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Review

The physicochemical properties of cardiolipin bilayers and cardiolipin-containing lipid membranes ☆

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ABSTRACT

In this review article, we summarize the current state of biophysical knowledge concerning the phase behavior and organization of cardiolipin (CL) and CL-containing phospholipid bilayer model membranes. We first briefly consider the occurrence and distribution of CL in biological membranes and its probable biological functions therein. We next consider the unique chemical structure of the CL molecule and how this structure may determine its distinctive physical properties. We then consider in some detail the thermotropic phase behavior and organization of CL and CL-containing lipid model membranes as revealed by a variety of biophysical techniques. We also attempt to relate the chemical properties of CL to its function in the biological membranes in which it occurs. Finally, we point out the requirement for additional biophysical studies of both lipid model and biological membranes in order to increase our currently limited understanding of the relationship between CL structure and physical properties and CL function in biological membranes.

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Abbreviations: CL, cardiolipin (diphosphatidylglycerol); PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; TLCL, 1,2,-1′,2′-tetraoleoylcardiolipin; TMCL, 1,2,-1′,2′-tetramyristoylcardiolipin; TPCL, 1,2,-1′,2′-tertapalmitoylcardiolipin; TSCL, 1,2,-1′,2′-tetrastearoylcardiolipin; TOCL, 1,2,-1′,2′-tetraoleoylcardiolipin; DMPC, 1,2-dimyristoylphosphatidylcholine; DMPE, 1,2-dimyristoylphosphatidylethanolamine; DMPG, 1,2-dimyristoylphosphatidylglycerol; DMPS, 1,2-dimyristoylphosphatidylserine; DMPA, 1,2-dimyristoylphosphatidic acid; DM-TAP, 1,2-di-O-myristoyl-3-N,N,N-trimethylaminopropane; DPPC, 1,2-dipalmitoylphosphatidylcholine; DPPE, 1,2-dipalmitoylphosphatidylethanolamoline; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl 2-oleoyl phosphatidylethanolamine; POPG, 1-palmitoyl 2-oleoyl phosphatidylglycerol; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; ppm, parts per million; FTIR, Fourier-transform infrared; AFM, atomic force microscopy; L_α, lamellar liquid/crystalline; L_β, lamellar gel; L_c, lamellar crystalline; H_{II}, inverted hexagonal; Q_{II}, inverted cubic; T_m, midpoint temperature of the lamellar gel/liquid-crystalline phase transition

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1. Introduction

The anionic phospholipid cardiolipin (diphosphatidylglycerol) is found in the plasma membranes of many types of Gram-negative and Gram-positive bacteria and in the mitochondrial and chloroplast inner membranes of eukaryotes [1–5]. Cardiolipin (CL) is usually a relatively small component (≤10 mol%) of the total membrane lipid in such membranes, where its primary role appears to be supporting the function of key membrane proteins [5]. Indeed, CL plays an important role in the structural stabilization and activation of many mitochondrial enzymes, especially those involved in ATP synthesis and energy transduction [6–12], and in the maintenance of the structure and function of the Type II photoreaction center of photosynthetic bacteria and plants [13]. However, CL may also be found in substantially higher quantities (~55–60 mol%) in the membranes of some microbial organisms, wherein elevated levels of CL and its derivatives appear to enhance the tolerance of these organisms to halophilic and resource-depletion stress [5,14–17]. For such organisms, elevated CL levels may be reflecting a requirement for enhancement of the structural integrity of the cell membrane, or an increased requirement for the support of stress-related increases in energy procurement and transduction, or both [12,14]. In higher eukaryotes, CL is also known to be an effector of the cytochrome P-450-dependent cholesterol side-chain cleavage enzyme, as well as an activator of cytochrome *c* oxidase and the mitochondrial phosphate carrier protein [18–20], and it has been suggested that the capacity for structurally specific interactions with membrane CL may actually be highly conserved in such proteins [21]. The latter possibility was also suggested by the observation that within any given organism, the diversity of molecular species of mitochondrial CL is considerably narrower than is typical of most other membrane phospholipids, and by the observation that it is not unusual for CLs from any natural source to be highly enriched (up to 95 mole%) in a single fatty acid [22]. CL is thus an essential component of the mitochondrial membranes of higher organisms and significant depletion and/or chemical modification of this phospholipid component usually results in mitochondrial dysfunction ranging from mild to severe [[22, 23] and references cited therein].

Unlike most naturally occurring glycerophospholipids, CL is a quadruple-chained anionic amphiphile composed of two 1,2-diacyl phosphatidate moieties esterified to the 1- and 3-hydroxyl groups of a single glycerol molecule. The CL polar headgroup thus contains two phosphodiester moieties which, in principle, should both be negatively charged under physiologically relevant conditions. It also contains a single secondary hydroxyl group which, in principle, could be hydrogen bonded to suitable moieties of the CL headgroup (both inter- and intra-molecularly) and/or to water. Naturally occurring CL also contains three optically active glycerol moieties, two of which (the backbone glycerols) are 1,2-acylated with long chain fatty acyl groups, and the third (the headgroup glycerol) is 1,3-phosphorylated with the diacyl phosphatidate moieties described above. This combination of structural features is unlike that of all other naturally occurring membrane lipid components and undoubtedly confers upon CL a unique suite of physicochemical properties, which are probably vital for the normal function of CL-containing membranes. In this review, we examine how this unique suite of physiochemical properties are reflected in the physical properties of CL-containing lipid bilayer membranes.

2. Structural considerations

The major structural feature which distinguishes CL from all other common membrane glycerolipids is the fact that a single headgroup alcohol (a glycerol molecule) is shared by two phosphatidate moieties. This simple feature has important implications regarding the overall physical properties of CL within the context of a lipid bilayer. For example, because the two phosphatidate moieties essentially share a single, relatively small headgroup alcohol, the mean headgroup area per CL phosphatidate will be significantly smaller than those of diacyl phosphoglycerides such as PC, PS and PG. The relatively small size of the polar headgroup should thus promote greater cohesion between the CL hydrocarbon chains (especially in the gel-state), which should in turn result in an elevation of the lipid T_m relative to those of most other phospholipids. The small size of the polar headgroup should also enhance the propensity of CL to form inverted nonlamellar lipid phases [24,25]. Although this tendency will be attenuated by the presence of the mutually repulsive negative charges of the phosphate groups [26,27], the presence of CL should still provide a driving force towards an increase in membrane negative curvature stress. Moreover, under physiologically relevant conditions, the presence of two negatively charged phosphate groups in CL may even afford some potential for moderating such nonlamellar phase-forming tendencies through changes in local pH and by the binding of cationic species, which may be dissolved in the solvent (e.g., metal ions) or located on membrane-associated proteins and peptides.

Second, because the headgroup alcohol of CL is tethered to and in effect shared by two phosphatidate moieties, its mobility and conformational flexibility should be severely restricted, as has been confirmed experimentally [28]. Thus, one would expect that the impaired mobility of the headgroup alcohol of CL will diminish its capacity for both intra- and inter-molecular interactions with other headgroup moieties. This may be particularly important in CL-containing bilayer membranes, because the secondary hydroxyl group of the CL headgroup glycerol is actually the sole source of hydrogen-bonding donor groups available for both intra- and inter-molecular interactions. An examination of molecular models suggests that because of the combined effect of its restricted mobility and the diminished hydrogen-bonding reach of the CL headgroup hydroxyl, inter-molecular hydrogen-bonding between CL headgroups is probably unlikely. Moreover, although internal hydrogen-bonding to the headgroup phosphate(s) may be possible, only a few of the phosphate conformations that are feasible within the context of a lipid bilayer can enable such interactions. It thus follows that in lipid bilayer membranes, the capacity for steric self-shielding of CL phosphate groups by intra- and/or inter-molecular interactions with its headgroup alcohol is considerably smaller than that of virtually all other common phospholipids save PA. Consequently, the accessibility of CL phosphate groups at the surfaces of lipid membranes to interactions with water, metal ions and drugs, and with peptides and integral and surface bound membrane proteins, will probably greatly exceed those of most other membrane lipids. Given the seemingly specialized role of membrane CL, this property may be very important *in vivo*.

As noted above, naturally occurring CL also contains three optically active glycerol moieties, two of which (the backbone glycerols) are 1,2-acylated with long chain fatty acyl groups, and the third (the headgroup glycerol) is 1,3-phosphorylated with diacyl phosphatidate moieties. Powell and Jacobus [29] noted that because all metabolic pathways leading to the biosynthesis of CL are stereo-convergent [30,31], the stereochemical configurations of the diacyl phosphatidate entities esterified at the 1- and 3-positions of the headgroup glycerol are identical. The two phosphatidate moieties of natural occurring CL are thus diastereotopically *inequivalent* and as a result, the rates and thermodynamic equilibria of chemical reactions at comparable positions on the two phosphatidate moieties will differ under both chiral (e.g., enzyme-mediated) and achiral conditions. Although rarely considered in examinations of the physicochemical properties of CL, the diastereotopic inequivalence of the phosphatidate entities of CL is important, because it implies that the behavior of the two phosphate groups of CL will probably be somewhat different. This fact may form the physical basis for the experimental evidence for the inequivalence in the physicochemical properties of the two CL phosphate moieties. For example, Henderson et al. [32] observed that high-resolution ^{31}P-

NMR spectra of chloroform:methanol (2:1) solutions of CL exhibit a doublet of resonances centered near −0.9 ppm. Since the authors assumed that the phosphate moieties of CL are chemically equivalent, they suggested that the resonance doublet observed could be the result of internal hydrogen-bonding of the 2-hydroxyl proton of the headgroup glycerol to one of the phosphate groups. However, as noted by the authors, this explanation will hold only if the rate of oscillation of the hydroxyl proton between free and hydrogen-bonded forms, or between the two possible hydrogen-bonded forms, is slow on the ^{31}P-NMR timescale, a situation which seems unlikely for small molecules in isotropic solution. In another study, Kates et al. [33] reported that pH titration of CL suspensions in methanol: water (1:1) indicates that the apparent pK_a values of the two phosphate moieties of CL differ significantly ($pK_1 \sim 2.8$ and $pK_2 \sim 7.5$–9.5). These authors also assumed that the phosphate moieties of CL are chemically equivalent, and thus they rationalized their experimental observations on the basis of phosphate group inequivalence induced by intra-molecular hydrogen-bonding of the 2-hydroxyl proton of the headgroup glycerol to one of the phosphate entities. This argument was further supported by the demonstration that similar pH titrations of the corresponding 2′-deoxy CL analogue reveal that the pK_a values of the two phosphate groups ($pK_1 \sim 1.8$ and $pK_2 \sim 4.0$) are a lot closer to each other, as would be expected upon titration of two chemically equivalent groups in close proximity [33]. In principle, the diastereotopic inequivalence of the two phosphate moieties of naturally occurring CL can form a plausible physical basis for explaining the experimental observations noted above, without an *a priori* requirement for hydrogen-bonding of the 2-hydroxyl proton of the headgroup glycerol to one of the headgroup phosphates. It may also account for the differences between the pH titration curves of CL and its 2′-deoxy analogue, because replacement of the 2-hydroxyl group of the headgroup glycerol with hydrogen destroys the optical activity at that centre, thereby making the stereochemical relationship between the remaining optically active backbone glycerols diastereomeric and, for the most part, chemically equivalent. However, we stress that although the diastereotopic inequivalence of the phosphatidate entities of naturally occurring CL can explain the apparent physicochemical inequivalence of the two headgroup phosphates of CL without the requirement for selective hydrogen-bonding interactions with the headgroup hydroxyl proton, such considerations do not exclude the possibility that those interactions contribute significantly to this inequivalence. This is because inequivalent hydrogen-bonding between the CL headgroup glycerol and the headgroup phosphates, which follows naturally from the diastereotopic inequivalence of the CL headgroup phosphates, may accentuate the physicochemical manifestation of the underlying inequivalence of the these groups. It should also be noted that any manifestation of the diastereotopic inequivalence of the two CL phosphate groups will probably be most noticeable when CL is dissolved in isotropic solution, when it interacts with enzymes, or when it is specifically bound to the proteins which require CL for normal function. The extent to which the diastereotopic inequivalence of CL headgroup phosphates may be manifest in the physical properties of CL-containing membranes is currently unknown.

3. Thermotropic phase behavior and organization

Despite the biochemical and biomedical importance of CL, relatively few studies of the phase behavior and organization of CL bilayers have been performed. In part, this may be attributed to the fact that until fairly recently, the chemical synthesis of stereochemically pure CL has not been as straight forward as with most other naturally occurring phospholipids [for references to modern synthetic methods, see [34–41]], which is also probably the main reason why only a few species of stereochemically pure, synthetic CL are currently commercially available. Initially, attempts were made to characterize the thermotropic phase behavior of CLs obtained from natural sources, such as bovine cardiac muscle and *Escherichia coli* [42–44]. Such studies showed that under physiologically relevant pH and ionic strength conditions, the T_m's of CLs obtained from such sources are all quite low (≤−20 °C), consistent with the fatty acyl chain heterogeneity and high unsaturated fatty acid content of the membranes from which the CL samples were derived. However, those studies also established that the L_β/L_α phase transitions of CLs are very sensitive to the presence of divalent cations such as Ca^{2+}, for which cation-induced elevations in the L_β/L_α phase transition temperature can approach values between 20 °C and 30 °C. The high sensitivity of the T_m's of CLs to both monovalent and divalent cations was more clearly characterized in later studies using hydrogenated CLs initially obtained from natural sources [45] and synthetic acyl chain-homogeneous materials [46]. Indeed, it was demonstrated that CL L_β/L_α phase transition temperatures are strongly influenced by the binding of mono- and especially divalent counter ions to the headgroup phosphates and, judging from the effects of such ions on the lipid T_m, divalent cations (especially Ca^{2+}) seemed to be the more strongly bound (Fig. 1). As expected, the temperatures and enthalpies of the L_β/L_α phase transitions of the linear saturated CLs increase progressively with hydrocarbon chain length (see Table 1), but the enthalpy values measured are approximately twice that typically observed at simple hydrocarbon chain-melting phase transitions of diacyl glycerolipids of similar hydrocarbon structure. However, when normalized on a per chain basis, the observed enthalpy changes are comparable to those exhibited by the corresponding diacyl phospho- and glycoglycerolipids, indicating that the energetics of the hydrocarbon chain-melting process are essentially the same as those of the diacyl glycerolipids [49]. Interestingly, despite the variations in the absolute values of all measurements of the T_m's of CL bilayers, it is consistently observed that even in the absence of significant perturbation by the binding of divalent cations, the T_m's of linear saturated tetraacyl CLs are some 17–25 °C higher than those of the corresponding diacyl phosphatidylglycerols (e.g., 38.5 °C for TMCL *vs* 23.9 °C for DMPG). Indeed. when measured under physiologically relevant pH and ionic strength conditions and in the absence of divalent cations, the T_m's of CLs seem to be in the mid to higher end of the range of T_m's exhibited by common membrane phospholipids, for which T_m's decrease in the

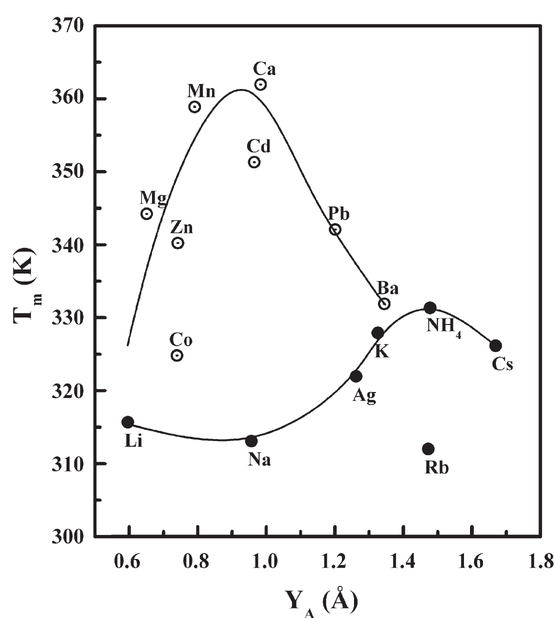

Fig. 1. Plot of transition temperatures of TPCL cardiolipin salts as a function of the unhydrated ionic radii of the cations. The monovalent (—●—) and divalent (—○—) cations appear to fall on distinct curves. (Redrawn from data published in Ref. [46]).

Table 1
Gel/liquid-crystalline phase transition temperatures (T_m) of synthetic cardiolipins.

Lipid (*n*:0)	T_m (°C)	Enthalpy (kcal/mol)	Method	Reference
TMCL (14:0)	47.0	n.d.	DSC	[47]
TPCL (16:0)	62.2	n.d.	DSC	[47]
TLCL (12:0)	25.5	8.7	DSC	[46]
TMCL (14:0)	40.0	12.5	DSC	[46]
TPCL (16:0)	58.0	17.7	DSC	[46]
TSCL (18:0)[a]	61.0	n.d.	FTIR	[45]
TPCL[b]	56.0	n.d.	FTIR	[45]
TMCL	35.6	10.5	DSC	[48]
TMCL	38.9	12.9	DSC	[49]

[a] Hydrogenated derivative of bovine cardiac muscle cardiolipin (~97% 18:0).
[b] 2′-Deoxy derivative of TPCL (~95.5% 16:0).

general order PE ~ PA > CL > PS > PC ~ PG. The possible basis for this observation will be examined later.

To date, the focus of most studies of the thermotropic phase behavior of CL bilayer membranes has been on a characterization of the L_β/L_α phase transition and of the sensitivity of that process to metal ions. Consequently, relatively little has been done to characterize the range of lamellar polymorphism possible in such membranes, and the overall structure and organization of CL lamellar polymorphs that may be formed. Currently, the only information available on this aspect of the behavior of CL bilayers is a recently published calorimetric, spectroscopic and X-ray diffraction study of synthetic TMCL [49]. The authors demonstrated that upon heating under physiologically relevant pH and ionic strength conditions (i.e., pH ~ 7.4 and ~ 100 mM salt), fully equilibrated aqueous dispersions of TMCL exhibit two thermotropic phase transitions, the transition temperatures of which are sensitive to the ionic strength of the dispersing buffer (see Fig. 2). The lower temperature transition is quite energetic but is much less cooperative, and exhibits a marked cooling hysteresis. The higher temperature transition is also highly energetic but is much more cooperative while exhibiting no appreciable cooling hysteresis. X-ray diffraction and FTIR and ^{31}P-NMR spectroscopy indicate that the lower temperature transition corresponds to L_c/L_β phase transition and the higher temperature endotherm to a L_β/L_α phase transition. These authors also presented a fairly detailed structural picture of the L_c, L_β and L_α phases of TMCL [for a more detailed description, see 49], which showed that the crystal-like L_c phase contains triclinically-packed all-*trans* hydrocarbon chains, partially dehydrated ester carbonyl groups, and highly immobilized polar headgroups residing in a fairly polar local environment. The conversion from the L_c to the L_β phase involves the formation of a less ordered gel-phase structure composed of orientationally disordered all-*trans* hydrocarbon chains, better hydrated ester carbonyl groups, and more mobile headgroup phosphate moieties. However, the increase in the polar headgroup mobility did not involve any noticeable change in the polarity of the local environments of the headgroup phosphates, and the phosphate mobility in the TMCL L_β phase as resolved by ^{31}P-NMR spectroscopy, was less than typical of the gel phases of most other membrane phospholipids, and phosphate reorientation in this phase was not fully axially symmetric about the bilayer normal (see ^{31}P-NMR powder patterns in Fig. 3). Finally, the formation to the L_α phase involved conversion to a more mobile lamellar structure containing conformationally disordered (melted) hydrocarbon chains, better hydrated ester carbonyl groups, and phosphate groups undergoing fast axially symmetric motion about the bilayer normal. However, as was observed in the L_β phase, the range of phosphate reorientations seemed to differ from those normally seen in the L_α phases of most other membrane phospholipids, and the L_β/L_α phase transition also did not involve appreciable changes in the polarity of local environments of the headgroup phosphates. The latter was inferred from the insensitivity of the O-P-O asymmetric stretching frequencies to structural changes occurring at the lipid L_c/L_β and L_β/L_α phase transitions (see Table 2), an observation atypical of the behavior of most other membrane phospholipids except PC, which does not contain headgroup hydrogen-bonding donor groups. The authors also noted that in all three lamellar phases, the phosphate O-P-O asymmetric stretching frequencies (~ 1215 cm^{-1}) occur in the lower end of the range of frequencies exhibited by most other phospholipids (see Table 2). These frequencies are also similar to those of hydrated bilayers composed of DSCL and the 2′-deoxy derivative of TPCL [45], and are consistent with the existence of hydrogen-bonded phosphates

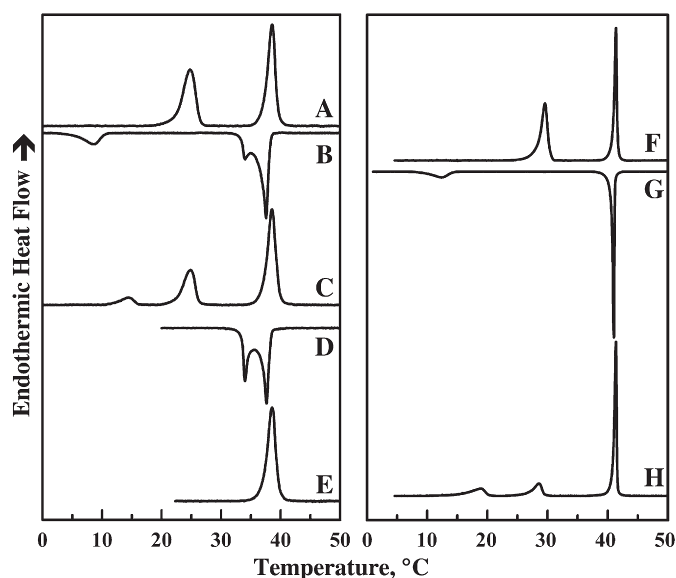

Fig. 2. DSC thermograms illustrating the polymorphic phase behavior exhibited by dispersions of TMCL. (Redrawn from data published in Ref. [49]). Left panel: Thermograms exhibited by samples dispersed in Tris buffer and recorded at scan rates of 10 °C/h. (A) Heating scan after low-temperature sample equilibration; (B) Cooling scan from high temperature to −7 °C; (C) Heating scan recorded immediately after cooling to temperatures near −7 °C; (D) Cooling scan from high temperature to 18 °C; (E) Heating recorded immediately after cooling to temperatures near 18 °C. Right panel: Thermograms exhibited by samples dispersed in phosphate buffer were recorded at scan rates of 60 °C/h. (F) Heating scan after low-temperature sample equilibration; (G) Cooling scan from high temperature to 0 °C; (H) Heating recorded immediately after cooling to temperatures near 0 °C.

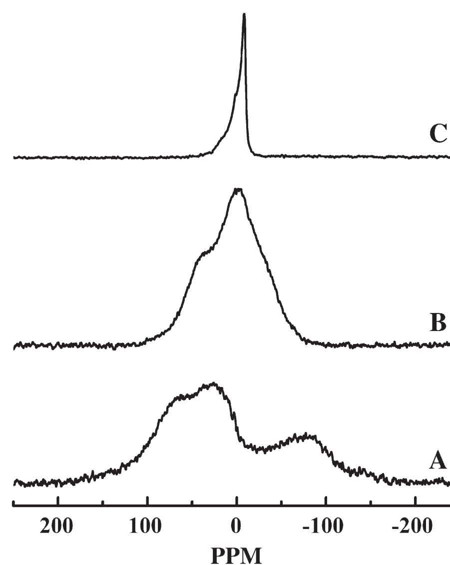

Fig. 3. Proton-decoupled ^{31}P-NMR powder patterns exhibited by aqueous dispersions of TMCL. (Redrawn from data published in Ref. [49]). The spectra shown were acquired in the heating mode after initial sample equilibration at low temperatures and are representative of: (A) L_c phase at 2 °C; (B) L_β phase at 32 °C; (C) L_α phase at 48 °C.

Table 2
Comparison of the phosphate O-P-O asymmetric stretching frequencies (cm^{-1}) in the L_c, L_β and L_α phases of hydrated membrane phosphoglycerolipids.

Phospholipid	L_c Phase	L_β Phase	L_α Phase	Reference
DMPC	1230	1230	1230	[a]
DMPG	1196	1208	1214	[50]
DMPE	1225	1220	1220	[51]
DMPS	1215	1227	1222	[52]
TMCL	1215	1215	1215	[49]

[a] Unpublished data from this laboratory.

residing in relatively polar local environments. This observation raises issues about the possible source of the hydrogen-bonding donor groups and is particularly relevant to the structural considerations discussed in the preceding section. As noted above, the impaired flexibility of the headgroup glycerol and resultant diminution of the hydrogen-bonding reach of the headgroup hydroxyl group, suggest that inter-molecular hydrogen-bonding to neighboring phosphate groups is highly unlikely within a lipid bilayer membrane, a suggestion consistent with the insensitivity of phosphate O-P-O asymmetric stretching frequencies to the changes in lipid packing density occurring at the L_c/L_β and L_β/L_α phase transitions of TMCL, and with the observation that similar frequencies are observed in hydrated bilayers composed of 2′-deoxy-TPCL, in which the headgroup hydroxyl group is replaced by hydrogen [45]. However, the apparent insensitivity of phosphate O-P-O asymmetric stretching frequencies to changes in bilayer structural organization is also compatible with the existence of an intra-molecular, headgroup glycerol-to-phosphate hydrogen bond which persists in all three lamellar phases of the lipid. However, as noted previously, the formation of long-lived hydrogen bonds to the phosphate groups markedly increases the longitudinal relaxation times of the ^{31}P nucleus [53] to such an extent that it may be impractical to record the ^{31}P-NMR spectra of unoriented phospholipid bilayers with hydrogen-bonded phosphate headgroups [50,51]. Moreover, because the ^{31}P resonance intensity of phospholipid L_c phases is dispersed over a very broad range, this effect will be further exacerbated, as has indeed been observed in previous attempts to obtain ^{31}P-NMR powder patterns of the L_c phases of PE and PG bilayers, where ^{31}P-NMR spectra of the stable L_c phases cannot be obtained [50,51]. Given this, the fact that the ^{31}P-NMR spectra of the lamellar and nonlamellar (and especially the L_c) phases of CL bilayers have been routinely acquired using direct excitation data acquisition methods (the methods most sensitive to the relaxation problems alluded to above) is thus inconsistent with the persistence of long-lived intra-molecular hydrogen bonds to CL phosphate groups. It thus appears that both intra- and inter-molecular headgroup glycerol-to-phosphate hydrogen-bonding is very unlikely in CL bilayers and that the predominant source of hydrogen-bonding donor groups to phosphate groups at the surfaces of CL layers is probably the solvent phase (water) and/or entities dissolved therein.

4. Headgroup conformation and dynamics in cardiolipin membranes

The current literature is also sparse as regards data on the conformation and dynamics of CL headgroups and glycerol backbone regions. To date, the experimentally developed picture of CL headgroup conformation and dynamics is derived entirely from a single ^2H-NMR spectroscopic study of the liquid-crystalline phase of *E. coli* CL bilayers in which the headgroup and backbone glycerol moieties were specifically deuterated at the C1, C2 and C3 positions [28]. The picture which emerges from those studies is as follows. First, regardless of whether CL is the major (only) or minor lipid component of the membrane, the orientations of the two CL backbone glycerols are indistinguishable from each other and are compatible with those determined for phospholipids such as PC, PE, PG and PS. The time-averaged orientation of the two CL backbone glycerols is thus parallel to the bilayer normal, and like those of other common membrane phospholipids, their conformational freedom is highly constrained by the requirements of the C1 and C2 acyl chains for optimal hydrophobic contact within the hydrophobic core of the bilayer and optimal interactions between the C3 phosphate groups and the aqueous phase. Second, regardless of whether CL is the major or minor lipid component of the model membrane, the deuterons located at C1 and C3 of the headgroup glycerol are orientationally equivalent within the resolution of the ^2H-NMR determination, and consistent with a ^2H-NMR time-averaged orientation of the headgroup glycerol that is essentially parallel to the bilayer surface. Third, the ^2H quadrupolar splitting and relaxation times of deuterons located on all positions of the headgroup glycerol are all consistent with motions with correlation times that are considerable longer (i.e., motions that are slower) than those of phospholipids such as PG, which have more flexible polar headgroups (see Table 3). Thus, regardless of whether CL is the dominant or a minor lipid component of the membrane, the CL headgroup glycerol is a conformationally and dynamically constrained, presumably because of the attachments of its C1 and C3 groups to the phosphate groups, which are themselves constrained to lie at the surface of the bilayer. This picture is entirely consistent with the structural considerations discussed above.

^2H-NMR spectroscopic studies have also been performed on CL-containing POPC membranes in which the ^2H observer probes were located on the PC headgroup choline moiety [56,57]. By definition, these types of studies evaluate the way(s) in which the membrane CL affects the conformation and dynamics of the PC choline headgroup, while providing relatively little information about the disposition of the CL headgroup itself. Nevertheless, both studies demonstrated that the ^2H quadrupolar splitting exhibited by headgroup ^2H-labeled POPC in POPC:CL membranes differs significantly from that observed in pure PC bilayers, indicating that the presence of CL alters the orientation and mobility of adjacent choline headgroups at the surfaces of these membranes, most probably because of interactions between the positively charged quaternary PC nitrogen with the negatively charged CL phosphates. Interestingly, however, a comparison of the results obtained with POPC:CL bilayers with those obtained in similar studies of POPC:POPG bilayers [58,59] indicates that although the choline headgroup quadrupolar splittings initially increase linearly as a function of the negatively charged lipid content of both membrane systems, the effects of CL are about twice that observed in the POPC:POPG system (see Fig. 4). Indeed, when plotted as a function of the nominal surface charge of the membrane (assuming that CL carries a

Table 3
Deuterium quadrupolar splittings and T_1 relaxation times of different lipid headgroups.

Cardiolipin	$P–O–CH_2–CH(OH)–CH_2–O–P$			Reference
Quadrupolar splittings (kHz)	13–17	17–20	13–16	[28]
T_1 relaxation time (ms)	5–7	3–9	5–8	
Phosphatidylglycerol	$P–O–CH_2–CH(OH)–CH_2OH$			
Quadrupolar splittings (kHz)	10–11	4–5	0.5–1	[54]
T_1 relaxation time (ms)	8–19	8–19	8–22	
Phosphatidylethanolamine	$P–O–CH_2–CH_2–NH_3^+$			[55]
Quadrupolar splittings (kHz)	10	4		
Phosphatidylcholine	$P–O–CH_2–CH_2–N(CH_3)_3^+$			[55]
Quadrupolar splittings (kHz)	6	5		
Phosphatidylserine	$P–O–CH_2–CH(COO^-)–NH_3^+$			[55]
Quadrupolar splittings (kHz)	2, 17[a]	15		

Phosphatidylglycerol and cardiolipin measurements were performed between 15 °C and 40 °C.
(Reproduced from Ref. [28], with permission).
[a] Two inequivalent deuterons.

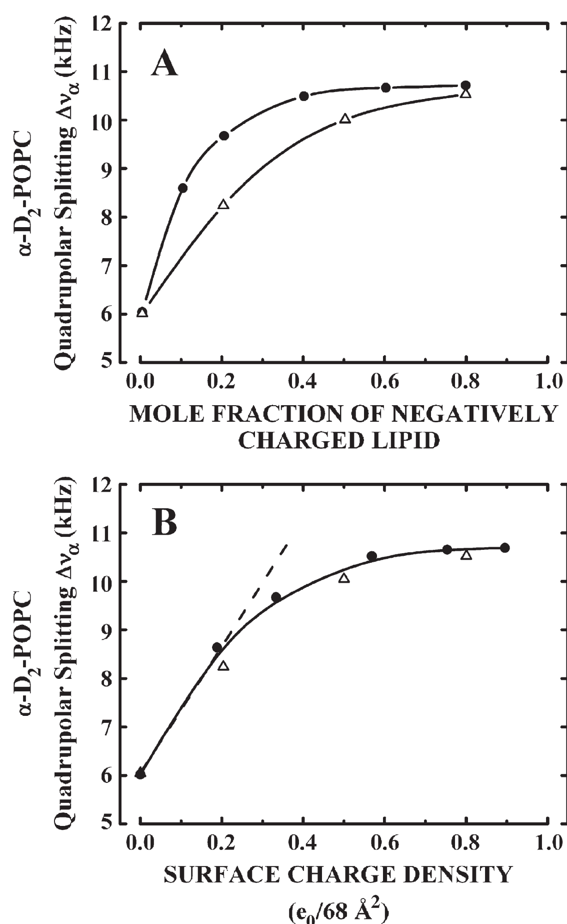

Fig. 4. Effect of negative surface charge on the ^{2}H quadrupolar splitting of the α-CD_2 groups of specifically ^{2}H-labeled choline headgroups of POPC membranes. Quadrupolar splitting were measured as a function of membrane composition in bilayers composed of POPC:POPG (–△–) and POPC:CL (–●–) and are plotted as a function of negative lipid mole fraction (panel A) and total surface charged density (panel B). (Redrawn from data illustrated in Ref. [60]).

charge of −2 at the membrane surface), the curves describing the effects of CL and PG are essentially the same (see Fig. 4), suggesting that from the perspective the positively charged polar headgroups located at the surfaces of CL-containing POPC membranes, the CL polar headgroup is actually behaving as a doubly negatively charged entity. In this respect, these results are compatible with those obtained in studies of the lamellar/nonlamellar phase behavior of membranes composed of mixtures of CL and cationic lipids (see below), but inconsistent with the inferences drawn from the pH titration studies described previously [33].

5. Lamellar/nonlamellar phase behavior of cardiolipin membranes

Lamellar/nonlamellar phase behavior is perhaps the best characterized of the physical properties of CL membranes. Early studies using naturally occurring CLs established that they exhibit a marked propensity to form H_{II} phases when dispersed in media containing high concentrations of monovalent cations or near equivalent amounts of divalent cations such as Ca^{2+} [61–63], and that nonlamellar CL phases may even be induced by a decrease in the pH of the dispersal medium [62] or by the binding of proteins and drugs [61,64]. Later X-ray diffraction and ^{31}P-NMR spectroscopic studies demonstrated that the propensity for salt-induced nonlamellar phase formation in CL membranes is dependent upon the length and degree of unsaturation of the lipid hydrocarbon chains, in a manner similar to that observed at the thermally induced lamellar/nonlamellar phase transitions which occur in membranes composed of PE or nonionic monoglycosyl glycerolipids [for a review of the latter, see [65]]. Thus, at room temperature, TOCL membranes exhibit a salt-induced L_α/H_{II} phase transition at NaCl concentrations near 3–3.5 M, the predominantly diunsaturated acyl chain bovine cardiac muscle CL (~90% linoleoyl chains) exhibits a similar transition at NaCl concentrations near 1 M (see Fig. 5), whereas membranes composed of the saturated, shorter-chain TMCL remain in the lamellar phase at NaCl concentrations up to at least 6 M [48,62]. Powell and Marsh [66] also examined the sensitivity of the salt-induced lamellar/inverted nonlamellar phase-forming propensity of CL to the number of hydrocarbon chains present on the CL motif. Their experiments involved a study of partially deacylated derivatives of bovine cardiac CL containing two and three fatty acyl chains and a CL derivative containing an additional acyl chain at C2 of the headgroup glycerol. The authors demonstrated that CL and its acylated derivative both exhibit salt-induced L_α/H_{II} phase transitions at NaCl concentrations below 3 M, suggesting that a minimum of 4 acyl chains are required for a salt-induced lamellar/inverted nonlamellar phase transition to occur. Their results also showed that when fewer than four acyl chains are esterified to the CL motif, stable lamellar phases cannot be formed under physiologically relevant conditions, though there was evidence of some lamellar phase formation at NaCl concentrations near 3 M. These results can be rationalized by considering the probable relative packing geometries or molecular shapes of these CL derivatives [24,25], as demonstrated by a recent coarse-grained molecular dynamics simulation study [67].

The common thread underlying virtually all demonstrations of inverted nonlamellar phase formation in CL membranes, however induced, seems to be the requirement for the neutralization and/or screening of the negatively charged phosphate groups at the bilayer surface. The possible role of surface charge in this regard was also investigated by an examination of inverted nonlamellar phase formation in CL-containing membranes, where the surface charge of the membrane was modulated by incorporating singly charged cationic lipids with identical fatty acyl chains [26,27]. These studies involved a characterization of the thermotropic lamellar/nonlamellar phase formation in such membranes at physiologically relevant pH and ionic strength. The authors demonstrated that although inverted

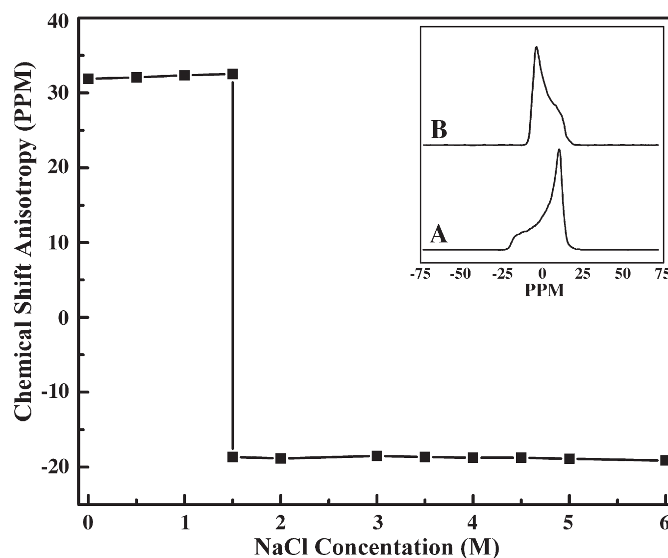

Fig. 5. ^{31}P-NMR characterization of the salt-induced L_α to H_{II} phase transition in beef cardiac cardiolipin. The main panel showing a plot of the ^{31}P chemical shift anisotropy as function of salt concentration indicates a discontinuity at NaCl concentrations near 1.5 M. The inset shows proton-decoupled ^{31}P-NMR powder patterns observed at NaCl concentrations below (A) and above (B) 1.5 M. (Redrawn from data published in Ref. [66]).

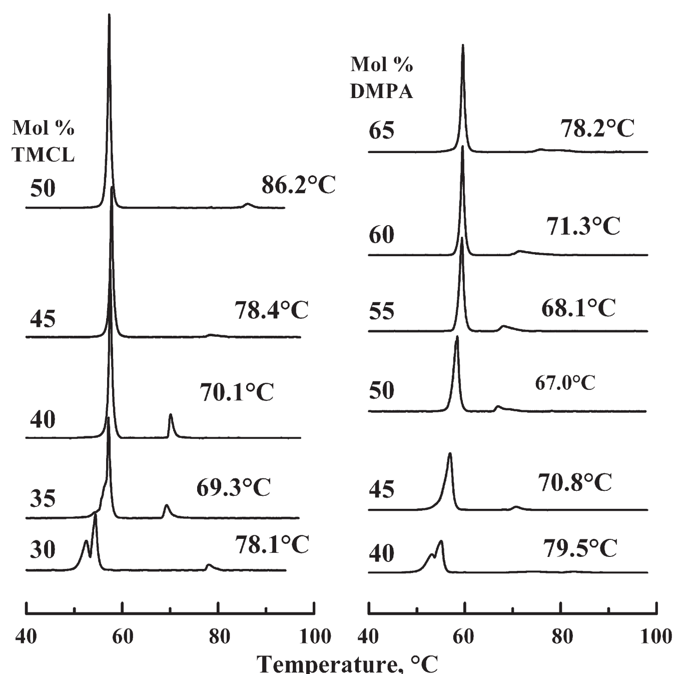

Fig. 6. DSC heating thermograms exhibited by binary mixtures of DM-TAP with TMCL (left panel) and DP-TAP with DMPA (right panel). The thermograms are shown for the compositions indicated and the temperatures indicate the estimated midpoint temperatures of the observed lamellar/nonlamellar transition endotherms. (Redrawn from data published in Ref. [26]).

nonlamellar phases are not formed in single-component membranes composed of either CL nor the cationic lipid, there was calorimetric and spectroscopic evidence for the formation of such phases in binary mixtures of the same lipids at compositions for which the nominal surface charge of the membrane approaches neutrality (for an example, see Figs. 6–7). Thus, with CL/cationic lipid mixtures, the formation of inverted nonlamellar phases was maximal in mixtures in which the CL:cationic lipid molar ratios are centered near 1:2, whereas with singly charged anionic lipids like PA, inverted nonlamellar phases are observed in mixtures with phospholipid: cationic lipid molar ratios centered near 1:1 (see Fig. 6). These authors also showed that although charge neutrality is not an absolute requirement for the formation of inverted nonlamellar phases, the lamellar/inverted nonlamellar phase transition temperatures are minimal in mixtures with nominally neutral membrane surface charge and increase rapidly when the surface charge of the membrane increases (see Fig. 6). Thus, for example, with TMCL/DM-TAP mixtures, significant inverted nonlamellar phase formation is only observed with mixtures with TMCL contents ranging from 30 to 50 mol% and the lamellar/nonlamellar phase transition temperatures approach a minimum (~69 °C) at membrane TMCL contents near 33 mol% (Fig. 6). This result also indicates that the effective charge of TMCL is −2 under these experimental conditions, and not −1 as may be inferred from pH titration studies [33]. It was also determined that the surface charge of the membrane affects the nature of the inverted nonlamellar phase that is thermodynamically stable at the lamellar/inverted nonlamellar phase transition temperature (see Fig. 7). Thus, the H_{II} phase is thermodynamically preferred under conditions approaching membrane charge neutrality, whereas inverted cubic phases are progressively preferred over the H_{II} phase as the nominal surface charge of the membrane deviates from neutrality [26,27]. These results of these experiments provided valuable insights into the relationship between membrane surface charge and CL lamellar/nonlamellar phase behavior.

6. Interactions of cardiolipin with other phospholipids

There have been a few studies published of the interaction of CL with other phospholipids in lipid monolayer or lipid bilayer model membranes. For example, the behavior of lipid monolayers composed of binary mixtures of bovine cardiac CL and egg PC at the air/water interface of a Langmuir trough has been examined [68]. These studies

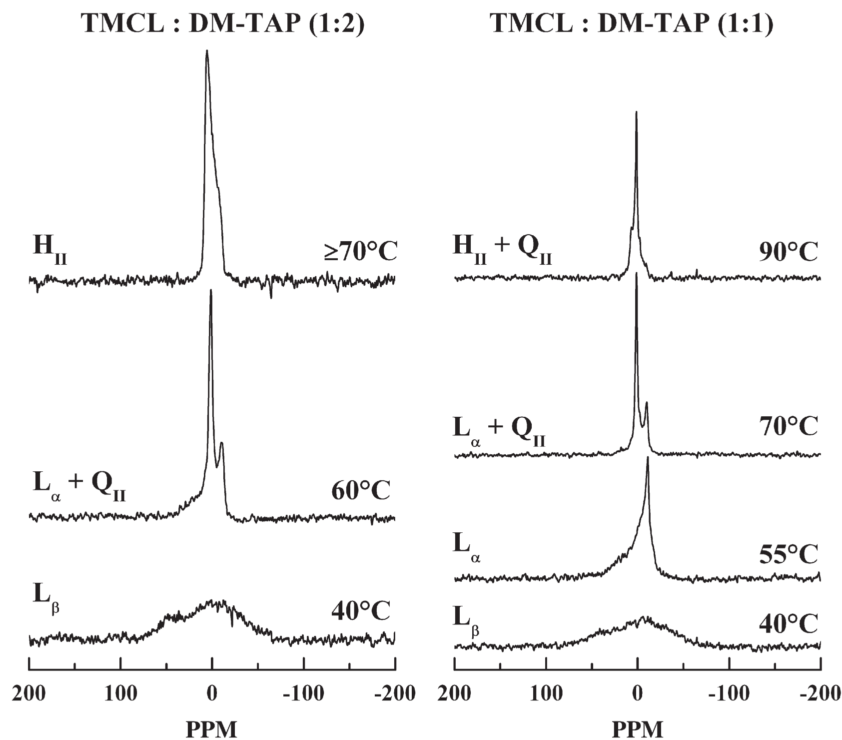

Fig. 7. Proton-decoupled ^{31}P-NMR powder patterns exhibited by aqueous dispersions TMCL with DM-TAP at the compositions indicated. For each spectrum, the acquisition temperature is indicated on the right and the phase assignments are indicated on the left.

indicate that the CL and egg PC are fully miscible with each other at all proportions tested and that the areas occupied by binary mixtures are less than the areas which would be occupied by each component alone. Thus, despite its more unsaturated fatty acid composition, bovine cardiac CL has a condensing or stabilizing effect on egg PC monolayers. As expected, this stabilizing effect is more pronounced when the monolayers are spread on a subphase containing 150 mM NaCl than with those spread on pure water, undoubtedly because of the screening of the two negatively charged CL phosphate groups through interaction with Na^+ in the subphase, and the resultant reduction of their mutual electrostatic repulsion. Very similar results were later presented in another monolayer film study of mixtures of bovine cardiac CL with POPC or POPE, although these workers claimed to detect the existence of CL-rich and DOPE-rich domains in monolayer formed supported on a mica substrate [69]. The net attractive interactions between CL and egg PC and POPC or POPE molecules were ascribed to favorable hydrogen-bonding interactions, although in the case of the CL/PC mixtures at least, a condensation of the monolayer due to a closer complementary packing of the small CL polar headgroups and the relatively large PC polar headgroups may also play a role. The former group also carried out a micropipette aspiration study of the mechanical properties of giant lipid bilayer vesicles composed of small amounts of bovine cardiac CL and larger amounts SOPC [68]. They reported that the compressibility modulus of SOPC vesicles was significantly reduced by the incorporation of CL, indicating that a lower stress is required to achieve a given increase in membrane area. This result suggests a weakening of the cohesive strength of the bilayer, in contrast to the results for monolayer films composed of bovine cardiac CL and egg PC. Similarly, the incorporation of CL also dramatically decreased the line tension of SOPC vesicles. Since the incorporation of anionic POPG into zwitterionic POPC vesicles has a similar effect, it was postulated that the repulsive electrostatic interactions between the incorporated CL molecules weakens the lipid bilayer membrane and renders them unable to withstand high stress. However, since SOPC contains a single saturated and a single monounsaturated fatty acyl chain whereas bovine cardiac CL contains four fatty acyl groups comprised primarily of fatty acids with two double bonds on each chain, these results can also be explained entirely by the differences in the degrees of unsaturation of these two lipids. This is because an increase in the degree of unsaturation of a series of PC's also significantly reduces both the area compressibility modulus and the lysis tension of vesicles. Thus, it is difficult to disentangle the potential effects of alteration in phospholipid polar headgroup structure from the known effect of variations in the degree of fatty acyl group unsaturation in all studies utilizing mixtures of highly unsaturated mammalian mitochondrial CL with either saturated, symmetric-chain phospholipids or mixed-chain phospholipids containing one saturated and one monounsaturated acyl chain.

The packing and interactions of TMCL with DPPC in pure and mixed Langmuir monolayers have recently been studied by grazing incidence X-ray diffraction [70]. The authors demonstrated that at surface pressures approaching those characteristic of phospholipid bilayers and at room temperature (~22 °C), the TMCL molecules in pure films exist in a solid (L_c-like) phase, with their aliphatic chains perpendicular to the monolayer surface, whereas DPPC alone exists in a liquid-condensed (gel-like) phase with tilted hydrocarbon chains. Thus, even in monolayers, TMCL seems to have a high propensity for condensing into a solid-like phase, as observed in studies of hydrated bilayers [49]. At higher temperatures (~30 °C), TMCL films convert to the liquid-expanded (liquid-crystalline-like) state and the TMCL and DPPC molecules appear to be fully miscible with each other up to TMCL contents near 50 mol%. Moreover, the presence of CL seems to increase the overall order and hydrocarbon chain packing of the mixed PC:CL monolayer, presumably by mechanisms comparable to those inferred from other studies (see above). In these experiments, the selection of a tetrasaturated CL and a disaturated PC was more likely to avoid ambiguities attributable to the poor miscibility between low-melting, highly unsaturated natural CLs and high-melting saturated synthetic phospholipids (see below). However, one should note that there still remains a hydrophobic mismatch between the tetradecanoyl chains of TMCL and the hexadecanoyl chains of DPPC and unfortunately, this study was performed on phospholipid monolayers spread on a subphase of pure water instead of a buffered physiological salt solution. The CL-containing layers used in these studies were thus essentially unbuffered and their negatively charged phosphates were certainly not as screened as they would be in biological membranes.

Studies have also been performed on the proton permeability of small unilamellar vesicles composed of bovine cardiac CL and DPPC [71]. These authors reported that at temperatures below the T_m of the pure DPPC vesicles (i.e., in the gel-state), the incorporation of increasing quantities of CL (0–20 mol%) markedly increased proton permeability, but had little effect on proton permeability at temperatures above the vesicle T_m (i.e., in the liquid-crystalline state). Also, from indirect fluorescence decay experiments, they inferred that the effect of CL in increasing proton permeability in gel-state DPPC bilayers was due a CL-induced increase in hydration at the bilayer surface. However, the marked increase in proton permeability and bilayer surface hydration at low temperatures is precisely what would be expected when small amounts of any highly unsaturated phospholipid are introduced into a gel-state bilayer, especially one composed of linear saturated fatty acyl chains. In this particular case, the highly unsaturated lipid will almost certainly drastically lower the phase transition temperature of the overall mixture and may also induce the formation of more fluid CL-rich and less fluid DPPC-rich domains, thereby markedly increasing the very low intrinsic proton permeability of the gel-state bilayer. These authors also noted that the presence of CL does not result in significant increases in the proton permeability of DPPC vesicles at temperatures above the L_β/L_α phase transition temperature of the vesicles, a result which seems counter-intuitive to the known tendency for membrane proton permeability to increase as the degree of unsaturation of the membrane lipids increases. Although these results could be attributed to an increase in membrane cohesion arising from the marked propensity for strong interactions between choline headgroups and CL phosphates [58–60], it is difficult to interpret these experimental results unambiguously, because the lipid polar headgroup, the degree of fatty acyl chain unsaturation and the acyl chain length are all being varied simultaneously when mammalian CLs are mixed with saturated lipids such as DPPC (see below).

The interactions of bovine cardiac CL with DPPC and DPPE in lipid monolayer and supported bilayers have also recently been studied using AFM techniques [72]. The main conclusion of this study was that CL forms domains in DPPC and DPPE monolayers and supported bilayers, consistent with the poor miscibility of the highly unsaturated bovine cardiac CL with the higher-melting saturated straight-chain phospholipids. Although this finding is doubtlessly valid, its physical and biological significance are probably very limited, because the naturally occurring differences in the degree of unsaturation between CL and the other membrane phospholipids would be considerably smaller in the mitochondrial inner membranes of animals and organisms such as *E. coli*. Moreover, even in a series of lipids in which the structure of the polar headgroup is invariant (e.g., PCs), the miscibility of molecular species is inversely proportional to the difference in their T_m's, which in turn depends markedly on their degrees of fatty acyl group unsaturation. One therefore expects that highly unsaturated mammalian CLs, which have T_m's well below 0 °C, will not be very miscible with fully saturated DPPC or DPPE, which have T_m's near 41 °C and 65 °C, respectively, which are actually in the gel-state at physiological temperatures. In order to minimize such effects and other ambiguities attributable to differences in hydrocarbon chain length and structure (mainly unsaturation), experiments such as these

and many of those described previously in this section should ideally be performed in mixtures with identical hydrocarbon chain composition. Unfortunately, however, this option is currently not practical because synthetic CL's with widely varying fatty acid compositions are not commercially available. Nevertheless, in lieu of this, model membranes made from mixtures of CL's from organisms such as *E. coli* (N.B. These CLs do not contain polyunsaturated fatty acid chains and usually contain predominantly saturated hydrocarbon chains at C1 and a monounsaturated chain at C2 of their backbone glycerols.) with mixed-chain lipids such as POPC, are better suited to providing more accurate insights into the biologically relevant interactions of CL with other phospholipids.

7. Molecular dynamics simulations of cardiolipin and cardiolipin-containing membranes

Recently, attempts have been made to model the behavior of CL bilayers and mixed CL:PC bilayers using both coarse-grained [67] and atomistic molecular dynamics simulations [73]. In these studies, the current shortage of basic physical data on CL and CL-containing membranes presented a problem, because it impeded the authors' capacity to construct force fields that reflect the various nuances of the structure and dynamics of the CL molecule. The force fields used for these studies were ultimately derived from those developed for PC bilayers and the CL molecules generated *in silico* were obtained by adapting molecular models of PE (coarse-grained) and PG (atomistic). The lower-resolution, coarse-grained simulations were performed over the 10^{-7}–10^{-6} s timescale and were primarily intended to model the salt-induced phase preferences of CL and way in which it is affected the number of hydrocarbon chains present on the CL motif, as characterized by Powell and Marsh [66]. Despite the limitations noted above, the coarse-grained model was remarkably adept at capturing the salt-induced phase behavior of CL and the various derivatives studied by Powell and Marsh [66], as well as the way in which the polymorphism of these compounds are likely to be affected by changes in pH, ionic strength and surface charge. As illustrated in Fig. 8, these simulations accurately modeled the nature of the phases formed by the various CL derivatives at both neutral and low pH and provided useful insights into the mechanistic aspects of interconversions between the various phases, especially the lamellar and nonlamellar forms of these CL derivatives (e.g., note the stalks in Fig. 8-3B). However, because of the low-resolution inherent in coarse-grained molecular dynamics simulations [74], these studies provided very little insight into the molecular details of the process.

The higher-resolution atomistic simulations were performed on the $1–3\times10^{-7}$ s timescale and were intended to model the behavior of membranes composed of CL alone and mixtures of CL with POPC [73]. These studies suggest that CL bilayers should be more ordered than those of PC bilayers, and that the incorporation of CL into PC bilayers should have a significant ordering effect, even at CL concentrations near 9 mol%, predictions largely supported by experimental observations [56,59]. In both CL and CL-containing PC membranes, these simulations seemed to have captured the essence of the electrostatic features of the surfaces of such bilayers, as regards the magnitude of the surface potential and the way in which this property is affected by interactions with ions, in a manner consistent with previously published experimental observations. However, some of the results of these simulations seem to be counter-intuitive. For example, although the simulations adequately predict that dissolved cations (in this case Na^+) should interact preferentially with the CL component of POPC:CL membranes, they also predict that the carbonyl groups in the bilayer polar/apolar interfaces are the preferred binding sites for such ions, instead of the phosphates. Also, in both CL and CL-containing membranes, the hydrocarbon chain segmental order parameters calculated for CL are remarkably constant (except in the vicinity of the C=C bonds and at the very end of the acyl chain), and do not show the order plateau followed by a graded decrease in segmental order towards the bilayer center which typifies the segmental order profiles of most lipid bilayer membranes [75,76]. These aspects of the simulations run counter to both expectations and experimental observations and, as noted by the authors, they likely reflect shortcomings in the particular force fields used in these

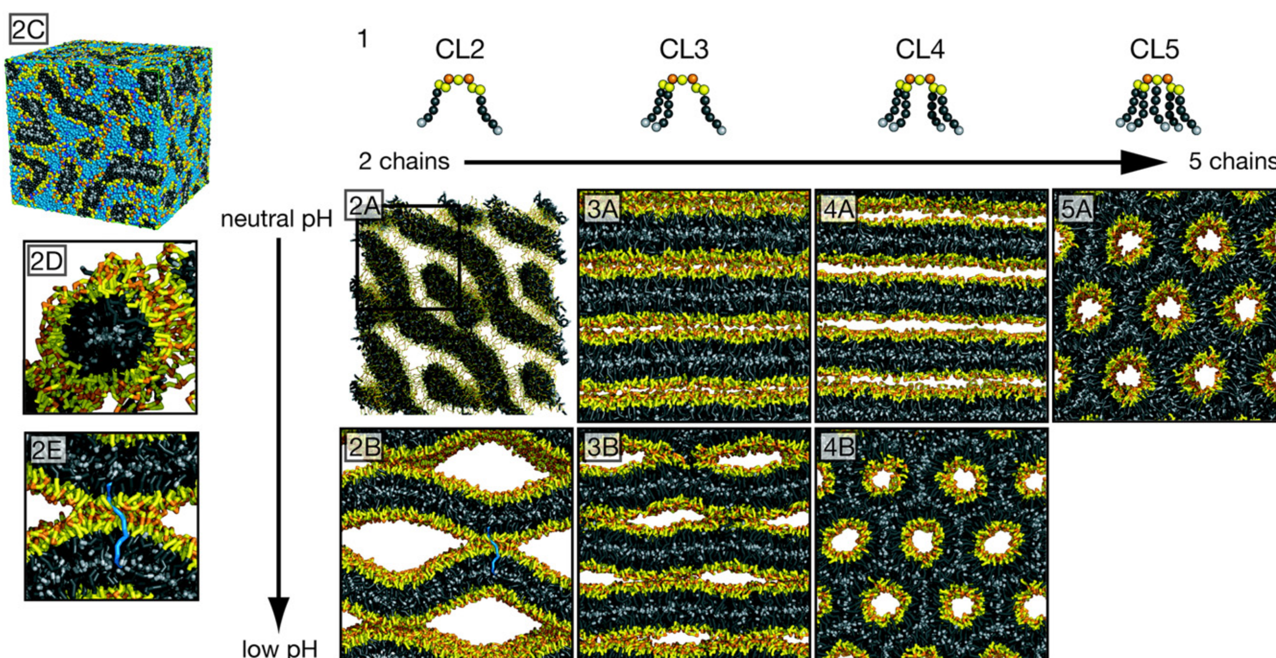

Fig. 8. Polymorphism of cardiolipin derivatives as modeled by coarse-drained molecular dynamics simulations. Water and ions have been removed for clarity: 1. Coarse-grained representation of the lipids. (2A) CL2 in micelles, with the head groups rendered thinner in order to show the hydrocarbon core. The periodic box is shown in black. 2B Lamellar CL2 with splayed-chain lipids. (2C) Micellar phase with 8000 CL2 lipids. Water and ions are shown in blue. (2D) Cross section of a cylindrical CL2 micelle. (2E) Close-up of (2B) with a splayed-chain lipid shaded in blue. (3A) Lamellar CL3 phase. (3B) Lamellar CL3 phase with stalks. (4A) Lamellar CL4 phase. (4B) Inverse hexagonal CL4 phase. (5A) Inverse hexagonal CL5 phase. Note the lighter shaded chain particle at the end of the acyl tails. Slightly different scales are used to show the characteristics of each phase. (Reproduced from Ref. [67], with permission).

studies. Further advancements the modeling of CL bilayers will thus be dependent on the capacity to construct force fields that more adequately reflect the nuances of the CL molecule, which will in turn require the acquisition of considerably more physical data on CL and CL-containing membranes than is available at present.

8. Concluding remarks

In principle, virtually all of the experimental data on the physicochemical properties of pure CL bilayers, and the demonstrable and/or presumed effects of CL on both model and biological membranes, can be directly or indirectly attributed to the restrictions placed on the flexibility and mobility of its polar headgroup by the tethering of the two phosphatidate moieties to a single glycerol molecule. Notwithstanding the obvious sparseness and lack of depth in the current database of knowledge about CL's and especially about CL-containing lipid bilayers, a fairly consistent picture of CL has emerged. The current consensus is that in the biologically relevant liquid-crystalline state, the impairment of the flexibility and reorientational mobility of the CL headgroup effectively reduces the effective cross-sectional area of the CL polar headgroup relative to that of its hydrocarbon chains, which in turn enhances its propensity for forming inverted nonlamellar phases, especially when the negatively charged phosphates are screened by bulk ionic strength effects, or through direct interaction with mono- and divalent cations and perhaps also peptides or proteins. The relatively small size of the CL polar headgroup, along with the impairment of its flexibility and reorientational mobility, also promotes cohesive interaction between the CL hydrocarbon chains and in the polar/apolar interfacial regions, properties which probably enhance the structural rigidity of CL-containing membranes. Finally, because of the small size and impaired flexibility of the CL headgroup glycerol, its capacity for steric self-shielding of the charged phosphate groups is diminished and as a result, the CL of cell membranes are probably more “exposed” to interaction with the solvent and entities dissolved therein. Most probably, this makes the CL component of cell membranes more prone to specific interactions with proteins, peptides and other entities, a property which may be important *in vivo*. Thus, the presence of CL seems to impart cell membranes with a unique suite of physical and chemical characteristics which may play an important role in the normal functioning of such membranes. However, this aspect of the functional role of CL is not well understood at present, a situation which is doubtlessly exacerbated by the current shortage of basic biophysical data on CL and CL-containing model membranes. Thus, further biophysical studies of the behavior and organization of CLs and CL-containing model membranes, as well as CL-containing biological membranes, would seem to be a priority.

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