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Increasing levels of cardiolipin differentially influence packing of phospholipids found in the mitochondrial inner membrane

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ABSTRACT

It is essential to understand the role of cardiolipin (CL) in mitochondrial membrane organization given that changes in CL levels contribute to mitochondrial dysfunction in type II diabetes, ischemia–reperfusion injury, heart failure, breast cancer, and aging. Specifically, there are contradictory data on how CL influences the molecular packing of membrane phospholipids. Therefore, we determined how increasing levels of heart CL impacted molecular packing in large unilamellar vesicles, modeling heterogeneous lipid mixtures found within the mitochondrial inner membrane, using merocyanine (MC540) fluorescence. We broadly categorized lipid vesicles of equal mass as loosely packed, intermediate, and highly packed based on peak MC540 fluorescence intensity. CL had opposite effects on loosely versus highly packed vesicles. Exposure of loosely packed vesicles to increasing levels of CL dose-dependently increased membrane packing. In contrast, increasing amounts of CL in highly packed vesicles decreased the packing in a dose-dependent manner. In vesicles that were categorized as intermediate packing, CL had either no effect or decreased packing at select doses in a dose-independent manner. Altogether, the results aid in resolving some of the discrepant data by demonstrating that CL displays differential effects on membrane packing depending on the composition of the lipid environment. This has implications for mitochondrial protein activity in response to changing CL levels in microdomains of varying composition.

1. Introduction

Cardiolipin (CL) is a unique anionic glycerophospholipid that is predominately localized to the inner mitochondrial membrane. CL has a variety of functional roles in the mitochondria, which range from direct binding to proteins of the electron transport chain, releasing cytochrome c in apoptosis, facilitating leakage of protons, and regulating mitochondrial structure and dynamics [1,2]. The importance of CL in mitochondrial bioenergetics is highlighted by the notion that changes in CL peroxidation, mass, and acyl chain composition is associated with numerous pathologies [1]. As an example, in the type I diabetic heart, the levels of tetralinoleoyl CL decrease at the expense of increasing levels of long chain polyunsaturated fatty acids [3]. Similarly, heart failure, Barth Syndrome, ischemia–reperfusion injury, aging, and breast cancer show decreased CL mass or remodeling that is driven by numerous factors including a reduction in CL biosynthesis and an increase in its degradation [1,4–10].

Many labs have focused on the structural role of CL as it relates to membrane phase behavior, fusion, fission, curvature, and morphology [11–13]. A combination of biophysical experiments, predominately in model membranes, show that CL's structural role is driven by its unique cone-shape, which is a result of four acyl chains with a relatively small headgroup [11,14]. As a consequence, CL has a propensity to form inverted hexagonal phase in synthetic bilayers, particularly in the presence of divalent cations,

Abbreviations: CL, cardiolipin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPI, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoinositol; DOPE, 1-2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-L-serine; LUV, large unilamellar vesicles; MC540, merocyanine 540; PC, phosphatidylcholine; PDPC, 1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphocholine; PDPE, 1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphoethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

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which is relevant for membrane fusion and curvature and ultimately mitochondrial function [15]. In contrast, much less is known about the role of CL on membrane packing. Earlier studies showed that CL served to decrease the area per molecule when interacting with other phospholipids, as calculated by percent condensation and elastic compressibility of monolayers on the air–water interface [16,17]. Similarly, electron spin resonance studies revealed that CL stabilized the bilayer by lowering fluidity [18]. However, these findings are not in agreement with a recent study in which fluorescence correlation spectroscopy measurements demonstrated CL increased the fluidity of supported bilayers made of phosphatidylcholine [19].

Given the lack of information and disparity in the literature on CL and membrane packing, the objective of this study was to determine if increasing levels of CL decreased packing in large unilamellar vesicles of equal mass but differing lipid composition. We approximated the relative mass of the major phospholipids of the inner mitochondrial inner membrane, which include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) [2]. We first categorized a range of primarily binary PC/PE mixtures and a select ternary (PC/PE/PS) and quaternary (PC/PE/PS/PI) lipid mixture as loosely packed, intermediate, or highly packed based on peak fluorescence intensity of merocyanine (MC540). We then studied the effects of increasing CL levels on these mixtures. The results reveal a complex picture, which shows that the effects of CL on membrane packing are not uniform and vary depending on the types of lipid vesicles that are interacting with CL. Overall, our data support the notion that CL generally decreases packing but in select cases can have no effect or even increase packing, which resolves some of the discrepant results in the field.

2. Materials and methods

2.1. Materials

Bovine heart cardiolipin (CL), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoinositol (DOPI), 1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphocholine (PDPC), and 1-hexadecanoyl-2-(4Z, 7Z,10 Z,13Z,16Z,19Z-docosahexaenoyl)-*sn*-glycero-3-phosphoeth an olamine (PDPE) were purchased from Avanti Polar Lipids (Alabaster, AL).

The composition of each batch of heart CL was verified with gas chromatography, using methods as previously described [20]. The composition of CL was predominately linoleic acid (~85–90%), oleic acid (~3–6%) and the remaining portion a mixture of several saturated and unsaturated fatty acids. HPLC-grade organic solvents were from Fisher Scientific. Merocyanine (MC-540) was acquired from Life technologies.

2.2. Synthesis of large-unilamellar vesicles (LUV)

All vesicles were made above the phase transition temperature of the highest melting phospholipid using stringent precautions to prevent oxidation. Multilamellar vesicles were first generated, as previously described [21]. Briefly, lipids containing MC540 (5×10^{-9} moles) were co-dissolved in chloroform and dried under a gentle stream of nitrogen gas. Subsequently, residual chloroform was removed in the dark with vacuum pumping for a minimum of 2 h. We ensured that 2 h of vacuum provided the same results as overnight vacuum pumping. Lipids were hydrated in 2 ml of 10 mM sodium phosphate buffer (pH 7.4) and subjected to three freeze–thaw cycles followed by extrusion with a 1.0 μm membrane (Avanti Polar Lipids) to generate LUVs. All studies were conducted to assess the impact of increasing CL mass on MC540 fluorescence. Thus, all PC/PE, PC/PE/PS, PC/PE/PS/PI mixtures (in the absence of CL) contained a total mass of 800 μg. CL levels were then increased from 200 to 800 μg. The ratios of PC, PE, PS, and PI approximated ratios found in the heart inner mitochondrial membrane [2].

2.3. Fluorescence measurements

MC540 fluorescence was measured at 23 °C on a PTI QuantaMaster 6000 Fluorometer and analyzed using PTFlex Software. Fluorescence excitation was set at 495 nm and emission scans were recorded from 540 to 660 nm.

2.4. Statistical analyses

All data sets are from multiple independent experiments, with most samples acquired in duplicates per experiment. The data displayed normalized distributions, which allowed for parametric analyses. The peak fluorescence intensity was analyzed for statistical significance with GraphPad Prism with a one-way ANOVA followed by a post hoc Bonferroni *t* test. $P < 0.05$ was considered significant.

3. Results

3.1. Membrane packing varies in lipid vesicles of differing composition

We first studied packing in different lipid vesicles that ranged in complexity from mostly two-component mixtures with different PCs and PEs to a select three and four component mixture that models the composition of the mitochondrial inner membrane (Fig. 1). We relied on MC540 fluorescence given that it an established probe for detecting lipid packing [22]. The vesicles displayed a range of packing, as expected, which we categorized as loosely packed (fluorescence greater than 1×10^6), intermediate (fluorescence in the range of 5×10^5–1×10^6), and tightly packed (fluorescence less than 5×10^5). DOPC/DOPE (40:30 w/w) vesicles showed the least packing whereas DOPC/DOPE/DOPS (40:30:10 w/w/w) vesicles displayed the tightest packing. The rest of the lipid mixtures of DOPC/PDPE (40:30 w/w), PDPC/DOPE (40:30 w/w), DOPC:DOPE:DOPS:DOPI (40:30:6:4 w/w/w/w), and DPPC:DOPE (40:30 w/w) displayed intermediate packing. Within the range of

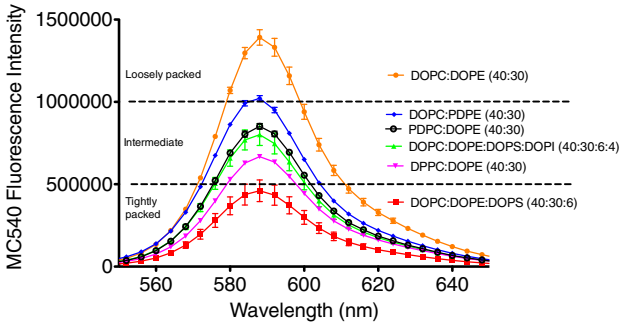

Fig. 1. Membrane packing in vesicles of equal mass varies in binary, ternary, and quaternary lipid mixtures. MC540 fluorescence was measured in vesicles of DOPC/DOPE (40:30 w/w), DPPC/DOPE (40:30 w/w), DOPC/PDPE (40:30 w/w), PDPC/DOPE (40:30 w/w), DOPC/DOPE/DOPS (40:30:6 w/w), and DOPC/DOPE/DOPS/DOPI (40:30:6:4 w/w/w/w). The dotted line indicates the designation for vesicles of high, medium, and low packing based on MC540 fluorescence. Data are average + S.E. from a minimum of 3 independent experiments.

intermediate packing, DOPC/PDPE was the least packed and DPPC/DOPE was the most packed.

3.2. CL exerts opposite effects on membrane packing in a dose-dependent manner

We next measured the effects of increasing levels of CL on the most loosely packed vesicles (Fig. 2A). The addition of 200 μg of CL to DOPC/DOPE (40:30 w/w) decreased MC540 fluorescence by 23% relative to the absence of CL (Fig. 2B). Increasing CL levels to 400 and 800 μg, respectively decreased fluorescence by 30% and 50% compared to the absence of CL (Fig. 2B). In comparison, the addition of CL to tightly packed vesicles of DOPC/DOPE/DOPS (40:30:6 w/w/w) increased MC540 fluorescence (Fig. 2C). Addition of 200 μg of CL increased MC540 fluorescence on average by 25% but failed to reach statistical significance. Addition of 400 and 800 μg increased fluorescence by 39% and 49%, respectively, compared to the absence of CL (Fig. 2C).

3.3. CL either has no effect or decreases packing in a dose-independent manner in vesicles of intermediate packing

We next tested the effects of increasing CL on vesicles of varying composition that displayed intermediate packing (Fig. 3). We present the results with the least packed to most packed vesicles in the category of intermediate packing. Addition of 200 μg CL to DOPC/PDPE (40:30 w/w) (Fig. 3A) increased peak MC540 fluorescence by 20% but had no effect at 400 and 800 μg of CL (Fig. 3B). In PDPC:DOPE (40:30 w/w) vesicles (Fig. 3C), inclusion of 200, 400, and 800 μg respectively increased fluorescence by 21%, 50%, and 18% (Fig. 3D).

CL increased fluorescence intensity at all three doses of CL with vesicles made of DOPC:DOPE:DOPS:DOPI (40:30:6:4 w/w/w/w). This model system was the most physiologically relevant given that it approximates the composition of the major phospholipids of the mitochondrial membrane (Fig. 3E). The addition of 200 μg of CL had the largest increase in fluorescence by 30% with the higher doses increasing fluorescence by 26% (Fig. 3F). Finally, in the more tightly packed vesicles of DPPC:DOPE (40:30 w/w) (Fig. 3G), CL increased fluorescence values by 25–27% for all of the doses of CL (Fig. 3H). Overall, in vesicles of intermediate packing, the effects of CL were not dose-dependent as observed for the loosely packed and tightly packed vesicles.

4. Discussion

4.1. CL has both condensing and fluidizing effects on model membranes

We initially hypothesized that CL would dose-dependently decrease packing in all of the lipid mixtures that we tested for two reasons. One, increasing the levels of membrane polyunsaturation, which is abundant in heart CL, generally lowers membrane packing due to increased conformational flexibility [23]. Second, a recent study elegantly showed with measurements of lipophilic dye diffusion coefficients at the single-molecule level that CL

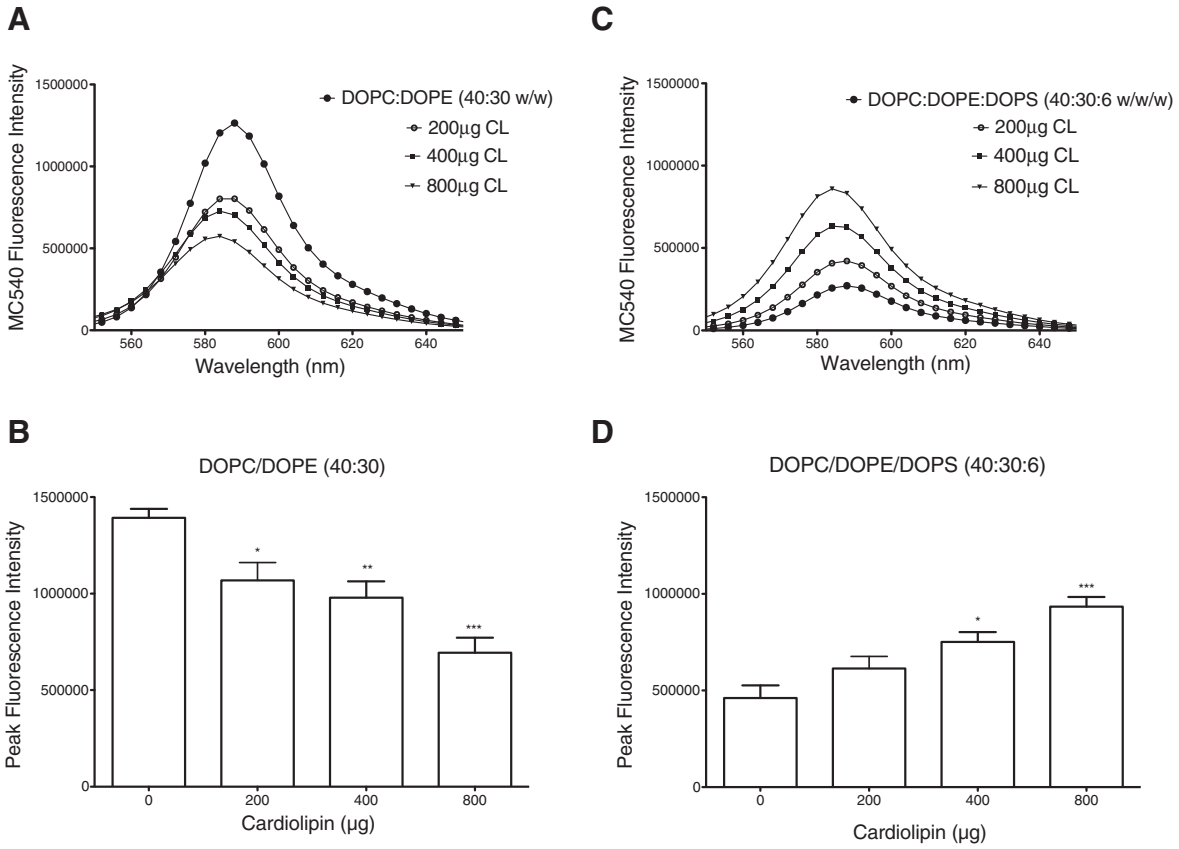

Fig. 2. CL exerts opposite effects on MC540 fluorescence in loosely packed compared to tightly packed vesicles in a dose-dependent manner. (A) Sample fluorescence MC540 emission spectra for DOPC/DOPE (40:30 w/w) vesicles with increases CL levels. (B) Peak MC540 intensity with increasing levels of CL for DOPC/DOPE vesicles. (C) Sample fluorescence MC540 emission spectra for DOPC/DOPE/DOPS (40:30:6 w/w/w) vesicles with increasing amount of CL. (D) Peak MC540 intensity with increasing levels of CL for DOPC/DOPE/DOPS vesicles. Data in B and D are average + S.E. from 3 independent experiments. Asterisks represent statistical significance from no CL controls, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

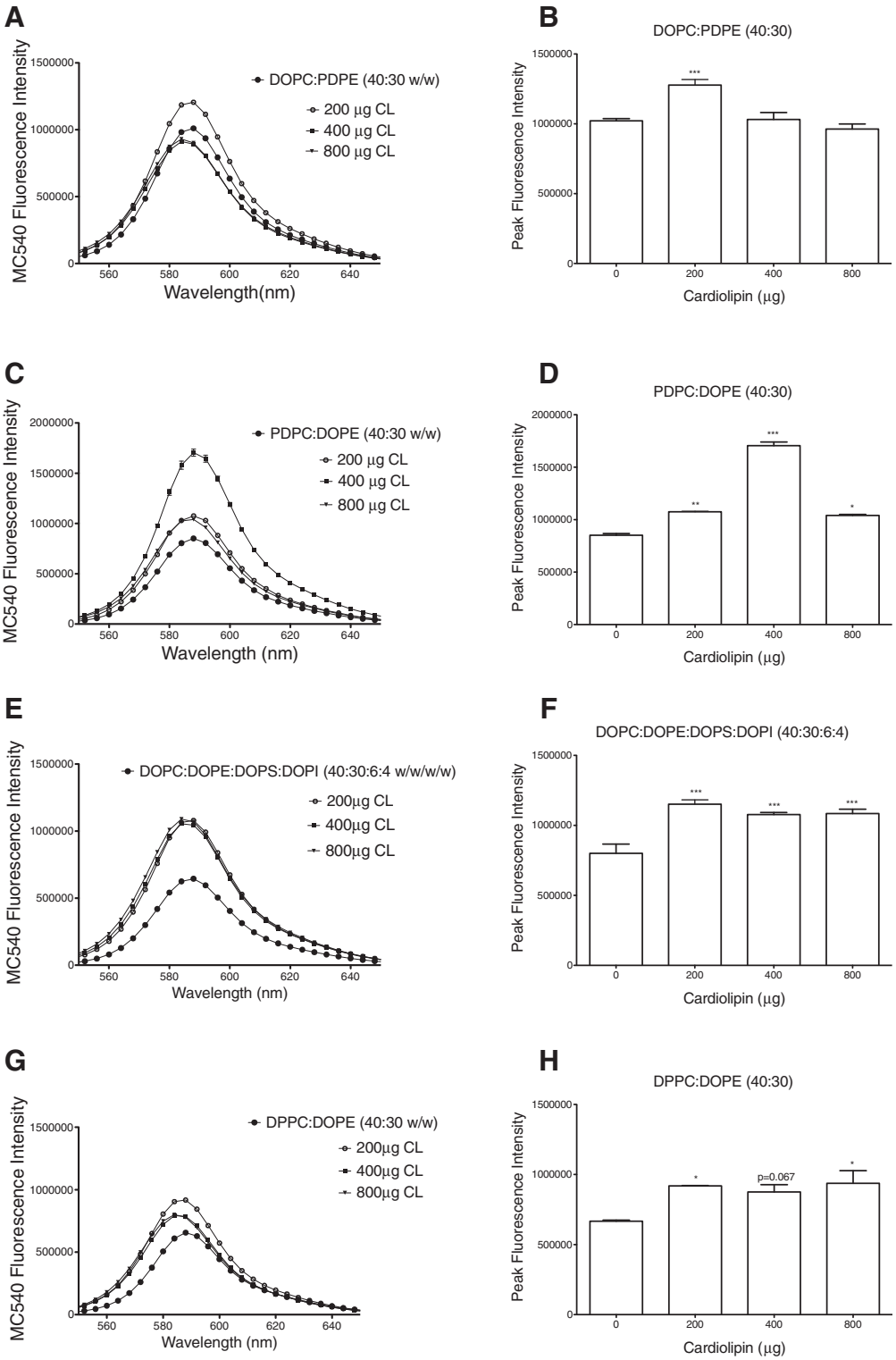

Fig. 3. CL has no effect or increases MC540 fluorescence in vesicles that display intermediate packing in a dose-independent manner. Sample fluorescence MC540 emission spectra and corresponding peak intensities for (A and B) DOPC/PDPE (40:30 w/w), (B and C), PDPC/DOPE (40:30 w/w), (D and E) DOPC/DOPE/DOPS/DOPI (40:30:6:4 w/w/w/w) and (F and G) DPPC/DOPE (40:30 w/w) vesicles with increasing amount of CL. Data in B, D, F, and H are average + S.E. from a minimum of 3 independent experiments. Asterisks represent statistical significance from no CL controls, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

increased membrane fluidity in a one-component egg PC bilayer and in a more complex model that approximated the mitochondrial inner membrane lipid composition [19]. However, we discovered that CL does not uniformly decrease packing in even simple two component vesicles.

The most striking result, contrary to our hypothesis, was that in DOPC/DOPE vesicles, CL increased packing (Fig. 2). Although this did not agree with the aforementioned single molecule diffusion study, the data are in agreement with monolayer studies and grazing incidence X-ray diffraction measurements that demonstrate CL

effectively condenses with PC molecules [17,24]. The stability of CL/PC mixtures is more effective when monolayers are formed in the presence of a sodium chloride buffer as opposed to water since the cation neutralizes the negatively charged CL phosphate groups. In our studies, we also relied on buffer containing sodium, which likely suppressed electrostatic repulsions with CL.

We did not find that CL increased packing when the PCs were changed from DOPC to DPPC or PDPC in the presence of DOPE. The rationale for using DPPC and PDPC in our two component studies was to address the impact of CL on two opposite extremes of PC in the presence of DOPE. DPPC is a high melting gel-phase lipid whereas PDPC is a low melting highly fluid phase phospholipid. Furthermore, PDPC is of biological significance given that omega-3 fatty acids can incorporate into phospholipids of the mitochondrial membrane [25]. In both cases (PDPC/DOPE and DPPC/DOPE), CL decreased packing. Therefore, the results show that within PC/PE mixtures, the effects of CL can be entirely opposite depending on the degree of unsaturation.

One possibility to explain why CL had no effect at select doses or a lack of dose-dependency in certain mixtures of lipids could be due to CL phase separating and sequestering MC540 within a CL-rich or CL-poor microdomain. As a result, the probe was unable to detect any additional changes in packing. While this could be a possibility, we do not favor this interpretation. A previous study demonstrated that MC540 could detect differences in the formation of domains based on its emission at two different wavelengths (between 593 and 625 nm) [22]. While we observed some small spectral shifts in the emission scans, there was no clear indication of the formation of distinct phase separated domains as measured by MC540 fluorescence in any of the lipid mixtures that were tested. An additional possibility could be that CL is inducing some unique structural arrangements in the bilayer that are not detectable with MC540 fluorescence. Indeed, there is precedence for CL to induce surface roughness at select doses and formation of flower-like domains [19].

4.2. Implications for the lateral organization of the mitochondrial membrane

The lateral distribution of lipids in the mitochondrial membrane is not homogenous [2]. In particular, it is likely that phospholipids in the mitochondrial membrane form microdomains of PC, PE, and CL of varying complexities. There is compelling evidence for the formation of CL and PE domains, particularly in bacterial membranes, which are sterol-poor and similar to mitochondrial membranes [26,27]. Furthermore, there are supporting data to show that CL microdomains are localized to regions of high curvature, which is critical for inner membrane morphology [27]. Recently, there is discussion that CL microdomains may facilitate the formation of respiratory supercomplexes in oxidative phosphorylation [28,29]. Our data suggest that local changes in CL levels, driven by a variety of pathologies, may differentially impact the packing of differing microdomains that regulate protein activity. In other words, it is plausible that localized changes in packing with a change in CL may serve to selectively disrupt protein activity, which could then prevent the formation of supercomplexes and impede electron flow and ATP production.

We acknowledge the limitations of this study, which was primarily the use of protein-free model membranes. Our rationale for conducting these studies in LUVs was to use a model system that allowed extreme control over the composition of the membrane and to maintain a constant mass of lipid. Nevertheless, future studies will have to address how lipid packing with different lipid mixtures are influenced by CL in the presence of select proteins and its influence on the movement of protons.

4.3. Summary

In conclusion, our data present a complex snapshot of how changes in CL levels impact membrane packing. Previous studies have yielded opposing results in which CL decreased membrane fluidity and had a condensing effect in monolayers and bilayers whereas a more recent report indicated that CL served to increase fluidity and destabilize the membrane in supported bilayers. Our results aid in resolving some of these discrepancies by demonstrating that although CL generally decreased packing to varying degrees in most vesicle systems, it is capable of increasing packing as well.

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