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Comparison of the effects of NaCl on the thermotropic behaviour of *sn*-1′ and *sn*-3′ stereoisomers of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol

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The phase behaviour of liposomes of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol (1′-DMPG) and the corresponding *sn*-3′ stereoisomer (3′-DMPG) were studied by DSC as a function of NaCl concentration. The melting of the metastable gel phase to the liquid-crystalline phase was similar for both lipids. However, in the presence of salt and at 6 °C ($T < T_p$) the gel phase of both stereoisomers of DMPG was shown to be metastable and a new phase nominated here as the highly crystalline phase was formed as the stable state. However, significant differences in the formation and melting of the highly crystalline phase were evident between the two polar headgroup stereoisomers. For 3′-DMPG in the presence of 300 mM NaCl the melting enthalpy of this phase is approx. 82 kJ/mol and the transition temperature about 11 degrees higher (at 33.6 °C) than for the gel to liquid-crystalline phase transition (25 kJ/mol at 23.0 °C). In the presence of 0.15–1.2 M NaCl at 6 to 10 °C the formation of the highly crystalline phase of 3′-DMPG is complete within 2 to 5 days, increasing [NaCl] facilitates the rate. For a 1:1 mixture of 1′- and 3′-DMPG the formation of the highly crystalline phase requires several weeks and melts at about 20 degrees higher than the gel phase (at approx. 40 °C). For 1′-DMPG partial conversion into the highly crystalline phase requires several months. For 3′-DMPG several intermediate phases appeared as endothermic peaks between the main phase transition temperature and the melting temperature of the highly crystalline phase. In contrast, for 1′-DMPG and the 1:1 mixture the subgel phase appears to be the only metastable intermediate phase. Different monovalent cations differ in their effect on the metastable behaviour.

Introduction

The phase behaviour of negatively charged phospholipids strongly depends on pH and the ionic environment. Accordingly, the properties of membranes containing acidic phospholipids can be regulated electrostatically [1–4,28,62–64].

The effects of divalent cations on the phase behaviour of phosphatidylglycerol (PG) are complex [18], whereas the effects of pH and monovalent cations are less dramatic [6,13,19]. The influence of monovalent cations on the phase behaviour of PG has been shown to depend on the cation species differing in the degree of their binding and their effects on the degree of hydration and hydrogen bonding at the polar headgroup level [6–10].

Metastable phase behaviour has been shown for PE and PC. When PC is incubated for several days at 4 °C, a new transition, nominated as subtransition, is observed below the main phase transition [38,39]. Prolonged incubation of PE at low temperatures results in the formation of a crystalline phase which has an elevated transition temperature T_c and enthalpy compared to the main transition [48,54,55]. As the transition temperatures of these phases (subtransition and melting of the crystalline phase) are exceeded the reappearance of the more crystalline phases requires a repeated incubation in the cold. The crystalline phase of PE is formed also without incubation in the cold if the liposomes are hydrated below the transition temperature of the crystalline phase [49,53]. A subtransition similar to that of the phosphatidylcholines has been observed also for DMPE [50] and DPPG [56].

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In studies on synthetic PGs the phospholipids used have generally been racemic with respect to the polar headgroup glycerol structure [11]. The naturally occurring PGs are stereochemically pure *sn*-1′ stereoisomers [12]. No indications of different phase behaviour of the stereoisomers of DMPG have been observed so far for the natural or the stereochemically pure synthetic phospholipids [7,13–16]. However, we recently reported the salt-induced aggregation of the *sn*-1′ and *sn*-3′ stereoisomers of DMPG to be different [17]. Na^+-induced structural differences between these two stereoisomers could be verified by Fourier transform infrared spectroscopy [58].

In the present study we describe the monovalent ion dependent phase behaviour of *sn*-1′- and *sn*-3′-DMPG polar headgroup stereoisomers, as revealed by DSC.

Materials and Methods

The ammonium salts of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol (1′-DMPG) and the corresponding 3′-stereoisomer were from the Department of Chemistry of Liquid Crystals of KSV Chemical Corporation (Helsinki, Finland). No impurities were detected in these lipids upon thin-layer chromatography on silica gel (Merck, Darmstadt, F.R.G.) developed with chloroform/methanol/water/ammonia (65 : 20 : 2 : 2, by vol.). The water used was freshly deionized in a Milli-RO/Milli-Q (Millipore, U.S.A.) water filtering system. For equimolar mixtures of 1′-DMPG and 3′-DMPG we used here the notation (1′ : 3′, 1 : 1)-DMPG. The compounds from Sigma (*rac*′-DMPG and *rac*′-DPPG) were non-specified for the molar proportions of the stereoisomers and may also contain the *sn*-2′ form.

Lipids were mixed in chloroform at a concentration of 2 mg/ml and dried under N_2 flow prior to exposure to low-temperature vacuum for two days. Liposomes were prepared by hydrating 2 mg of dry, solvent-free lipid in 2 ml of buffer at 45 °C with 1 min sonication on a bath type sonicator (Bransonic 220) followed by a 30 min incubation interrupted by vortexing (5 to 8 periods of approx. 15 s. Unless otherwise stated, the buffer used was 50 mM Tris-HCl, 0.5 mM EDTA (pH 7.4) containing different concentrations of the given salt. After dispersing, the lipid preparations were allowed to cool to room temperature and the vials were sealed under nitrogen. Samples were equilibrated at temperatures of 6, 10, or −18 °C before loading into the DSC cuvette maintained at about 6 °C. When indicated, samples were also incubated in the cuvette of DSC instrument.

DSC scans were performed using two high-sensitivity differential scanning calorimeters, the Privalov DASM-1M (Mospribintorg, Moscow, U.S.S.R.) and Microcal MC-2 (Microcal Inc., Northampton, U.S.A.). Scanning rates were 1.0 and 0.5 C°/min, respectively. Transition temperatures were determined as the temperature with maximal deviation from the baseline. The calorimetric enthalpies were determined using a Tamaya Planix 6 digital planimeter (Tamaya Inc., Japan) and by paper weighing or by a planimeter and a computer operated by software provided by Microcal or written in-house. Unless otherwise stated , the baseline was approximated by one straight line. All techniques gave fully consistent results.

Temperature dependency of light scattering of incident light at 500 nm and at an 90 ° angle was measured using a Kontron SFM-23 (Tegimenta AG, F.R.G.) fluorometer. The temperature was measured with a thermocouple inserted in the cuvette using an Exacon MC 9200 digital thermometer (Exacon, Denmark). Due to the rather high lipid concentrations the intensity of the scattered light was measured at 450 nm.

No indications of lipid degradation were observed for samples incubated for periods up to several months, as suggested by the complete reversibility of the changes in DSC behaviour and thin-layer chromatography on silica gel.

Transition which are observed only after an incubation in the cold we call subtransitions if they occur below the main phase transition and crystalline phase transitions if they occur at temperatures above the main transition. The crystalline phase transition with the highest transition temperature we nominate here as the highly crystalline transition. The transition which is observed without an incubation in the cold and appearing above the main phase transition we call posttransition.

Results

Dependency of the thermotropic phase behaviour of 1-DMPG on [NaCl]

Fig. 1 shows DSC traces for 1′-DMPG at varying NaCl concentrations. Three different transitions can be observed. At ≥ 250 mM NaCl a pretransition below the

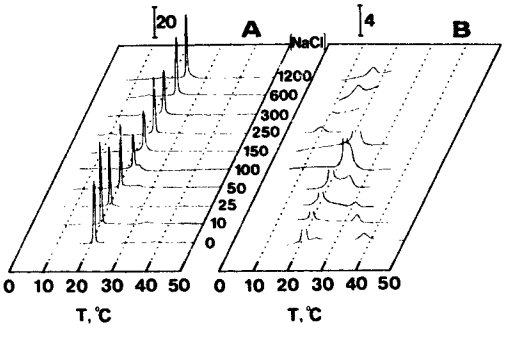

Fig. 1. (Panel A) DSC traces for liposomes of 1′-DMPG. The concentration of NaCl is indicated. The incubation times were 3 to 8 days at 6 ° C or 10 ° C. (Panel B) depicts the data in panel A in more detail. The magnitude of the calibration bar is given in 4.2 kJ/C ° per mol.

main transition is evident. At ≤ 100 mM NaCl a third transition can be observed above the main phase transition. Upon increasing [NaCl], T_m first decreases and then monotonically increases. This behaviour is qualitatively in accordance with the simple electrostatic theory for melting of charged membrane explaining the behaviour of monomethylphosphatidic acid [1–3]. The minimum in T_m for DMPG was observed at ≈ 25 mM NaCl, whereas for DPPG this was at ≈ 100 mM NaCl [19].

Fig. 1 also reveals the dependency of T_p on [NaCl]. At 300 mM NaCl $T_p \approx 11°C$ and at 1.2 M NaCl $T_p \approx 19°C$. In addition ΔH_p increases from ≈ 2.1 to ≈ 2.9 kJ/mol. Below 250 mM NaCl no pretransition is observed. This dependency of T_p and ΔH_p on [NaCl] has been observed for DPPG [20] and is also evident in Ref. 56. Similar although weaker dependency of the pretransition temperature on [NaCl] has been described for the neutral (zwitterionic) PC [29].

A third transition can be observed above the main transition at salt concentrations between zero and 100 mM (Fig. 1). This transition is observed in samples immediately after their preparation and does not require an incubation in the cold. It is reversible in immediate rescans also when the lipid samples were not cooled below the main phase transition after the first scan. This transition we have nominated the posttransition. As [NaCl] is increased the temperature of this transition decreases and its enthalpy increases. At 150 mM NaCl it appears to fuse with the main phase transition and is never observed below the main transition. At ≈ 150 mM NaCl the main transition consists of two peaks (with $\Delta T \approx 0.3$ C°, Fig. 1), whereas at higher and lower NaCl concentrations only one sharp peak is seen. The two components of the transition were observed only when the samples were incubated long enough, for approx. 24 h, at 6 °C or 10 °C.

Tables I, II and III summarize the main transition, pretransition and posttransition temperatures and enthalpies for 1′-DMPG at different NaCl concentrations. At salt concentrations of 50–100 mM the main and posttransition overlap and the determination of the individual enthalpies is ambiguous. At lower salt concentrations the baselines could be approximated individually with a single descending baseline. In the case of overlapping main transition and posttransition only one horizontal baseline was used (cf. Ref. 31). Increasing [NaCl] to 50 mM the baseline between these transitions seems to become more descending. Salt dependency of the pretransition parameters is shown in Table II. At low content or in the absence of buffer the pretransition temperatures are 1–2 C° higher. Yet, T_p depends on [NaCl] and can be observed also at 100 mM salt. No clear [NaCl] dependency for the pretransition enthalpy was observed.

When 1′-DMPG liposomes were incubated for 16 to 84 days at 6 or 10 °C (below T_p), melting of crystalline states (above the main phase transition) and a subtransition (below the main phase transition) were observed Fig. 2. Increasing [NaCl] decreases the time required for the formation of both the subgel and crystalline phases.

The subgel phase of 1′-DMPG is metastable and precedes the formation of the crystalline phase. However, up to 1.2 M NaCl the crystallization of 1′-DMPG was incomplete. Highest subtransition enthalpy (21 kJ/mol) was seen for 1′-DMPG (see Table IV). We also observed subtransitions composed of one or two components, in accordance with Ref. 56. The salt concentration dependency of the subtransition temperature is similar to that of pretransition. Our preliminary studies indicate that T_s increases with longer incubation times. Compared to the incubation times necessary for detect-

TABLE I

The dependency of T_m and ΔH_m of non-incubated 1′-DMPG-liposomes on [NaCl]

NaCl (mM)	T_m (°C)	ΔH_m (kJ/mol)
0	21.1 ± 0.1	27.2 ± 0.8
50	20.8 ± 0.1 [a]	26.4 ± 1.3
150	22.2 ± 0.3 [b]	25.1 ± 1.3
300	22.9 ± 0.1	24.3 ± 0.8
1200	24.7 ± 0.2	23.9 ± 0.8

[a] Overlapping with the posttransition.
[b] Transition consisting of two peaks.

TABLE II

The dependency of T_p and ΔH_p for 1′-DMPG on [NaCl]

NaCl (mM)	T_p (°C)	ΔH_p (kJ/mol)	Medium
100	≈ 11.8	≈ 1.7	H_2O
300	11.3 ± 0.4	2.2 ± 0.6	buffer
600	14.9 ± 0.6	2.4 ± 0.4	buffer
1200	18.7 ± 1.2	2.8 ± 0.4	buffer

TABLE III

The dependency of T_{post} and ΔH_{post} for 1′-DMPG on [NaCl]

NaCl (mM)	T_{post} (°C)	ΔH_{post} (kJ/mol)	Baseline [a]
0	38.8 ± 0.5	1.8 ± 0.2	HB,DB
10	33.9 ± 0.3	2.1 ± 0.2	HB,DB
25	29.7 ± 0.2	2.1 ± 0.3	DB
50	25.3 ± 0.3 [b]	2.1 ± 0.4 5.0 ± 0.8	DB HB
75	24.2 ± 0.3 [b]	6.1 ± 0.5	HB
100	23.3 ± 0.2 [b]	5.8 ± 0.5	HB

[a] HB, horizontal baseline; DB, descending baseline.
[b] Overlapping with main phase transition.

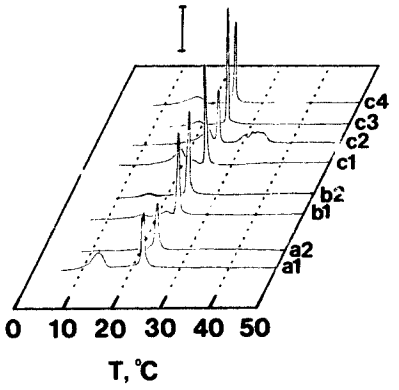

Fig. 2. Formation of the subgel and crystalline phases of 1′-DMPG upon prolonged incubations. The rescans were recorded immediately after the preceding scan. (a1) Liposomes stored for 60 days at −18°C, [NaCl] = 150 mM. (a2) rescan of a1. (b1) Liposomes stored for 84 days at +6°C, [NaCl] = 600 mM. (b2) rescan of b1. (c1) Liposomes stored for 16 days at +6°C. (c2) Liposomes stored for 75 days at +6°C. (c3) rescan of c2. (c4) Liposomes stored for 24 days at −18°C. In c1 to c4, [NaCl] = 1200 mM. The calibration bar is for 21 kJ/C° per mol.

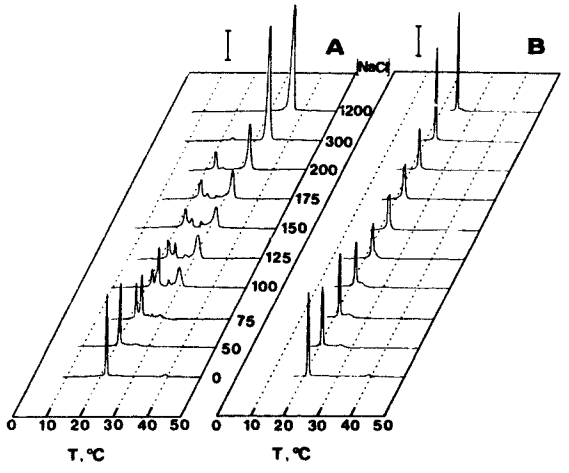

Fig. 3. (Panel A) The effects of different NaCl concentrations (in mM) on 3′-DMPG upon incubation for 3 days (69–76 h) at +6°C. (Panel B) Immediate rescans of the samples are shown in panel A. The calibration bar represents 21 kJ/C° per mol.

ing the subtransition for the first time, the formation of the subgel phase is faster for repeated incubation.

Dependency of the thermotropic behaviour of 3′-DMPG on [NaCl]

In Fig. 3A are shown DSC traces for 3′-DMPG liposomes incubated for three days at +6°C. At NaCl concentrations from 0 to 50 mM the gel to liquid-crystal transition is seen at 20–21°C. At [NaCl] between 75 and 200 mM several transitions are observed. Between 300 and 1200 mM NaCl only one peak (endotherm for the melting of the highly crystalline phase) is evident. T_{HC} is approx. 33 to 37°C, i.e. well above the gel to liquid-crystal transition temperature (23°C to 25°C) (Fig. 3B). Thus T_{HC} increases with increasing [NaCl] more than T_m. The intermediate transitions between T_m and T_{HC} at 75–200 mM NaCl seem to behave in a similar manner. As the NaCl concentration is increased the enthalpy content of the peak of the melting of the highly crystalline phase increases with a simultaneous gradual decrease in the enthalpy of the main transition peak. The formation of the highly crystalline phase occurs considerably faster for 3′-DMPG than for the 1′ stereoisomer. At the same NaCl concentration in the range of 300 to 1200 mM T_{HC} for 3′-DMPG was approx. two degrees higher than for 1′-DMPG. If 3′-DMPG liposomes were not incubated below T_p, the DSC traces and the phase characteristics are within experimental error both qualitatively and quantitatively equivalent to those for 1′-DMPG (Fig. 1 and Fig. 3B). For 3′-DMPG pretransition was readily observed at low (1 mM and 10 mM Tris) buffer concentrations similarly to 1′-DMPG. However, due to the metastability of the gel phase the pretransition of 3′-DMPG was not studied in more detail.

TABLE IV

Characteristics of the subtransition of 1′-DMPG at different NaCl concentrations

NaCl (mM)	$T_{incubation}$ (°C)	$t_{incubation}$ (days)	T_{sub} (°C)	ΔH_{sub} (kJ/mol)
150	−18	60	13.3	21
600	−18	40	16.0	17
1200	+3.5	3	17.8	17
1200	+6	75	23.0	14

The stability and metastability of 3′-DMPG in the presence of NaCl

The crystalline phase is stable at low temperatures also at low salt concentrations although longer incubation times are required. At 150 mM NaCl and at 6 or 10°C the formation of the crystalline phase is complete within one week. When the incubation times were prolonged from that necessary for crystallization (about 2–3 days), T_{HC} increased by 0.5–1.0 degree.

Samples of 3′-DMPG ([NaCl] = 300–1200 mM) were maintained below T_m for varying periods of time (up to 2 days). In order for the crystallization to occur the 3′-DMPG samples must be incubated in the lamellar gel phase, below T_p (more accurately, below the completion temperature of the pretransition, i.e. about 1.4 C° above T_p, Table II). For both DMPG polar headgroup stereoisomers the formation of the highly crystalline phase leads to the disappearance of both the pretransition and the main phase transition. No posttransition is observed

preceding a fully developed highly crystalline phase transition for 3'-DMPG at 50–100 mM NaCl. Yet, at 25 mM NaCl a posttransition is evident after the crystalline transition (data not shown).

The highly crystalline phase can be observed by visual inspection as a 'pearly' solution indicating macroscopic inhomogeneity and aggregation of liposomes [17]. Similar appearance has been previously reported for crystalline PE [49]. This is not observed for the subgel phase.

Light scattering measurements for 3'-DMPG incubated for 2 to 5 days at +10°C in the presence of 150 mM NaCl show that at the first heating scan the transition from the most scattering highly crystalline phase occurs directly to the least scattering fluid phase and upon cooling from the fluid phase to the gel phase. For 1'-DMPG similar temperature scans show a two-phase behaviour between gel and fluid phases (data not shown).

When the samples in highly crystalline phase were heated into the middle of the highly crystalline phase transition and then cooled and reheated, approximately half of the highly crystalline phase had remained. This is similar to the behaviour of the crystalline phase of PE [54]. The highly crystalline phase was stable and was observed for samples (at 300 mM NaCl) maintained in the $T_m > T > T_{HC}$ temperature range for three days. In addition, a 20 min sonication of 3'-DMPG in 300 mM NaCl with a bath sonicator below T_{HC} does not lead to the disappearance of the highly crystalline phase.

The formation of the subgel phase of DMPE and DPPC has been shown to begin at temperatures not

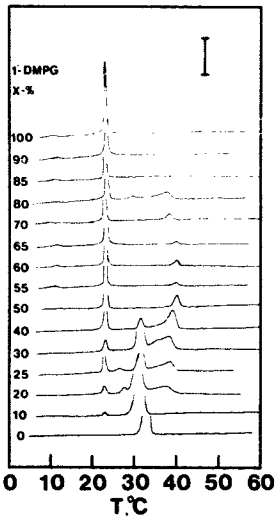

Fig. 4. The mixtures of 1'-DMPG and 3'-DMPG at different molar proportions incubated at 6°C in the presence of 300 mM NaCl. The incubation time was 10–24 days. The calibration bar denotes 21 kJ/C° per mol.

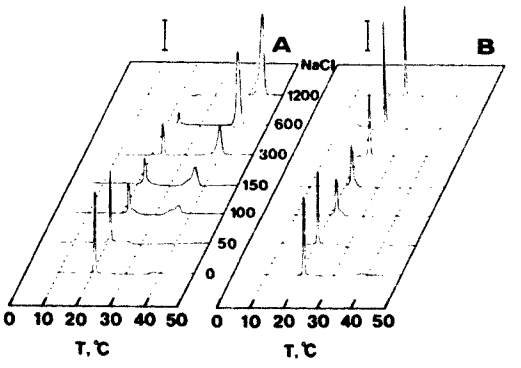

Fig. 5. (Panel A) The effect of NaCl concentration on the 1 : 1 mixture of 1'-DMPG and 3'-DMPG. The incubation times were 17 to 24 days at 6°C. NaCl concentrations are given in mM. (Panel B) Immediate rescans of the samples are shown in panel A. The calibration bar gives 21 kcal/C° per mol.

exceeding 6°C yet to continue up to the melting temperature of the subgel phase [52,54]. Indications of a transition of 3'-DMPG into the highly crystalline phase also at temperatures > T_p are seen in light scattering measurements. Upon heating, an increase in the light scattering efficiency is evident and approaches that of the highly crystalline state. In calorimetric scans this appears as exothermic transitions below T_{HC}. The temperatures and enthalpies of these transitions vary between individual DSC-runs as described for DPPE [53]. However, they seem to occur after the pre- and main phase transitions. Heating and cooling below T_{HC} did not have an effect on the time required for the formation of the highly crystalline phase. Samples of 3'-DMPG were also hydrated in 150 mM NaCl at temperatures of 0°C, 6°C and 26°C (with 1 min sonication and 30 min vortexing) and were scanned immediately from 16°C (data not shown). Samples prepared in this manner did not differ significantly from those hydrated at 45°C and then incubated at the above temperatures in an identical manner. This is in contrast to DPPE which can form the crystalline phase directly without incubation when hydrated below T_C [53].

Behaviour of mixed liposomes of 1'- and 3'-DMPG

The DSC traces for mixed DMPG liposomes prepared at varying molar proportions of the 1'- and 3'-enantiomers and incubated in the presence of 300 M NaCl are shown in Fig. 4. Clear asymmetry is evident. Based on these results it can be assumed that as the lipids crystallize they have a tendency to separate at certain molar proportions (0%, 50% and 100% of 1'-DMPG).

Fig. 5A illustrates DSC scans for (1' : 3',1 : 1)-DMPG incubated at +6°C for 20 days. T_{HC} of mixed liposomes is 5–7 C° higher than for 3'-DMPG. The incubation time required for the formation of the highly

crystalline phase is, however, about five times longer than for 3′-DMPG. No peaks between the main transition and the highly crystalline transition were detected at salt concentrations between 75 and 200 mM NaCl. Fig. 5B illustrates scans recorded immediately after the first scan, revealing now within experimental error behaviour similar to that of nonincubated 1′-DMPG and 3′-DMPG. The phase diagram in Fig. 6A summarizes the dependency of the phase behaviour of the three different kinds of DMPG-liposomes (1′,3′ and their equimolar mixtures) on [NaCl] (see also Figs. 1, 3B and 5B, as well as Tables I, II, and III). The melting temperature for the highly crystalline phase as a function of [NaCl] for 3′-DMPG and (1′:3′,1:1)-DMPG liposomes is summarized in Fig. 6B and 6C.

For (1′:3′,1:1)-DMPG, subtransitions similar to those of 1′-DMPG were observed. As for 1′-DMPG the reappearance of subtransition is faster after a repeated incubation thus resembling the crystallization behaviour of DPPE [53]. When the subtransition, main phase transition and highly crystalline transition were detected for DMPG in the same DSC-run, the enthalpy content of the subtransition was decreased compared to scans in which no highly crystalline transition was seen. No pretransition precedes the fully developed subtransition of (1′:3′,1:1)- and 1′-DMPG, similarly to the behaviour observed for DMPC [40]. However, pretransition can be seen after subtransition [39].

Preliminary conclusions can also be made on the kinetics of the formation of the highly crystalline phase by the different stereoisomers. At 1200 mM NaCl and at 6 °C the formation of the highly crystalline phase is complete for *sn*-3′ in one to three days. For *rac*′ one to two weeks are required. For *sn*-1′ several months (3 to 8) are necessary to see a partial transition. See Figs. 2, 3, and 5.

For comparison DSC curves for *rac*′-DPPG (from Sigma) incubated at 6 °C at 0.3 M and 1.2 M NaCl are shown in Fig. 7. Below the main transition a subtransition takes place and consists of two peaks. Above the main phase transition the highly crystalline transition is observed. The difference between T_{HC} and T_m is less for *rac*′-DPPG (5 to 7 C°) than for an equimolar mixture of 1′- and 3′-DMPG (17 to 20 C°).

Comparison of the effects by Li^+, Na^+, K^+ and Cs^+

The highly crystalline phase is observed also in the presence of monovalent cations other than Na^+. In Fig. 8 are shown DSC data on 1′-DMPG and 3′-DMPG at 300 mM Li^+, Na^+, K^+ and Cs^+ incubated at +6 or −18 °C. Incubation at the latter temperature was used to reduce the time of crystallization. Strong dependency of T_{HC} on the cation species is evident. The dependency of transition parameters for monovalent cations are summarized quantitatively in Table V. When crystallization was not complete ΔH_{HC} was approximated by

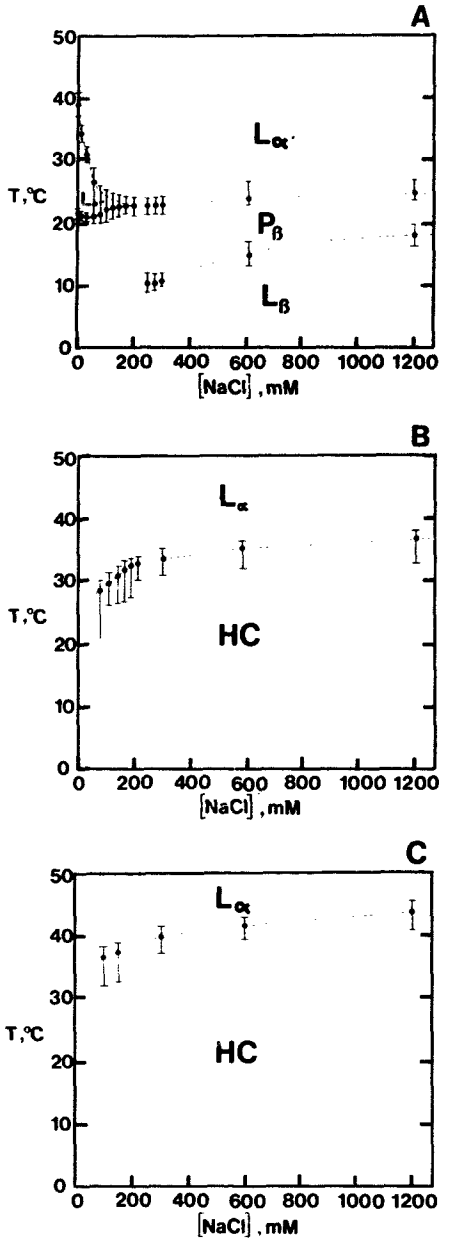

Fig. 6. The [NaCl]-dependency of some of the transition temperatures of DMPG. (Panel A) Nonincubated 1′-DMPG-liposomes. (Panel B) Melting of the highly crystalline phase of 3′-DMPG. (Panel C) Melting of the highly crystalline phase of an equimolar mixture of 1′-DMPG and 3′-DMPG.

comparing the main transition enthalpy and the sum of the crystal melting enthalpies from the first scan with the main transition enthalpy from a rescan [54]. The absolute enthalpy values may be slightly inaccurate due to the long incubation times. Accordingly, we have

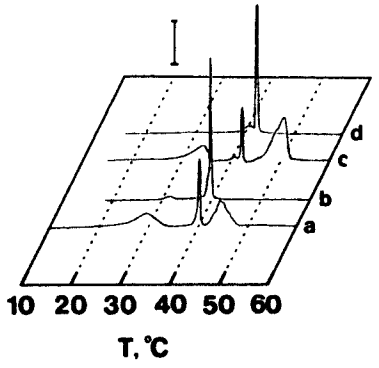

Fig. 7. Scans for *rac*'-DPPG-liposomes incubated for one month (a and c) at 6°C with immediate rescans (b and d) in the presence of 300 mM NaCl (a and b) and 1200 mM NaCl (c and d). The calibration bar gives 21 kJ/C° per mol.

taken the ratio of the enthalpies of these transitions ($\Delta H_{HC}/\Delta H_m$) for the different cations as the basis for comparison. The enthalpy difference between the highly crystalline phase and liquid-crystalline phase does not markedly depend on the cation in question. In KCl the crystalline and main transitions overlap. The rather small total enthalpy for the peak is probably due to incomplete crystallization. In the presence of monovalent cations the melting enthalpies of the crystalline phase of DMPG are somewhat higher (≈ 70–80 kJ/mol) than for DMPE and DMPG in the presence of Ca^{2+} (i.e., 67 kJ/mol, see Refs. 54 and 21). As for NaCl also in the presence of the other monovalent cations studied pretransition disappears as the crystalline phase is formed. When 3'-DMPG is incubated at 6°C in the presence of KCl for three days no evidence for the formation of a crystalline phase is observed, yet the pretransition temperature and enthalpy are higher than in the presence of NaCl. With LiCl no pretransition is

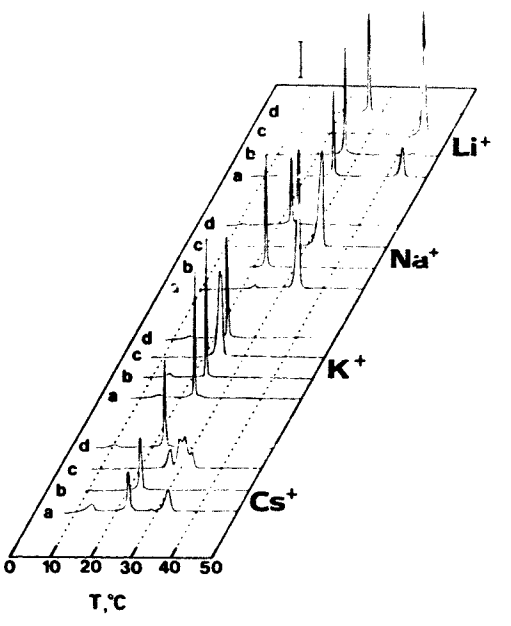

Fig. 8. The effects of the indicated monovalent cations at a concentration of 300 mM on the phase behaviour of 1'-DMPG and 3'-DMPG. (a) 3'-DMPG stored for 3–4 days at +6°C. (b) 3'-DMPG stored at −18°C for 7–10 days. (c) rescan for 3'-DMPG stored before the first scan for 3–4 days at 6°C. (d) 1'-DMPG stored at +6°C for 4–5 days. The bar shows 21 kJ/C° per mol.

seen, however, melting of the crystalline phase is observed. This could indicate that Li^+ abolishes the rippled phase similarly to H^+ [25]. The increase in T_m is most pronounced for Li^+. In the series of increasing cation radius (Li < Na < K) T_{HC} decreases nearly linearly. The crystallization of 3'-DMPG in the presence of the largest cation Cs^+ appears to be more complicated than the behaviour seen in the presence of the other cations studied and resembles the behaviour ob-

TABLE V

Effects of the different monovalent cations at a concentration of 300 mM on the transitions of 3'-DMPG

For comparison, a summary for an equimolar mixture of 1' and 3'-DMPG in the presence of NaCL is given.

Salt (300 mM)	T_p (°C)	ΔH_p (kJ/mol)	T_m (°C)	ΔH_m (kJ/mol)	T_{HC} (°C)	ΔH_{HC} (kJ/mol)	$\Delta H_m/\Delta H_{HC}$
3'-DMPG							
LiCl	– [a] (≈ T_m?)	–	26.6 ± 0.1	27.2 ± 0.8	43.8 ± 0.1	80 ± 4	2.9 ± 0.3
NaCl	[b]		23.0 ± 0.1	24.7 ± 1.3	33.6 ± 0.1	82 ± 0.1	3.4 ± 0.2
KCl	14.3 ± 0.3	3.7 ± 0.2	23.4 ± 0.3	24.3 ± 1.3	≈ 24.0	> 67 [c]	> 2.7 [c]
CsCl	10.3 ± 0.3	2.2 ± 0.2	22.8 ± 0.3	25.9 ± 0.8	32.6 ± 0.2 [d]	78 ± 8	3.0 ± 0.3
An equimolar mixture of 1'- and 3'-DMPG							
NaCl	11.0 ± 0.3	2.4 ± 0.7	22.9 ± 0.1	24.7 ± 1.3	39.8 ± 0.2	76 ± 5	3.0 ± 0.3

[a] See text for details.
[b] Not defined, see text for details.
[c] See text for details.
[d] The peak at highest temperature in Fig. 7.

served in the presence of NaCl. With CsCl, however, a subtransition is observed for 3'-DMPG but not for 1'-DMPG, in contrast to the data for NaCl. The excess heat difference between the liquid crystalline and the lamellar gel phases ($\Delta H_p + \Delta H_m$) is probably constant (25–28 kJ/mol) for all these cations, Table V. The ratio $\Delta H_{HC}/(\Delta H_p + \Delta H_m)$ for 3'-DMPG is about 2.8–3.1 for all the monovalent cations studied.

The effects of different monovalent cations on the posttransition were compared at 10 mM salt concentration. The addition of 10 mM LiCl decreases T_{post} from 38°C to 29°C and the other monovalent cations NaCl, KCl, CsCl decrease it to 32.5–34°C. Posttransition is also observed for the *rac'*-DMPG from Sigma. Addition of EDTA in the presence of 50 mM NaCl causes the posttransition to become more pronounced. As the concentration of Tris buffer in the absence of salt is decreased T_{post} increases and ΔH_{post} decreases and finally disappears. Decreasing the content of the buffer causes broadening also of the main transition. This might indicate the formation of smaller liposomes and other structures detected at low content or in the absence of buffer or salt [22].

At 300 mM NaCl no significant differences in the crystallization behaviour of 3'-DMPG in H_2O or in buffer were detected.

Discussion

Four distinct transitions have been described so far for disaturated phospholipids: pretransition and main transition (for reviews, see for instance Refs. 41 and 61), subtransition (for PC [38,39], for PE [50], and for PG [54]), and melting of the crystalline phase (for PE [48,49,53–55], for PS [59,60]. The formation of the subgel and crystalline phases generally requires prolonged incubation in the cold, whereas the other transitions are readily observed. Structural differences between the subgel and crystalline phases are, however, unclear. In the present DSC study we describe for DMPG incubated below T_p and in the presence of salt the formation of what we have nominated as the highly crystalline phase. Accordingly, including this study, a (highly) crystalline phase with a large enthalpy has now been reported for PE, PS and PG. It has not been found for PC which is the only polar headgroup without the ability to form intermolecular hydrogen bonds [61].

The enthalpy of the melting of the highly crystalline DMPG is much higher than for fully developed subtransition and higher than the sum of the enthalpies of the subtransition and main transition. This is in accordance with the data on DMPE [50,54]. The difference between the transition enthalpies of the crystalline phase and the gel phase does not depend on the chain length of the fatty acids and has been accounted for by hydration energy [49]. Compared to the gel phase the hydrocarbon chains have been shown to be more tightly packed and ordered in an orthorhombic subcell in both PC-subgels [39,44–46,51] and PE-crystals [49,55]. Our infrared studies on 3'-DMPG incubated in the presence of 300 mM NaCl reveal spectral features which are characteristic for rigid packing of the acyl chains and reflect reduced motional freedom of the interfacial and polar headgroup regions in the highly crystalline phase [58].

The shift of T_{HC} to higher temperatures with increasing [NaCl] is larger than the increase in T_m (see Fig. 6). Assuming the binding of Na^+ to occur with partial charge neutralization (see below) the increase in T_{HC} must be explained mainly by effects other than those due to electrostatics. One possibility could be decreased hydration. Addition of salt has been shown to decrease the hydration of the polar headgroups of negatively charged phospholipids [4]. Decreasing hydration has been shown to lead to increased transition temperatures for PE [5]. The degree of hydration of the polar headgroup is decreased in the subgel phase of DPPC [39,45]. Similar findings on hydration have been reported for the crystalline phase of DMPE [48]. However, against the concept of decreased hydration as a general cause for crystallization are the results in Ref. 5. Upon dehydration, the transition temperature of PE increases, whereas there is only a small increase in transition enthalpy.

The subtransition temperatures observed here for DPPG are approx. 10 C° higher than reported previously [56]. The higher scanning rate used in the present study could explain in part this difference. However, the salt concentrations used in Ref. 56 were lower than those used by us. In addition to the salt concentration dependency of the subtransition, we observed an increase in the subtransition temperature upon longer incubation times, similarly to a recent report for DPPC [43].

Characteristics of the association of inorganic cations to phospholipids are complex. Dehydration of the cation must preceed binding [7]. The possibility that the conformation and hydration of the polar headgroup changes upon penetration and binding of the cation to the polar headgroup has been discussed [9]. The degree of binding of the monovalent cations on the fluid PG membrane at neutral pH has been shown to be slightly less than one [8]. The behaviour of PG in the presence of 100 mM NaCl is comparable to PC and PE yet different from the acidic PS [15]. The present results indicate that also the crystallization of PG is similar to the zwitterionic PC and PE, however it only occurs in the presence of salt. The crystallization data for DMPS in LiCl [59] has been interpreted as complete cation binding to the lipid polar head thus forming an ion lattice in the plane of the bilayer [59].

It has been shown that the increase in the main phase

transition temperature of DMPG cannot be explained in detail by mere screening of the membrane surface charge according to the theory of diffuse double layer [6–10]. This is substantiated by our results. The highest observed value for T_{HC} in the present study was about 44°C at 300 mM LiCl which is 4 C° higher than for fully protonated DMPG [6,21,25]. In addition the large increase in the enthalpy content and the metastable phase behaviour cannot be accounted for by the electrostatic theory. A recent report from our laboratory on the NaCl-induced aggregation of small unilamellar liposomes of 3′-DMPG and 1′-DMPG revealed high affinity of 3′-DMPG for Na^+ [17]. This was suggested to be due to a favored conformation of the 3′-DMPG polar headgroup with the glycerol OH-groups closer to each other and providing a binding site for Na^+. The orientation of the hydroxyl groups might have an effect on hydration as well.

The glycerol backbone region of phospholipids has been shown to be involved in the crystallization of the subgel [42,47] and crystal [53] phases. The differences in the temperatures and enthalpies of the melting of the highly crystalline phase of 3′-DMPG and (1′:3′,1:1)-DMPG resemble those observed between L-DPPE and DL-DPPE [53]. The transition temperature of the racemic lipids is markedly higher and the transition enthalpy somewhat lower than for the pure lipid. The behaviour of DL-DPPE has been interpreted as pairing of the different stereoisomers [53]. The higher transition temperature of the highly crystalline phase of racemic DMPG compared to the enantiomerically pure stereoisomers and the apparent phase separation of the highly crystalline phase of the (1′:3′,1:1)-DMPG (Fig. 4) indicate strongly favoured interactions between the 1′ and 3′ stereoisomers of DMPG in the presence of Na^+. In support of this, it was shown in a recent X-ray diffraction study that in single crystals of Na^+ salt of the two DMPG polar headgroup stereoisomers in an equimolar mixture crystallize as pairs. For 1′-DMPG crystallization was not achieved [30]. The most important factor in the organization of *rac*′-DMPG in the bilayer plane in the dry crystal seems to be hydrogen bonding between the polar headgroups and the bonding of Na^+ to the polar headgroups with partially coordinated character between the adjacent bilayers. These interactions cause significant differences in the lipid conformation at the level of the acyl chains and backbone glycerol between the two headgroup stereoisomers. The degree of hydrogen bonding has been shown to influence the behaviour of PG monolayers on water [10].

Wilkinson and McIntosh [56] have shown that bilayers in DPPG-liposomes crystallize lamellarly and independently from each other to form a subgel. A condition for the crystallization to occur might be that the lipids in the two lamellae of the bilayer should be more or less identically packed. Observations on the ordering of the lipids in the subgel phase and with some mixed chain phospholipids have been interpreted in a similar manner so that phospholipids from opposing lamellae of the bilayer are strongly paired in a crystal-like manner [33,35,36,45]. PG-liposomes are stable at neutral pH in the presence of salt [23,24] and they have been shown to become larger when the salt concentration is increased [22]. Small PG-liposomes have been observed to have an elliptical shape [22,23]. This also indicates the flat bilayer structure to be favoured. If the acyl chains between lamellae couple strongly and pair with partial interdigitation in periodic crystalline structures in subgel and crystalline but not in the other phases, the difference in the amount of lipid in the inner and outer leaflets depending on bilayer curvature might be minimal for more ordered subgel and crystalline phases. In the present study we have shown that the crystallization of *rac*′-DPPG occurs more slowly compared to *rac*′-DMPG. Similar observation has been made for the PC subgel formed by lipids with increasing acyl chain lengths [40,41]. A simple explanation for this difference in kinetics might be the lower probability of flip-flop for the longer chain lipids. Moreover the probability of flip-flop is likely to be slower for the more ordered phases which could explain the slow rate of formation of these phases. The lower cooperativity of the crystal melting transitions might be due to crystal defects being dependent upon radius (or curvature) in the multibilayer liposomes.

We also describe here a new transition for DMPG, nominated as posttransition. Posttransition was observed in the presence of Tris buffer. Binding at pH 7.4 of the positively charged Tris to PG membranes has been proposed [9,37]. However, we observed posttransition also at 50 mM sodium acetate. In a recent study on *rac*′-DPPG it was shown that in 50 mM Tris-HCl buffer the acyl chains are interdigitated in the gel phase, whereas in the same buffer containing 100 mM NaCl there is no interdigitation. In this case interdigitation was not accompanied by higher degree of organization of the acyl chains [37]. However, the transition enthalpy and temperature are somewhat increased for interdigitated DPPG [34,37]. The enthalpy change per methylene unit and transition temperature are also increased for partially interdigitated PCs with saturated mixed fatty acids [32,33]. We observed ΔH_m and T_m for DMPG to decrease with increasing [NaCl] from 0 to 50 mM (Table I). A higher melting component similar to posttransition has been observed for DLPG and DLPC [14,26,27,31]. It could be identical to the posttransition of DMPG observed in the present study (Fig. 1).

The biological significance, if any, of the 'highly crystalline' phase described here for DMPG remains uncertain. The high degree of order and stability of this phase also above T_m could be significant in the regu-

lation of membrane functions [48,54]. Only the 1'-DMPG stereoisomer which has a slower rate of crystallization is found in Nature [12]. This could indicate that the highly crystalline phase is not favoured in natural membranes or that its formation is strictly controlled.

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