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CARDIOLIPIN FORMS HEXAGONAL STRUCTURES WITH DIVALENT CATIONS

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SUMMARY

The structures formed by precipitating acidic phospholipids, particularly cardiolipin, with divalent ions are described, with their structural dimensions. Ca^{2+}, Mg^{2+} and Ba^{2+} form hexagonal phases with cardiolipin, the phase being of the H_{II} type with water cylinders packed hexagonally in a hydrocarbon matrix. At 40° the distance between cylinder axes for Ca^{2+}, Mg^{2+} and Ba^{2+} is 50, 60 and 60 Å, respectively. The tendency for the transition from hexagonal to lamellar phase, by lowering the temperature of the precipitate for example, is in the order $Ba^{2+} > Mg^{2+} >> Ca^{2+}$. The Ca^{2+}/cardiolipin^{2-} mole ratio is 1 and this and the dimensions of the phase are practically independent of the concentration of the precipitating solution of $CaCl_2$ from 0.001 to 1.0 M. Ca^{2+} precipitates of two other acidic phospholipids, phosphatidylionistol and phosphatidylserine are lamellar structures. Ca^{2+} precipitates of various mixtures of lecithin and cardiolipin display a complex polymorphism between the hexagonal and lamellar phases. The potential relevance of these studies to those membranes that contain cardiolipin, particularly inner mitochondrial membranes, is briefly outlined.

INTRODUCTION

The ubiquitous nature of divalent cations, particularly Ca^{2+} and Mg^{2+}, in the biology of membranes is well documented. Ca^{2+} is required to maintain both the structural and functional integrity of cell membranes and functional changes are often entirely dependent on changes in divalent ion concentration. Until more is known about the structure of cell membranes however, the ways in which Ca^{2+} acts remain obscure. Of the number of components of cell membranes that interact with divalent cations, the acidic phospholipids do so strongly and these interactions have been studied in many ways, such as binding studies of the bulk lipid, interactions with monolayers of lipids and effects of divalent ions on thin lipid membranes (for example refs. 1–3). Although different divalent cations usually have the same qualitative effects in these model studies, for example the condensing of monolayers[1], they do so to different degrees and this is usually attributed to different affinities of the acidic groups of the phospholipid for the ion, or different screening effects of the ions.

Phospholipids in water form most often the familiar lamellar phase composed of alternating parallel layers of water, and bimolecular lipid leaflets. The thickness of

the water layer usually depends on the nature of the phospholipid and can reach large values when the bilayer contains charged groups providing electrostatic repulsion between leaflets that can overcome a long range van der Waals attraction between leaflets[4]. Ions, by screening the electrostatic charges on the acidic phospholipids reduce the electrostatic repulsion and the lamellar phase condenses to one that has a thin layer of water. Often the effect of divalent cations is not related to its concentration or ionic strength, showing that the ion is bound to the acidic group rather than screening it and indeed structural complexes, of Ca^{2+}–phosphatidylserine for example, have been described[1,2].

This report describes the structures formed by the interaction of divalent cations with one particular phospholipid, cardiolipin, which, while varying slightly in structure in each organelle, occurs in large amounts in bacteria membranes, mitochondria and chloroplasts, all of which contain respiratory assemblies. These studies show that Ca^{2+} instead of simply condensing the lamellar phase of cardiolipin in water, as it does for the acidic lipids phosphatidylserine[9] and phosphatidylinositol, converts it to a hexagonal structure. This observation has in fact been referred to[5] and the hexagonal structure was used in freeze-fracture studies of electron microscopy. This report is a detailed structural description of the system, with different divalent cations, using X-ray diffraction to determine the structural parameters. A brief discussion of the possible relevance of the hexagonal structure to the highly organized respiratory assembly of inner mitochondrial membranes is given.

MATERIALS AND METHODS

Cardiolipin (beef heart) and lecithin (pig liver) were obtained from Serdary Research Laboratories, stored in ethanol solution at −20°, assayed by thin-layer chromatography and used without further purification, always working under nitrogen. Ca^{2+} concentration was determined by atomic absorption spectrophotometry. Samples of phosphatidylserine and phosphatidylinositol were a generous gift of Dr. D. O. Tinker of the University of Toronto. All other reagents were of analytical grade and all solutions were made in double glass-distilled water.

X-ray samples were prepared in two ways. First, control samples (the lipid in water) of varying concentration (dry weight percent of lipid) were prepared by weighing the dry lipid and adding the required amount of water. Second, lipid was suspended in water (0.1 % by weight) by a Virtis homogenizer and 8 ml was added dropwise and with constant stirring to 75 ml of the divalent ion solution. The heavy precipitates that resulted were concentrated by centrifugation at 12000 × g for 15 min and mounted immediately as X-ray samples in equilibrium with the supernatant. In order to determine the water content of the structure formed, the precipitates had to be dried under a stream of nitrogen until the dimensions of the structure just began to change. At that point, drying was removing water from the structure and not from pools of excess supernatant. At this point the composition of the sample was determined; water by lyophilization of the sample[6], lipid by phosphorus analysis[7] and Ca^{2+} by atomic absorption spectrophotometry.

The X-ray technique, camera, and the two structures of concern in this paper have been described in detail before[8]. Basically the X-ray diagrams give the symmetry of the sample and the dimensions of that symmetry. The composition of the sample

determines how the molecules, lipid and water, must be packed into the structure. The two structures are shown in Fig. 1 and the various structural dimensions of these, given in the results, are calculated as previously described. We have used 1.00 as the partial specific volume of the lipid. For the purposes of comparison with other phospholipids we have considered the molecular weight of cardiolipin as 750 which is one half of a cardiolipin molecule containing one phosphate group and two fatty acyl chains although it is clear that cardiolipin is two of these 750 units covalently bonded at the polar end. The mol. wt. of lecithin is taken as 790.

RESULTS

Cardiolipin precipitates as a pure hexagonal phase at all concentrations of $CaCl_2$ from 0.001 to 1.0 M. The dimensions of the phase, shown in Table I, change very little over this range of $CaCl_2$ concentration. The hexagonal structure is maintained over the temperature range 4 to 40° with the usual change in dimension with temperature[8]. The Ca^{2+}/cardiolipin^{2-} mole ratio is one indicating the formation of stoichiometric combination of the ions in the precipitate. The internal packing within the hexagonal structure is determined from the composition of the hexagonal phase

TABLE I

PHASES OF PRECIPITATES OF CARDIOLIPIN WITH Ca^{2+}, Mg^{2+}, Ba^{2+}, AT THE DIVALENT ION CONCENTRATIONS AND TEMPERATURES INDICATED

Numbers in table give the repeat distance of the lamellar phase (L) or the distance between cylinder axes of the hexagonal phase H. L/H, lamellar phase predominates. H/L, hexagonal phase predominates.

$CaCl_2$ (M)	*4°*	*20°*	*40°*
	H	*H*	*H*
0.001		54.2	
0.01	58.9	54.2	50.4
0.05	59.2	54.5	50.4
0.07	58.9	53.4	50.4
0.1	58.5	53.3	50.4
0.3	58.2	52.9	49.9
0.7	57.7	52.9	49.8
1.0	55.4	52.9	47.6
$MgCl_2$ (M)		*H*	*H*
0.003	70.9/55.1 *H/L*	64.4	60.3
0.5	66.0 *H*	61.0	57.5
$BaCl_2$ (M)	*L/H*	*H/L*	*H*
0.01	47.5/65.3	63.5/47.7	61.8
0.05	47.2/65.1	61.9/46.2	60.8
0.07	47.2/65.3	61.9/46.3	60.1
0.1	47.6/65.1	62.6/—	60.6
0.3	47.3/65.9	62.6/47.1	59.4
0.7	—/61.9	59.5/—	—

and, as with all diacyl phospholipids, is as shown in Fig. 1. The phase is H_{11} with water cylinders and hydrocarbon matrix. The composition and structural dimensions of the phase are given in Table II for the three temperatures 4, 20 and 40° including the distance between cylinder axes, the diameter of the water cylinder, the polar to polar distance of the cardiolipin through the hydrocarbon matrix and the surface area S available to each half cardiolipin molecule on the water cylinder. All the dimensions of the hexagonal phases in Table II are consistent with hexagonal phases observed with other diacylphospholipids[8].

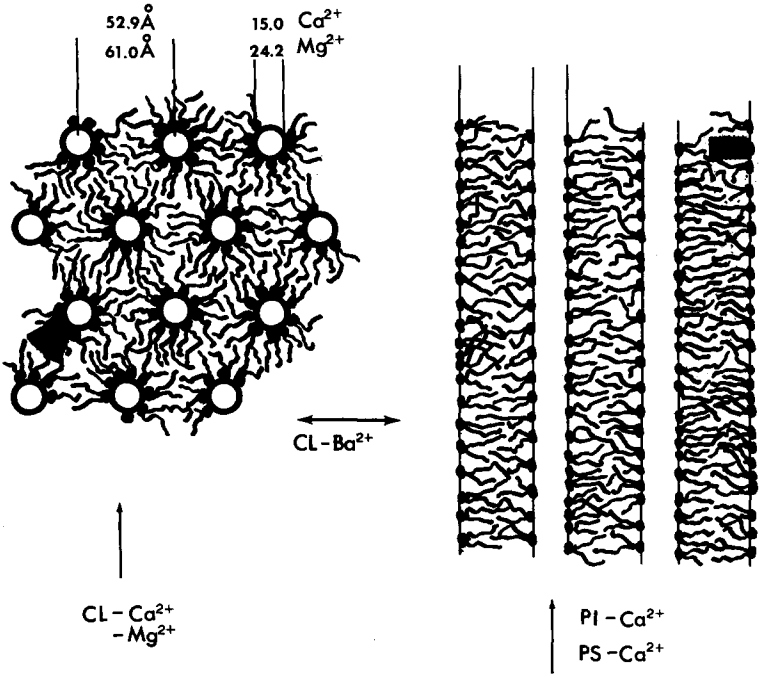

Fig. 1. Polymorphism of phospholipid–divalent ion complexes. Hexagonal and lamellar phases of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol (PI). Details of the structural dimensions and transitions are given in RESULTS.

Table I shows that $MgCl_2$ precipitates of cardiolipin are hexagonal phases over the concentration range like that of $CaCl_2$. However, for low temperatures and for the precipitates formed at low $MgCl_2$ concentrations a lamellar phase coexists with the hexagonal. The composition and structural dimensions within the Mg^{2+} hexagonal phase are shown in Table II. The water content of this phase and the diameter of the water cylinder are larger than those of the Ca^{2+}–cardiolipin precipitate but the polar to polar distances, indicating twice the average length of cardiolipin molecule, are identical.

Table I shows that $BaCl_2$ precipitates of cardiolipin, over the concentration range of $BaCl_2$ from 0.01 to 0.7 M, are coexisting lamellar and hexagonal phases, the relative quantity of the former decreasing as the temperature is raised until a pure hexagonal structure exists at 40°. The composition and structural dimensions of the hexagonal phase are shown in Table II and are similar to Mg^{2+}–cardiolipin precipates.

TABLE II

COMPOSITION AND STRUCTURAL DIMENSIONS OF THE HEXAGONAL PHASES OF TABLE I

Divalent ion	*Temp.* (°)	*Dry wt. and vol. % of lipid in precipitate*	*Distance between cylinder axes* (Å)	*Diameter of water cylinder* (Å)	*Polar to polar distance* (Å)	*S, surface area available to polar group at water interface* ($Å^2$)
Ca^{2+}	4	92.6	58.0	16.6	41.4	29.4
	20	92.6	52.9	15.0	37.9	27.4
	40	92.6	49.8	14.2	35.6	29.2
Mg^{2+}	4	85.7	66.0	26.1	39.9	33.1
	20	85.7	61.0	24.2	37.8	36.1
	40	85.7	57.5	22.8	34.7	38.0
Ba^{2+}	40	84.5	59.4	24.6	34.8	38.9

TABLE III

PHASE FORMED BY CARDIOLIPIN IN WATER

Dry wt. % lipid	*Phase*	*Dimensions*	
39.3–53.4	Lamellar	d_1	34.5–37.1 Å
≈53.4–85.7	Lamellar and hexagonal	—	
85.7–≈95	Hexagonal	Distance between cylinder axes	52.2–40.5 Å
		Diameter of water cylinders	23.8–11.0 Å
		Polar to polar distance	28.4–29.5 Å
		S	34.7–24.0 $Å^2$

Table III shows, as control studies, the phases formed by cardiolipin at various water concentrations. At 39 % to approx. 53 % a single lamellar phase exists, the thickness of its lipid leaflet varying from 34.5–37.1 Å. From approx. 85–95 % a single hexagonal phase exists whose distance between cylinder axes varies from about 52–40 Å. The concentration range over which the lamellar and hexagonal phases coexist varied from batch to batch of cardiolipin and may have resulted from a small but variable divalent ion content of the lipid. Typical Ca^{2+}/cardiolipin ratio of the cardiolipin was 1/30. Nevertheless on either side of this region of mixture of the two phases the single phases exist.

Table IV shows that Ca^{2+} does not give precipitates of hexagonal phase for two other acidic phospholipids common to membranes, phophatidylserine and phosphatidylinositol. The precipitates are lamellar and their dimensions are given in the table.

TABLE IV

PHASE OF Ca^{2+} PRECIPITATES OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLINOSITOL FROM 0.1 M $CaCl_2$

Phosphatidylserine	Lamellar	d = 51.8 Å	d_1 = 40.4 Å
Phosphatidylinositol	Lamellar	d = 51.3 Å	d_1 = 44.5 Å

TABLE V

MIXTURES OF LECITHIN AND CARDIOLIPIN AT VARIOUS LECITHIN/CARDIOLIPIN MOLE RATIOS

Lecithin/cardiolipin		*75/25*	*70/30*	*60/40*	*50/50*
In water	d	53.5	55.0	48.4	59.1
(all lamellar phases)	d_1	39.5	41.5	37.6	41.4
Precipitates in 0.1 M $CaCl_2$		Lamellar (d = 68.0 Å) and other phases	Lamellar (d = 65.7 Å) and other phases	Hexagonal phases, distance between cylinder axes 79.2 Å	72.0 Å

Table V shows that, in a very restricted survey, mixtures of the zwitterionic phospholipid, lecithin, and cardiolipin at various mole ratios give single lamellar phases in water, even at concentrations where pure cardiolipin gives the hexagonal phase, and that 0.1 M $CaCl_2$ precipitates of these mixtures give coexisting phases at the lower cardiolipin contents and a single hexagonal phase at higher cardiolipin contents but of quite larger dimensions than those of the pure cardiolipin hexagonal phases.

DISCUSSION

The most interesting observation in these results is that the interaction of cardiolipin with divalent ions yields a precipitate of hexagonal structure. The Ca^{2+}/cardiolipin ratio is one indicating the formation of an insoluble salt of cardiolipin with Ca^{2+} bound to the two phosphate groups of the whole cardiolipin molecule. Except for the presence of Ca^{2+}, the composition, structure and dimensions of this precipitate are similar to those of cardiolipin with little water present, indicating that Ca^{2+}, in neutralizing the charge on the cardiolipin, collapses the swelled lamellar phase to the extent of removing all but 7.4 % of the solution from the lipid although it is still in equilibrium with it: *i.e.* the structural effect of Ca^{2+} is equivalent to dehydrating the lipid. The Ca^{2+}–cardiolipin affinity is sufficient for the complex to form equally well in 0.001 as in 1.0 M $CaCl_2$. In general, the formation of the hexagonal phase with phospholipids, and indeed the reversible transition between hexagonal and lamellar phases, is understood on the basis of the shape and interactions of the amphiphilic molecules as determined by the relative volumes of the hydrophilic and hydrophobic parts of the molecule. If the shape is approximately cylindrical, as one molecule is outlined in the lamellar phase of Fig. 1, the molecules can pack in a plane. If the molecules are conical or pie-shaped (Fig. 1) they cannot pack in a plane thus accounting for the curvature around the water cylinders in the hexagonal phase. Cardiolipin either at low water content, Table III, or "dehydrated" by Ca^{2+} results in a tighter packing of the hydrophobic head groups, giving a low value for S, the area available per polar group at the water interface, compared to the lamellar phase where it is about 60°Å^2, and the hexagonal phase forms. Even lowering the temperature to 4°, which reduces the thermal motion and therefore volume of the hydrocarbon chains, does not result in transition to the lamellar phase as it does in some other systems. One reason for this apparent bulkiness of the hydrocarbon chains in cardiolipin may

well be their particularly high degree of unsaturation compared to phosphatidylinositol and phosphatidylserine[11]. In order to ensure that the formation of a hexagonal structure was not a general property of the acidic phospholipids, phosphatidylserine and phosphatidylinositol were precipitated with 0.1 M $CaCl_2$ and both gave lamellar structures confirming the results of others[9].

In the Mg^{2+}, Ca^{2+}, Ba^{2+} series, Ca^{2+} appears to be optimal in forming the single hexagonal structure; in condensing the structure thereby forming the smallest diameter water cylinders. Both a smaller ion, Mg^{2+}, and a larger one, Ba^{2+}, form more hydrated structures with larger water channels and larger area per polar group at the water interface (Table II), and these ions result in structures with higher probability of hexagonal–lamellar transition (Table I). This series, $Ca^{2+}>Mg^{2+}>Ba^{2+}$, is paralleled in other systems, for example, their condensing effect of monolayers of other acidic phospholipids[1], where Ca^{2+} has a greater effect than either Mg^{2+} or Ba^{2+}, and this is often attributed to differences in binding constants between the divalent ion and the phospholipid. The present results would suggest that, compared to Mg^{2+} or Ba^{2+}, Ca^{2+} is optimal in reducing the size of the polar group of the cardiolipin molecules, and thereby changing the shape of the molecule, by binding to, and reducing the distance between, two phosphate groups either within or between cardiolipin molecules.

The various mixtures of lecithin and cardiolipin as model membranes would represent variation in acidic phospholipid content of membranes. They show that in water the cardiolipin could be stabilized into the lamellar structure at much higher dry weight concentrations than could pure cardiolipin. Ca^{2+} precipitates of lecithin/cardiolipin mixtures showed a number of co-existing phases for low cardiolipin content and pure hexagonal phases at higher cardiolipin contents but of larger dimensions than those of pure cardiolipin.

Although the relevance of the lipid hexagonal phase to biological membranes is still speculative, much evidence from electron microscopy is accumulating showing that hexagonal arrays do exist in some cell membranes. The present results suggest that one might expect such arrays in mitochondria where there are high levels of cardiolipin and Ca^{2+}. LUZZATI *et al.*[10] and others have suggested that if the hexagonal structure, such as described here, is stabilized even transiently in the plane of a membrane so that the aqueous channels are perpendicular to its plane, the membrane would have vastly different permeability properties than those of a bimolecular lamellar layer. In order to traverse the membrane the channels, which are lined with the polar groups of the cardiolipin molecules whose long axis is perpendicular to the channel axis (Fig. 1), would have to be about 20 cardiolipin molecules long. The results reported here suggest more factors that could effect both changes in the dimensions of the hexagonal spacing and transitions between lamellar and hexagonal structures. These transitions could take place on the whole membrane or more likely on patches whose size vary with the conditions of the media. For example, as cardiolipin competes with other membrane components for Ca^{2+}, or if Ca^{2+} concentration changes as it does in mitochondria, then the relative amount of the hexagonal and lamellar phases could vary. Also as Ca^{2+} and Mg^{2+} concentrations change the size of the aqueous channels would change. Finally, as the relative concentration of cardiolipin to other phospholipids may vary over the surface of the membrane, the structure over the surface would again vary.

The aqueous channels of all these hexagonal phases are very large compared

to what is normally considered a membrane "pore". However, these channels might well be the site of attachment for membrane proteins. This raises a number of interesting possibilities that will be discussed in terms of inner mitochondrial membranes. Although these hypotheses are purely speculative, they are suggested by the experimental observations of (a) the coincidental occurrence of cardiolipin and respiratory assemblies in membranes[11,12], (b) the affinity of cardiolipin for divalent ions and the structural polymorphism that results, and the requirement for a discrete spatial arrangement of the electron transport components[13], (c) the intimate connection between the energy of electron transport and that of oxidative phosphorylation and Ca^{2+} accumulation in mitochondria.

First, the hexagonal arrangement of sites would provide a lipid substratum for an orderly arrangement of proteins and it may not be coincidental that cardiolipin is a major component of those membranes that have respiratory assemblies. Indeed, the inner membrane of mitochondria, wherein lies all the cardiolipin of that organelle[14] as well as the respiratory assemblies, show occasionally a regular arrangement of subunits in the plane of the membrane which may reflect the underlying lipid structure[15,16]. The centre to centre spacing of the channels with pure cardiolipin, 54Å for Ca^{2+} and 64 Å for Mg^{2+}, would be the right order for the spacing between cytochromes, 40–60 Å. Furthermore it has been reported[17] that arrangement and the distance between membrane core particles, of 50–100 Å diameter, undergo changes when the respiratory state of the mitochondria is changed, the distance decreasing for example, during oxidative phosphorylation. These changes may well reflect or indeed result from changes in the lipid matrix in which the particles are found; changes brought about by a variation in Mg^{2+}/Ca^{2+} ratio for example.

The second noteworthy aspect of the channels as sites of protein attachment is that the channel provides a means for a protein to penetrate the membrane gaining access to both sides. One prime direct use of the energy of electron transport of the respiratory chain of mitochondria is the active transport of ions, particular Ca^{2+}, into the inner compartment, an alternative use of the energy to oxidative phosphorylation. Since the proteins of the respirator assembly are coupled to Ca^{2+} movement across the membrane, having access to both sides of the permeability barrier by means of the "channel", would give the protein, such as cytochrome *c*[18], access to outside Ca^{2+} and may represent the site of Ca^{2+} movement. The control of whether the energy of electron transport is directed to oxidative phosphorylation or to Ca^{2+} transport is obscure but changing levels of Ca^{2+} and Mg^{2+}, in changing the dimensions of the substratum, might well influence the arrangement in the respiration assembly affecting the energy flow.

Finally, any transition from hexagonal to lamellar phase would destroy the regularity of the lipid substratum and would confine the attached protein to one side of the bilayer, unless hydrophobic penetration of the protein through the molecular bilayer occurred.

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