately lay down in the NMR tubes upon Cd(II) addition, forming a solid precipitate. The same is observed with DMPA after a few hours, but to a lesser extent. Increasing the temperature greatly reduces the broadening of DMPC, DMPA and DMPS spectra (Fig. 3d). Cd(II)–EPE systems

remain in the hexagonal H_{II} phase (not shown), while the isotropic line still dominates the DMPG spectrum.

Chemical shift anisotropies ($\Delta\sigma$) were measured between low-field shoulder and high-field peak of powder spectra, and are reported in Table 2. As expected for pure systems, $\Delta\sigma$ values are lower for lipids in fluid phase than in gel phase. Upon Cd(II) addition at 24°C, one observes a significant $\Delta\sigma$ increase for DMPG (powder pattern part) and DMPS spectra, while $\Delta\sigma$ is unaffected for DMPC and DMPA bilayers and is reduced for EPE (as a result of the lamellar fluid-to-hexagonal phase transition). The cadmium-induced $\Delta\sigma$ increase is cancelled in the fluid phase for DMPS and reduced for DMPG. A very broad component is detected on the DMPA spectra at high temperature, which is possibly linked to a precipitate formation.

3.3. ^{113}Cd-NMR

In preliminary studies we monitored the effect of nature of buffer used, cadmium and chlorine concentrations on the ^{113}Cd-NMR isotropic chemical shift (δ_{obs}). Addition of a Tris buffer caused significant changes in the Cd(II) isotropic chemical shift, revealing formation of soluble Cd(II)–Tris species, while increasing concentrations of MES, Hepes and MOPS (up to 0.25 M, at constant pH) induced no chemical shift changes (data not shown). Addition of phosphate buffer resulted in the formation of a precipitate and therefore to the concomitant disappearance of the isotropic NMR line. We thus conclude that MES, Hepes and MOPS do not form complexes with aqueous cadmium under our experimental conditions: they do not modify the free metal ion concentration and are therefore adequate for Cd(II) speciation studies, unlike Tris or phosphate buffers. Increasing progressively the concentration of cadmium in the solution (as $Cd(NO_3)_2$ or $CdSO_4$) resulted in a linear downfield shift of δ_{obs}, easily accounted for by the formation of the nitrated or sulfated cadmium species of chemical shift lower than that of free Cd^{2+} in solution (Fig. 4, top). Further experiments were then conducted with a fixed Cd concentration of 50 mM $Cd(NO_3)_2$ in MOPS buffer (pH 7.0). At 24°C, increasing the concentration of NaCl resulted in the progressive formation of $CdCl_n$ complexes which shifts the ^{113}Cd-NMR isotropic signal upfield by more than 200 ppm at 3 M NaCl (Fig. 4, bottom).

Phospholipids multilamellar vesicles were added to $Cd(NO_3)_2$ (50 mM), both at 24°C and 5°C above T_m of the cadmium–lipid systems (R_i from 0 to 2.0). Three general effects were observed.

(i) The Cd(II) isotropic chemical shift and linewidth ($\Delta\nu_{1/2}$) remained nearly constant during all experiments conducted in the presence of phospholipids, for a given set of pH, salinity, cadmium concentration and temperature (data not shown). Maximal δ_{obs} changes are within 2 ppm.

(ii) The ^{113}Cd-NMR isotropic signal progressively disappeared upon addition of acidic phospholipids (Fig. 5). At 24°C, addition of DMPG, DMPA or DMPS resulted in a complete loss of signal at R_i=1.7, 1.85 and 1.35, respectively. The peak area is not significantly affected by DMPC or EPE addition, up to R_i=2. The NMR peak areas varied quasi-linearly with R_i. Increasing the temperature above

Table 2
^{31}P-NMR chemical shift anisotropy ($\Delta\sigma$) values of phospholipid multilamellar vesicles in the presence and absence of cadmium

	DMPC	EPE	DMPA	DMPS	DMPG
Controls (24°C)	49	39	43	59	31
Controls (5°C above T_m)	42	$18_{(H_{II})}$	28	47	30
Controls+Cd (24°C)	49	$19_{(H_{II})}$	43	77	49
Controls+Cd (5°C above T_m)	43	n.d.	28	46	42

Chemical shift anisotropy values are measured on powder spectra in ppm, with a 3 ppm accuracy. Cd(II) was added at $R_i = 2$. All lipids are in the gel phase at 24°C, except for DMPC whose T_m is about 24°C. Fluid phase temperatures (5°C above T_m at $R_i = 2$) are 35°C for DMPC, 60°C for DMPG, and 70°C for DMPA and DMPS. EPE controls at high temperatures are recorded at 40°C, that is, in the hexagonal H_{II} phase since the lamellar-to-hexagonal phase transition occurs at 35°C.
n.d., not done.

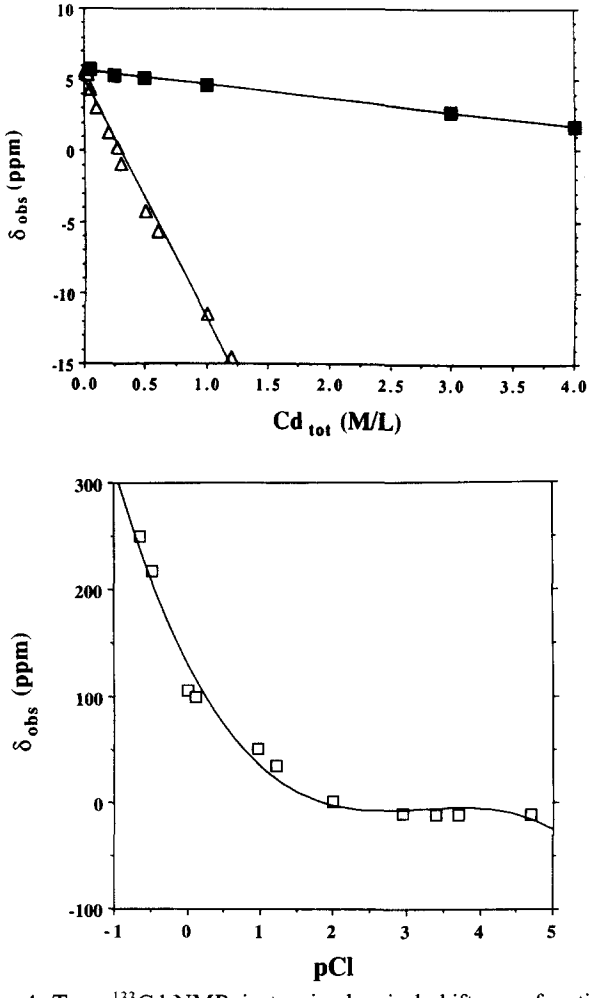

Fig. 4. Top: ^{133}Cd-NMR isotropic chemical shift as a function of total Cd concentration (in mol/l). (□) $Cd(NO_3)_2$ or (■) $CdSO_4$ dissolved in MOPS buffer, pH 7, $T=24$°C. Bottom: ^{133}Cd-NMR isotropic chemical shift of 50 mM $Cd(NO_3)_2$, as a function of $pCl=-\log[Cl^-]$, $T=24$°C. Salinity is modified by NaCl addition to $Cd(NO_3)_2$ in MOPS buffer at pH 7. ^{113}Cd-NMR chemical shifts are expressed relative to 0.1 M $Cd(ClO_4)_2$, with a 1 ppm accuracy.

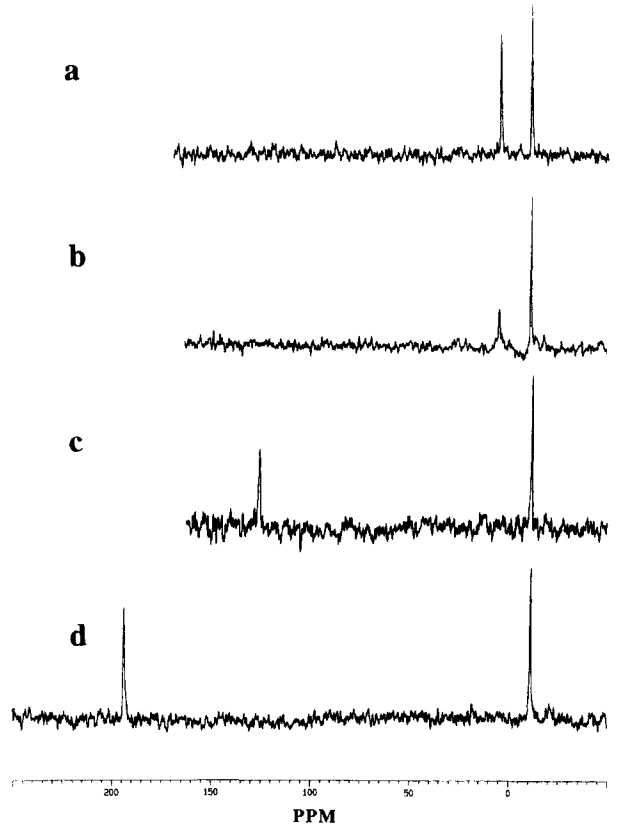

Fig. 5. ^{133}Cd-NMR spectra of a Cd(II)–DMPG sample in MES buffer (pH 6) as a function of NaCl concentration in the presence of an external reference ($Cd(NO_3)_2$ 1 M in water, right-hand-side line, $\delta=-11.5$ ppm). DMPG-to-cadmium molar ratio is 0.7 and temperature 24°C. Spectrum a is a lipid- and salt-free control, (b) +DMPG and NaCl = 0, (c) +DMPG and NaCl = 0.8 M, (d) +DMPG and NaCl = 1.8 M. Corresponding peak areas and chemical shift values are reported in Table 3. ^{113}Cd-NMR chemical shifts are expressed relative to 0.1 M $Cd(ClO_4)_2$.

Table 3
Salinity effects on the ^{113}Cd-NMR parameters of phospholipid–Cd(II) interactions

	Control[a]		Control+0.8 M NaCl		Control+1.8 M NaCl	
	δ^b_{obs}	peak area[c]	δ_{obs}	peak area	δ_{obs}	peak area
DMPG–Cd ($R_i=0.7$)	+4.5	59%	+125.5	70%	+193.0	100%
DMPG–Cd ($R_i=1.66$)	n.m.	0%	+107.0	69%	+199.0	100%
DMPA–Cd ($R_i=0.75$)	+4.0	59%	+103.0	58%	+173.0	100%
DMPS–Cd ($R_i=0.7$)	+4.0	34%	+104.0	26%	+159.0	52%

n.m., not measurable (no peak).

[a]Control is 50 mM $Cd(NO_3)_2$ in buffer+phospholipid (DMPG and DMPA at pH 6.0 and DMPS at pH 7.0). In the absence of phospholipid, δ_{obs} is +4.0 and +4.5 ppm, at pH 6 and 7, respectively.

[b]δ_{obs} is the ^{113}Cd-NMR isotropic chemical shift expressed relative to 0.1 M $Cd(ClO_4)_2$, with a 1 ppm accuracy.

[c]Peak area is given in percentage compared to control (100%), with 10% accuracy.

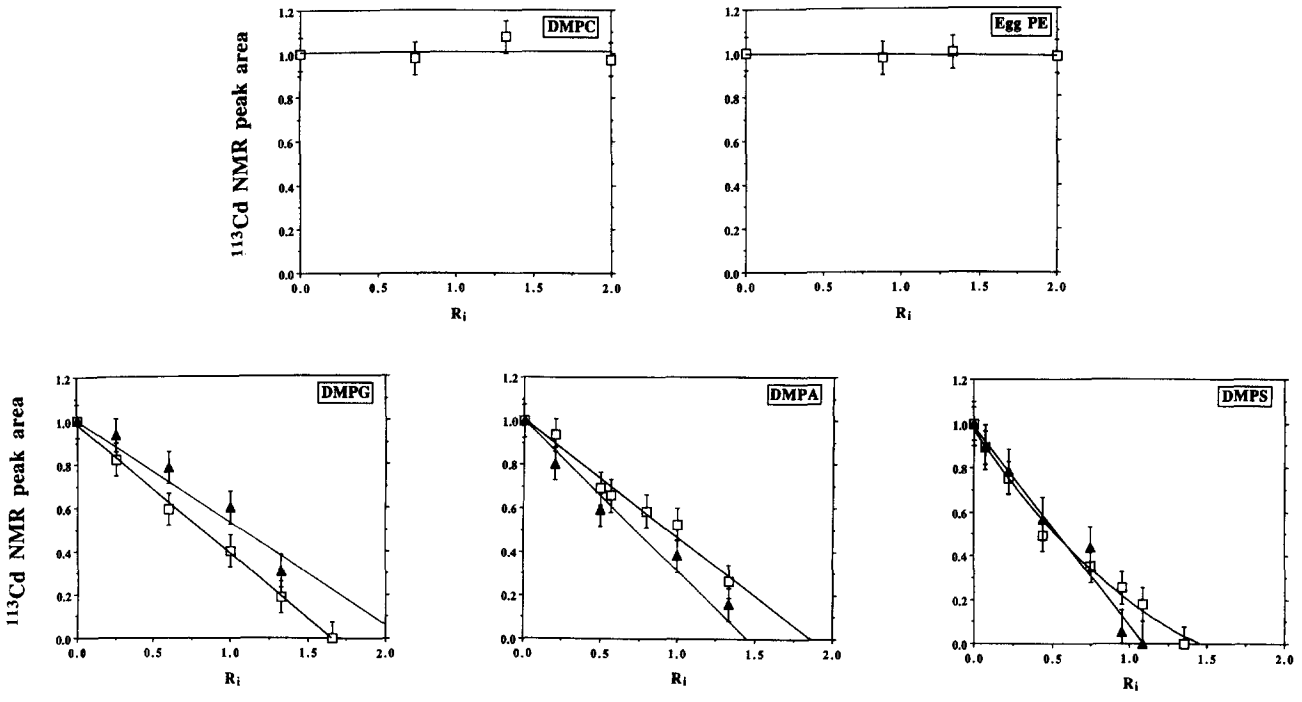

Fig. 6. ^{113}Cd-NMR peak area decrease of 50 mM $Cd(NO_3)_2$, as a function of lipid-to-metal molar ratio (R_i). Control area is normalized to 1 at $R_i = 0$. Nature of added phospholipid is indicated on graph. Cd(II) peak area decrease was measured by comparing with an external reference of constant peak area. Temperature is 24°C (□) or +5°C above the T_m of the lipid–cadmium system (▲), as determined by DPH fluorescence polarization, i.e., 60°C for DMPG and 70°C for DMPA and DMPS. Data points are the means of 2–4 independent measurements, giving an average 10% accuracy on peak areas. Solid lines are drawn to help reading the figure.

the T_m of the lipid–cadmium system slightly reduced the effect of DMPG on Cd(II) isotropic signal area, but enhanced the DMPA and DMPS effect, resulting in a loss of detectable signal at $R_i = 1.45$ and 1.1, respectively. A high temperature did not change the DMPC and EPE binding curves (data not shown).

(iii) Upon NaCl addition (0.8 M or 1.8 M) to the Cd–DMPG, Cd–DMPA and Cd–DMPS samples, the isotropic signal rises again and is shifted upfield, as shown in Fig. 6. Peak area recovery follows the order: DMPG > DMPA > DMPS at a given NaCl concentration (Table 3).

4. Discussion

In this study, we investigated the interactions between cadmium and model membranes by probing both the metal and the phospholipids. This dual monitoring approach yields information on the nature, stoichiometry and binding parameters of the metal–lipid complexes, as well as on the cadmium-induced membrane perturbations. On the one hand, ^{113}Cd-NMR quantitatively describes Cd(II) mobility and complexation, both in solution and at the membrane interface, without inducing perturbations of thermodynamic equilibrium conditions. On the other hand, fluorescence polarization reveals structural changes at the level of the hydrophobic chains of the phospholipid bilayers, whereas ^{31}P-NMR is indicative of the phosphate headgroup mobility at the interface and allows detection of non-bilayer phases [23]. All of these parameters will be discussed below.

4.1. Cadmium binding to membranes

Three readily observable experimental parameters on the ^{113}Cd-NMR spectra are sensitive to the nature of Cd(II) interactions with phospholipid bilayers.

(i) The isotropic line chemical shift (δ_{obs}) is quite sensitive to cadmium complexation in the aqueous phase and exchange with the bound species [24]. When the exchange rate with various ligands is fast in the NMR time scale (i.e., exchange rate greater

than 1 kHz), δ_{obs} is the weighted mean of the individual chemical species δ values. Changes in relative concentrations of cadmium soluble complexes thus result in chemical shift changes of several ppm and are therefore easily detectable [24–26].

(ii) The isotropic signal area is proportional to the amount of cadmium in solution. This is simply related to the fact that the ^{113}Cd-NMR chemical shift anisotropy in solids is on the order of kHz wide [27]. The signal of cadmium bound to slowly tumbling large lipid assemblies, such as MLV, is therefore broadened beyond detection under high-resolution ^{113}Cd-NMR conditions, resulting in a decrease in peak area of the isotropic signal, which allows quantitative determination of bound cadmium.

(iii) Finally, line broadening of NMR signals is indicative of restricted mobility of the observed nucleus, or may arise from intermediate exchange rate between the observed signals.

In our experiments, no significant line broadening of the isotropic signal was observed in the presence of phospholipids, and the δ_{obs} values were nearly constant at any R_i (maximal shifts are of 2 ppm). Bound and free cadmium pools are therefore in slow exchange in the NMR time scale (exchange rate $\ll 1$ kHz), indicating the formation of stable complexes of Cd(II) with phospholipid headgroups, which exhibit long lifetimes. This is unusual, since Cd(II) is a labile ligand and most ^{113}Cd-NMR studies of cadmium binding to proteins show fast or intermediate exchange rates [28]. These results also demonstrate that free Cd(II) remains mostly uncomplexed in the bulk solution as Cd^{2+}. Kinetics of cadmium binding to bilayers is directly proportional to the initial rates of peak area decrease shown in Fig. 4. At 24°C; i.e., near or in the gel phase, DMPC $\approx$ EPE $\ll$ DMPA $<$ DMPS whereas in the fluid phase they rank as DMPC $\approx$ EPE $\ll$ DMPG $<$ DMPS. On going through the gel-to-fluid phase transition, cadmium binding to DMPG decreases (Fig. 5). Therefore, increased membrane fluidity frees Cd(II) from PG bilayers or reduces Cd(II) interactions with PG, possibly by favoring formation of (1:2) $Cd(DMPG)_2$ versus (1:1) Cd(DMPG), as suggested from the disappearance of the ^{113}Cd-NMR signal for R_i values between 1.0 and 2.0 (Fig. 5), indicating the formation of (1:1) and (1:2) cadmium–phospholipid complexes. This is coherent with increased phosphate mobility, as shown by ^{31}P-NMR (see below). Conversely, PA bilayers bind more cadmium in the fluid phase. Such a phase dependence of PA binding could result from a deeper inclusion of Cd(II) in fluid bilayers, i.e., close to the beginning of the acyl chains.

Metal partitioning into a membrane can be defined by the bound-to-free metal ratio [29,31]. Assuming that 10 mM phospholipids in water occupy about 1% of total sample volume and using the ^{113}Cd-NMR peak area decreases, we can calculate K_{lw} values for Cd(II)–phospholipid systems:

$$K_{lw} = (\text{watervol./lipidvol.}) \times ([Cd(II)]_{bound}/[Cd(II)]_{free})$$

Bound-to-free molar ratio is estimated from the Cd(II) peak area at saturation or peak disappearance depending on the shape of curves in Fig. 5 (accuracy $\pm 5\%$). It must be mentioned that in the case of DMPC and EPE only rough estimates can be given because the ^{113}Cd-NMR line never disappears in our range of R_i. This leads to the following results: at 24°C, $K_{lw}(DMPC) \approx K_{lw}(EPE) \approx 2 \pm 2$; $K_{lw}(DMPA) = 392 \pm 20$; $K_{lw}(DMPG) = 428 \pm 21$; $K_{lw}(DMPS) = 544 \pm 27$. In the fluid phase one finds $K_{lw}(DMPC) \approx K_{lw}(EPE) \approx 2 \pm 2$; $K_{lw}(DMPG) = 352 \pm 17$; $K_{lw}(DMPA) = 505 \pm 25$; $K_{lw}(DMPS) = 672 \pm 34$. This affinity order is the same as that obtained by measuring the initial rates of binding (vide supra).

Obviously, Cd(II) binds strongly to the negatively charged phospholipids and shows no apparent affinity to the zwitterionic ones, within the sensitivity limits of the method. These results are in agreement with various studies demonstrating selective Cd(II) binding to acidic phospholipids, but not to neutral PC and PE [8–10]. However, large structural changes of DMPE/EPC and EPE bilayers have been observed in the presence of Cd(II) (see Section 3), and both the Cd–glycerophosphocholine and Cd–DPPC (1:1) complexes have been previously described [10,30]. Consequently, cadmium interacts to some extent with PC and PE. Under the conditions prevailing in our experiments, less than 10% of total Cd is bound to PC or PE, so that the affinity constants of these complexes are very low compared to those formed with anionic phospholipids. Cd(II) has a high

affinity to inorganic phosphate, thus forming the solid $Cd_3(PO_4)_2$ complex [14]. The phosphate group therefore stands as the most likely Cd-binding site in phospholipids (and is indeed the only possible site in PA).

NaCl addition to Cd–lipid samples promoted a release of bound cadmium and leads to the formation of chloride complexes, yielding an isotropic signal upfield, compared to the controls (Fig. 6 and Table 3). In this respect, Fig. 4, bottom, is a calibration curve of the δ_{obs} shift induced by NaCl in the absence of phospholipids. Our curve is in agreement with the results of similar experiments performed by Drakenberg et al. [25]. It is noteworthy that Ackerman et al. [26] found higher δ_{obs} values for corresponding chloride concentrations. Because they employed a higher ionic strength (4.5 M), their values probably reflected Cd(II) complexation with both chloride and other, undetermined anions. Close inspection of Table 3 reveals that some δ_{obs} values, in the presence of NaCl, are significantly higher or lower than those expected for free $CdCl_n$ complexes in solution, as shown in Fig. 4, bottom ($\delta_{obs} = +100$ ppm for [NaCl] = 0.8 M and $\delta_{obs} = +172$ ppm for [NaCl] = 1.8 M, respectively). This is especially the case for Cd–DMPG ($R_i = 0.7$) and Cd–DMPS ($R_i = 0.7$). Some phospholipids therefore affect both the speciation of 'free' cadmium and the formation of chloride complexes, either directly (by forming soluble complexes with Cd, though no such complexes were detected in the absence of Cl^-) or indirectly, by binding $CdCl_n$ complexes as well as Cd^{2+}. It has indeed been reported that the choline phosphate group binds $CdCl_2$ and not the ionic species [30]. At 0.8 M NaCl, Cd removal from DMPG MLV is 70% complete, while no release was detected from DMPA or DMPS. At 1.8 M, complete removal was observed from DMPG and DMPA, while only 50% of the total cadmium were freed from DMPS. The order of Cd(II) affinities for phospholipid headgroups can thus be confirmed from these salting out experiments: PS ≫ PA > PG. Two molecular mechanisms may contribute to the NaCl effect: chloride competition with phospholipids for Cd(II) binding or sodium competition with Cd(II) for phospholipid binding. NaCl-induced cadmium release from DMPG appears not to depend on R_i, which favors the Cd-speciation hypothesis, but these two mechanisms are not mutually exclusive. The uptake and toxicity of cadmium to aquatic organisms are known to decrease with increasing water salinity and decreasing activity of the free Cd^{2+} ion [31–34]. Because cadmium adsorption at the membrane surface is commonly described as the first step of Cd uptake by live cells [6], it is tempting to ascribe this toxicity decrease obtained in vivo to the cadmium release from acidic phospholipid bilayers observed in our experiments at high salinity.

4.2. *Consequences of cadmium binding on membranes structure*

Cd(II) binding has drastic effects on the thermotropism of acidic phospholipids (Fig. 1). This is in agreement with earlier works reporting that cadmium decreases the fluidity of PS-containing PC membranes [8,9], of erythrocyte ghosts [7] and of whole cell membranes and tissues [3,35]. Cd(II) also forms solid phases with DMPA [36] and fatty acids [37]. The large T_m increase of DMPG in the presence of Cd was therefore predictable. The disappearance of the main phase transition of pure DMPA and DMPS bilayers, somewhere between $R_i = 2$ and 0.5, is more remarkable but not unexpected since cadmium effects are very similar to those induced by calcium on negatively charged phospholipids: Ca^{2+} binds to the lipid phosphate and is able to bridge several headgroups, forming cylindrical lamellar phases (organized somewhat like myelin sheets) with very high T_m values due to dehydration of the polar heads [38]. For instance, the $Ca(PS)_2$ cochleate has a T_m of 155°C, effectively masking the phase transition [39], $Ca(PG)_2$ melts at 70°C [40] and calcium affinities to DMPA are intermediate, exhibiting the same pH dependency as Cd–PA interaction [41]. The Ca–lipid affinity order is exactly the same as observed for Cd(II), suggesting that formation of (1:1) or (1:2) Cd–phospholipid cochleate phases could account for the observed fluorescence polarization results. The decrease of P values in the gel phase observed for PA and PS is specific of cadmium, however. Interestingly, inorganic mercury has a similar effect on P values of DPH embedded in phospholipids, but with different headgroup specificity [16].

The ^{31}P-NMR spectra of Ca and Cd complexes with phospholipids are also very different: $\Delta\sigma$ varia-

tions reveal changes either in the phosphorus motions or in the headgroup tilt angle relative to the normal to the bilayer [18,42]. The phosphate group is completely immobilized when bound to calcium at $R_i = 0.5$, yielding very broad spectra (170 ppm wide) that can only be detected using ^1H cross-polarization techniques to enhance sensitivity. Only the PG spectrum remains unaffected at lower R_i [40]. The cadmium-induced $\Delta\sigma$ increases of PG and especially PS membranes at 24°C (Table 3) are probably related to reduced headgroup mobility and general membrane fluidity decrease, as revealed by fluorescence polarization, due to steric hindrance caused by direct phosphate–Cd(II) binding. However, $\Delta\sigma$ values are smaller than those observed in the presence of calcium and spectra are easily detectable with a standard Hahn-echo sequence. Also, $\Delta\sigma$ of DMPA is unchanged, despite the strong bilayer rigidification, so individual phosphorus motions may be greater in Cd–lipid phases than in Ca complexes. Besides, broadening of PA and PS spectra reflect a decrease of the lipid motions that are slow in the NMR time scale (collective motions and lateral diffusion [18]). Possible mechanisms involved could include bridging of adjacent phospholipids and/or charge neutralization at the membrane surface by ionic Cd^{2+} (resulting in increased headgroup packing), depending on the nature of the interactions, i.e., covalent or purely electrostatic. Unlike Ca, Cd is indeed considered as a relatively 'soft' cation in Pearson's classification, with a tendency to form electrostatic bonds with oxygen-donor ligands, including phosphate [43]. Stronger covalent character of interactions could explain that Ca establishes tighter bonds with lipid headgroups than does Cd(II), in spite of similar effects on membrane fluidity at the chains level. Above T_m, cadmium-induced changes on $\Delta\sigma$ disappear or are reduced, possibly because increased phospholipid mobility suppresses contact between adjacent headgroups and related Cd(II) steric effects.

Besides modifications of membrane fluidity, ^{31}P-NMR isotropic line is also observed upon cadmium addition to DMPG. Isotropic lines account for the presence of micelles or small vesicles ($\varnothing < 500$ Å), with rotational correlation times (τ) less than the nanosecond [21], and reveal membrane destructuration in the presence of Cd(II).

Cadmium-induced hexagonal phase formation on EPE at 24°C is especially interesting on a toxicological point of view, since the H_{II} phase is supposed to be a necessary intermediate during membrane fusion, exo- and endocytose, and for lipid turnover [44]. Hexagonal phase formation in vivo would be a possible mechanism to explain blebbing and leakage of contents observed upon Cd addition to lipid vesicles [8]. It is noteworthy that weak Ca–PE interactions generally do not result in H_{II} phase formation, though this has been observed with EPE following Ca-induced PE–PS phase separation [45].

KCl induces a reversal of the cadmium-induced rigidification of phospholipid bilayers, as shown by fluorescence polarization. This can be logically attributed to release of bound cadmium upon formation of $CdCl_n$ complexes. However, one notices that at 0.8M NaCl, cadmium release is only 70% from DMPG and 0% from DMPA or DMPS. KCl effects can thus only be explained by the formation of ternary (1:1:1) $CdCl^+$–lipid complexes and concomitant increase of the membrane charge surface (or breaking of the lipid–Cd–lipid bridges, if considering the covalent binding hypothesis). Note that $CdCl^+$ would be the dominant Cd(II) species at this salinity in the absence of phospholipids. At 0.5 M KCl, Cd effects on bilayer fluidity are near completely cancelled for DPPG, markedly reduced for DPPA and mostly unchanged for PS, confirming once more the affinity order PS ≫ PA > PG for Cd(II) binding.

pH increase from 6.0 to 7.0 also reduces cadmium effects on DPPA fluidity (Fig. 1). This can result of reduced binding, due to partial neutralization of the phosphate group and decrease of the charge density at the membrane surface. It is specific of the PA headgroup since PG and PS do not possess ionizable functions with pK_a between 6.0 and 7.0. This observation confirms the basically electrostatic nature of the Cd–PA interactions. Interestingly, decreasing pH values from 7.5–8.0 to 5.0–6.0 also reduced cadmium uptake in vivo, and competition with H^+ for the Cd binding sites has been evoked to interpret these results [46,47].

4.3. *Possible toxicological implications of the cadmium–phospholipid interactions*

Cadmium displays various toxicological properties at the cell level, but the biomembrane seems to be a

primary site of action. Besides its rigidification effects, Cd(II) disrupts lipid distribution between membrane leaflets, causes leakage of cellular contents at micromolar concentrations [7,9] and induces endodermic tissue necrosis in kidneys and gills [4,5]. Binding to surface proteic thiols may also occur but cannot be the only rationale of such extended membrane structural damages. Complexation to PE or acidic phospholipids and consequent formation of rigid or non-bilayer phases, together with increased lipid degradation [11], could be obvious mechanisms to account for Cd(II) toxicity at the membrane level. The lipid composition of biomembranes is modified upon prolonged exposition to cadmium, with an increase of PE and a decrease of PS and sterols. This could correspond to a defensive adaptation of organisms, both to reduce Cd binding (PS decrease) and to maintain membrane fluidity in the presence of Cd (sterols decrease) [48].

Cadmium also interferes with the physiological calcium cycle, most certainly because the two ions share many chemical properties and compete with each other for binding sites, resulting at the cellular level in the inhibition of calcium-binding proteins and in perturbations of the calcium functions as a second messenger [49]. Among these Ca-regulated properties, the cellular apoptosis induction is of critical importance. At higher integration levels, decalcification and bone malformations were observed upon cadmium exposure [50]. Ca and Cd competition for phospholipids certainly is to play a role in these toxicity events, given the apparently higher cadmium affinity for acidic lipid sites.

The first step of cadmium uptake into cells is adsorption at the membrane surface and was described as electrostatic binding on the lipid headgroups, which is coherent with our results. Then a limited part of the bound cadmium pool is translocated across the bilayer through many possible pathways, including protein-mediated transport. Non-saturable cadmium transport has also been described in several cell types and was attributed to free diffusion of uncharged Cd(II) species. However, the diffusion coefficient of neutral $CdCl_2$ through lipid bilayers is very low, due to high polarity of the Cd–Cl bond [51]. Depending on membrane charge and composition, mechanisms of interfacial electrostatic attraction and binding of cationic Cd(II) species, followed by translocation of the neutralized lipid–cadmium complexes across the bilayer, could display kinetics much faster than the rate of $CdCl_2$ diffusion from the bulk solution. This 'shuttle' uptake mechanism was demonstrated to occur with Ca^{2+} in PA and PS liposomes and has been proposed to account for non-saturable calcium uptake into cells [52,53], and we suggest it could intervene in cadmium uptake as well, since Ca and Cd display nearly identical interaction properties with phospholipids.

5. Conclusion

The use of three complementary analytical techniques yields an overall view of the cadmium–lipidic membrane interactions. Metal binding and its structural consequences on the bilayer fluidity and organization were simultaneously detected, enabling us to relate the observed macroscopic effects to specific molecular mechanisms. We demonstrated this way that electrostatic cadmium binding is predominant and can be reversed in high salinity or low pH conditions. Large, potentially toxic, perturbations of bilayer structure are induced upon Cd(II) binding, so that phospholipids may be considered as a major target for cadmium complexation at the biomembrane level.

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