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THERMAL ADAPTATION OF *TETRAHYMENA* MEMBRANES WITH SPECIAL REFERENCE TO MITOCHONDRIA

ROLE OF CARDIOLIPIN IN FLUIDITY OF MITOCHONDRIAL MEMBRANES

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During temperature acclimation of *Tetrahymena pyriformis*, the changes in fluidity and composition of total lipids from three membrane fractions, mitochondria, pellicles and microsomes were studied by a spin-label technique using a stearate probe and thin-layer and gas-liquid chromatography. The increase of fluidity observed in microsomal and pellicular lipids following the temperature shift from 39 to 15°C corresponds with the increase of the ratio of total unsaturated to saturated fatty acid content. However, despite the increase of this ratio, the fluidity of mitochondrial lipids was found to be constant up to 10 h after the temperature shift. The fluidity of total lipids of mitochondria isolated from *Tetrahymena* cells grown at 39°C was not changed by removal of cardiolipin, whereas cardiolipin-depleted lipids of mitochondria from 15°C-acclimated cells showed a decrease in fluidity. The re-addition of cardiolipin to the mitochondrial lipids depleted of cardiolipin restored the fluidity to the initial level, thereby confirming the rigidifying effect of cardiolipin in cold-acclimated cells. These results suggest that cardiolipin may be implicated in maintaining consistent fluidity of mitochondrial membranes against change in thermal environment.

Introduction

Recent studies have shown that many membrane-bound enzymes are closely associated with lipids [1] and their activities are particularly affected by the physical state of the surrounding lipid environments [2]. Therefore, a variety of microorganisms strive to adapt to changed growth conditions by modifying the membrane lipid composition. It is well known that changes in temperature cause alterations in the composition of membrane lipids, especially fatty acyl chains.

As reported earlier [3], a thermotolerant strain of *Tetrahymena pyriformis* NT-1, has proved an attractive system for studying the molecular mechanism of temperature adaptation of cells. This free-living eukaryotic cell has been observed to undergo changes in the fatty acyl chain as well as phospholipid composition in response to growth temperature variation [4–7] We have conducted extensive studies using *Tetrahymena* to elucidate the regulatory mechanism of unsaturation of phospholipid fatty acid composition and the effect of altered lipid composition upon membrane fluidity during temperature adaptation. It has been shown that the fluidities of various subcellular organelles of *T. pyriformis* adjust in response to a new thermal environment by modifying fatty acid composition [6]. Earlier studies gave evidence which suggests that palmitoyl-CoA desaturase activity is somehow correlated with membrane fluidity [8–10]. Two possible mechanisms were proposed for temperature-associated enhancement of fatty acid desaturases: induction of palmitoyl-CoA desaturase [11–13] and activation due to altered fluidity

[14,15]. We reported the correlation between fluidity and fatty acid composition for three major phospholipid species of *Tetrahymena* whole cells during temperature acclimation [16].

In the present study, we have made attempts to obtain further information as to how lipid composition corresponds with the fluidity of various membrane fractions (mitochondria, pellicles, microsomes) isolated at different acclimating stages of *Tetrahymena* cells. The results obtained from this work lead us to propose a concept that cardiolipin may play an important role in the regulation of membrane fluidity of mitochondria

Materials and Methods

Cell culture. Cells of a thermotolerant strain of *Tetrahymena pyriformis* NT-1 were grown in an enriched medium at 39°C as described previously [7] Cells in the logarithmic growth phase were used in all experiments

Conditions for temperature shift. All procedures were carried out according to the previous paper [11] For the temperature shift-down experiments, cells were grown at 39°C in 200 ml medium to a cell density of approx. $(2.0–3.0) \cdot 10^5$ cells/ml, and were cooled to 15°C over 30 min by swirling the flask in an ice-water slurry. The rate of cooling was essentially linear (0.8 K/min) and was monitored by placing a sterile thermometer directly into the medium. After cells were cooled to 15°C, cell division did not occur for approx. 10 h.

Isolation of membrane fractions from Tetrahymena pyriformis. Various membrane fractions, mitochondria, pellicles and microsomes, were isolated according to the method of Nozawa and Thompson [17] using a phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/3 mM EDTA/0 1 M NaCl, pH 7.2).

Lipid extraction and analysis. Lipids were extracted from individual membrane fractions or whole cells by the method of Bligh and Dyer [18], and the resultant lipids were stored in $CHCl_3/CH_3OH$ (6 1, v/v) at −20°C. Phospholipid phosphorus was determined by the method of Bartlett [19] modified by Marinetti [20]. Neutral lipids and phospholipid species were separated by silica gel H thin-layer chromatography developed by $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (75 · 25 : 5 · 2.2, v/v). Each spot was identified from standard phospholipids (egg-yolk phosphatidylcholine, human platelet phosphatidylethanolamine and bovine heart cardiolipin). The desired phospholipid fraction(s) was scraped off the plate and extracted by the method of Bligh and Dyer [18]. Quantitative analysis of fatty acids was carried out by GLC as described previously [8]. Bovine heart cardiolipin was purchased from Sigma Chemicals Co. and used without further purification.

ESR spectroscopy. The stearic acid spin probe, *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, was purchased from Syva Associates (Palo Alto, CA). For preparation of spin-labeled liposomes, the lipid extract dissolved in $CHCl_3/CH_3OH$ (6 1, v/v) was mixed with 1 mol% of the spin probe in benzene and the solvent was evaporated first under a stream of N_2 gas and then by evacuation To this lipid mixture, 0 1 ml of Tris-buffered saline (150 mM NaCl/50 mM Tris-HCl, pH 7.5) was added, and the mixture was sonicated for 30 s with the microtip of a Branson sonifier (B-12) set at 1.

The spin-labeled liposomes thus prepared were taken into a glass capillary and ESR spectra were measured at various temperatures using a commercial X-band spectrometer (JEOL FE-2X) equipped with a temperature controller. The parallel ($T'_{/\!/}$) and perpendicular ($T'_{\perp}$) principal values of the hyperfine tensor of an axially symmetrical spin Hamiltonian were estimated from the ESR spectra and the order parameter, S, was calculated using the following relation.

$$S = \frac{T'_{/\!/} - T'_{\perp}}{T_{zz} - \frac{1}{2}(T_{xx} + T_{yy})} \cdot \frac{a}{a'}$$

where $a = (T_{xx} + T_{yy} + T_{zz})/3$, $a' = (T'_{/\!/} + 2T'_{\perp})/3$, $T_{xx} = T_{yy} = 5.9$ G and $T_{zz} = 32.9$ G are the hyperfine principal values of the nitroxide radical.

Results

Changes during the cold acclimation in membrane lipid composition

Mitochondria, pellicles and microsomes were isolated from *Tetrahymena* cells at different time intervals in the course of thermal adaptation and their lipid compositions were analysed. Phosphatidylcholine, phosphatidylethanolamine and 2-aminoethylphosphonolipid (glycerol type) are the major compo-

TABLE I

ALTERATIONS IN PHOSPHOLIPID COMPOSITION OF VARIOUS MEMBRANE FRACTIONS FROM *TETRAHYMENA PYRIFORMIS* NT-1 CELLS DURING TEMPERATURE ACCLIMATION

Various membrane fractions were isolated from cells harvested at indicated intervals according to the procedure of Nozawa and Thompson [17], and their lipids were extracted by the method of Bligh and Dyer [18]. Individual phospholipids were separated by silica gel H TLC and the phosphorus content of each phospholipid was determined Times (h) are those elapsed after the shift down to 15°C The values in parentheses are the number of experiments performed. Values in the table are relative percentages of total phospholipids

Phospholipid	Mitochondria				Pellicles				Microsomes			
	(39°C) (2)	2 h (2)	6 h (2)	10 h (2)	(39°C) (3)	2 h (2)	6 h (2)	10 h (2)	(39°C) (3)	2 h (2)	6 h (3)	10 h (2)
Phosphatidylcholine	27.7	26.3	26.0	35.7	20.0	17.5	16.8	18.1	28.5	27.8	28 2	26 3
Phosphatidyl-ethanolamine	39.7	44 0	40.1	42.4	48 9	45 1	49 9	50 3	46.0	45.9	45 9	49 2
2-Aminoethyl-phosphonolipid [a]	11.6	10.2	10.6	12 2	15.2	16 3	16 8	16.0	14.4	12 5	13.0	12.4
Cardiolipin	13 8	11.3	11.1	12.6	4.2	4.3	5.7	2 4	2 2	2.5	2.1	2.9

[a] Glycerol-type.

nents in these membranes. Cardiolipin is richest in mitochondria. Little or no change was seen in the phospholipid composition of any membrane fraction within 10 h after temperature shift to 15°C (Table I). On the other hand, a marked alteration in temperature changes was observed to occur in fatty acid composition (Table II). Upon shifting 39°C-grown cells to 15°C, there was a gradual decrease in palmitic acid (16 . 0), a principal saturated fatty acid, in all three membrane fractions. Coincidently, γ-linolenic acid (18 : 3), which is the most abundant unsaturated fatty acid, was observed to increase in relative percentage. Although it has been suggested in the previous study using whole cells that the increase in palmitoleic acid (16 : 1) within 4–6 h after the cold shift would be implicated in the quick thermal adaptation of cell membrane lipids [11], the enhanced level of this fatty acid was seen with pellicles and microsomes but not with mitochondria, which are most unsaturated as indicated by the *U/S* (unsaturated/saturated) ratio.

Changes during the cold acclimation in the membrane lipid fluidity

The changes in the *U/S* ratio following the temperature shift were displayed in Fig. 1A, which indicates its progressive increment in all three membranes. In order to examine changes in physical states of membrane lipids during the cold acclimation, ESR spectrometry using a stearate spin probe was carried out at 15°C for liposomes derived from mitochondria, pellicles and microsomes. The time-course of change in the order parameters for liposomes prepared from membrane fractions isolated from 39°C-grown cells (Fig. 1B) showed that initially the parameters were in the order mitochondria < microsomes < pellicles. After the temperature shift, the order parameter remained constant for mitochondrial lipids but decreased gradually for pellicular and microsomal lipids, the latter showing an order parameter even lower than that of mitochondrial lipids at 10 h after the shift. This was confirmed by another experiment using a fluorescent probe, diphenylhexatriene (data not shown).

Effects of cardiolipin upon the membrane lipid fluidity

It is generally accepted that the membrane fluidity depends on the proportional content of unsaturated fatty acids in membranes. As can be seen in Fig. 1A and B, changes in the order parameter of pellicular and microsomal membranes appeared to be correlated in an inverse manner with changes in the *U/S* ratio. But in mitochondrial membrane this was not the case.

TABLE II

ALTERATIONS IN FATTY ACID COMPOSITION OF VARIOUS MEMBRANE FRACTIONS FROM *TETRAHYMENA PYRIFORMIS* NT-1 CELLS TEMPERATURE ACCLIMATION

Various membrane fractions were isolated from cells harvested at indicated intervals according to the precedure of Nozawa and

Fatty acid	Mitochondria					Pellicles	
	(39°C)	2 h	4 h [a]	6 h	10 h	(39°C)	2 h
14 0	5.4 ± 0.7	5.3 ± 1.0	5.8	4.8 ± 1.3	4 2 ± 0 8	8.1 ± 1.2	8.1 ± 1.0
16 0	12.4 ± 0.9	10.9 ± 1.2	10.1	7.7 ± 1 4	6.6 ± 0 8	17.6 ± 1.1	15.4 ± 0 9
16 1	10 0 ± 0 6	11.8 ± 1 2	11 5	11.5 ± 0.9	10.5 ± 1.1	9.6 ± 0.6	12 3 ± 0.7
16 2 [b]	4 9 ± 1.1	5.8 ± 1.4	4.6	5.6 ± 0.4	5.6 ± 0 8	4.5 ± 0 7	5.5 ± 1 1
18 1	9 4 ± 1.7	7.4 ± 0.9	6.8	7.0 ± 1.1	6 0 ± 0 2	11.9 ± 1.7	8.5 ± 0 5
18 2	19.0 ± 0 9	19.4 ± 0 7	17.9	20.3 ± 0.9	20.3 ± 1.6	15.4 ± 0.5	16.2 ± 0 3
18 . 3 [c]	27.2 ± 0.5	28.9 ± 1.6	29.5	32.7 ± 3.1	36.2 ± 2.5	19.7 ± 2.3	20 9 ± 1.1
U/S	2 62 ± 0.21	2.92 ± 0 37	3 04	3 79 ± 0.72	4 38 ± 0.41	1 60 ± 0 23	1 76 ± 0 13

[a] Data from a single experiment

[b] Contains also small amounts of 17 0 which was not separable from 16 2 under conditions used .

[c] A ratio of unsaturated to saturated fatty acids

Despite a considerable increase in the *U/S* ratio, the fluidity was kept constant. A conspicuous difference in lipid composition between mitochondria and the other two membranes (pellicles and microsomes) was a much higher content of cardiolipin in mitochondria (Table I). One would then expect that this phospholipid might play a role in maintaining the consistent fluidity. Therefore, some experiments were done to examine effects of cardiolipin upon the physical state of mitochondrial lipids. Cardiolipin was removed by thin-layer chromatography from mitochondrial total lipids extracted from 39°C-grown cells and 15°C-acclimated cells, and the cardiolipin-depleted mitochondrial lipids were studied by ESR (Table III). It was found that removal of cardiolipin caused no change in the order parameter of mitochondrial lipids from 39°C grown cells, whereas it did reduce the parameter (0.651 → 0.637) of mitochondrial lipids from 15°C-acclimated cells. This implies that cardiolipin would exert a rigidifying effect in mitochondria of the acclimated cells.

TABLE III

EFFECTS OF CARDIOLIPIN ON FLUIDITY OF MITOCHONDRIAL LIPIDS FROM *TETRAHYMENA*

Mitochondria were isolated from *Tetrahymena* cells grown at 39°C or cells acclimated to 15°C for 10 h. Lipids were extracted by the method of Bligh and Dyer [18]. Cardiolipin was removed from total lipids by silica gel H TLC using a solvent system of $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (75 . 25 · 5 2.2, v/v) Order parameter, *S*, was calculated from ESR spectra measured at 15°C with 5-nitroxide stearate spin probe

	Mitochondrial lipids from		Cardiolipin-free mitochondrial lipids from	
	39°C-grown cells	15°C-acclimated cells	39°C grown cells	15°C acclimated cells
S	0.651	0 651	0.651	0 637

Then in order to see whether this cardiolipin effect is specific to mitochondrial lipids, ESR experiments were carried out with whole-cell phospholipids, giving rise to the comparable findings which confirm the rigidifying effect of cardiolipin in 15°C-acclimated cells. Table IV demonstrates the influence of added cardiolipin content upon the order parameter of cardiolipin-free phospholipids. The effect of lowering fluidity in 15°C-acclimated cells was observed to be dependent upon the concentration of cardiolipin which was extracted from either 39°C-grown cells or 15°C-acclimated cells Bovine heart cardiolipin exhibited a similar but less marked effect. Such difference might be due to the different fatty acid

Thompson [17], and their lipids were extracted by the method of Bligh and Dyer [18] Fatty acids were methylated and analysed by GLC. Values are averages of four experiments and are expressed as relative percentages of total fatty acids ± S D. Times (h) are those elapsed after shift to 15°C.

			Microsomes				
4 h [a]	6 h	10 h	(39°C)	2 h	4 h [a]	6 h	10 h
8.6	6 8 ± 1 3	7.5 ± 1.2	7 0 ± 1.3	7.8 ± 1 5	7 9	6.7 ± 1.3	6.7 ± 0.7
13.4	10 4 ± 1.5	10.9 ± 0 8	15.5 ± 0.6	12.7 ± 1.0	10.9	8.1 ± 1.2	7.6 ± 0.6
13.4	13.0 ± 0.5	11.7 ± 1.1	10.9 ± 0.7	14.5 ± 1.5	14 5	14.6 ± 0.8	13.6 ± 1 1
5.1	6 0 ± 0.2	5.7 ± 0.9	5.7 ± 1.1	6.8 ± 1.5	5.9	7.3 ± 0.7	7.0 ± 1.2
7.4	7.4 ± 0.9	7.8 ± 0.6	12.4 ± 2.7	6 5 ± 0.9	6.6	6.1 ± 0.7	6.0 ± 0.7
16 3	17 9 ± 1.0	17.9 ± 0.8	16.0 ± 1.0	16.4 ± 1.1	16.8	17.4 ± 1.5	18.4 ± 2.3
20 8	25.3 ± 3.3	25.1 ± 1.3	20.5 ± 1.2	21.8 ± 1.2	23.4	26.8 ± 1.9	27.9 ± 0.9
1.82	2 43 ± 0.41	2.24 ± 0.20	1.94 ± 0.26	2.00 ± 0.22	2.22	2.71 ± 0.40	2.97 ± 0 36

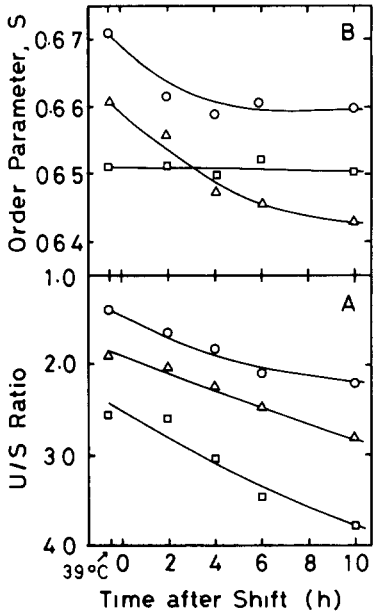

Fig. 1. Changes in fluidity and fatty acid composition of various membrane fractions of *Tetrahymena* during temperature acclimation. Pellicles, microsomes and mitochondria were isolated from *Tetrahymena* cells at the indicated times after shift-down, and their lipids were extracted. Order parameters were obtained from ESR spectra of 5-nitroxide stearate spin probe in liposomes prepared from extracted lipids. The order parameters at 15°C are plotted in the figure The ratio of unsaturated to saturated fatty acids was calculated from the fatty acid composition listed in Table II. ○, Pellicles, △, microsomes; □, mitochondria. *U/S*, ratio of total content of unsaturated fatty acid to total content of saturated fatty acids.

composition of cardiolipins from two sources (Table V). While bovine heart cardiolipin was composed almost exclusively of linoleic acid (approx. 87%), *Tetrahymena* cardiolipin contained linoleic (approx. 36%) and γ-linolenic (approx. 45%) acids. It is of interest to note that there was no change in fatty acid composition of *Tetrahymena* cardiolipin during the 10 h-period of acclimatization (Maruyama and Nozawa, unpublished data).

Discussion

When *Tetrahymena* cells are exposed to a lower temperature, they strive to overcome the uncomfortable situation by modifying membrane lipid composition [4,6,8]. In general, lowering the environmental temperature produces a higher ratio of unsaturated to saturated fatty acid in the cell membrane. A major purpose of temperature acclimation is thought to be the maintenance of optimal membrane physical properties [21]. Membranes rich in unsaturated fatty acids are generally in more fluid state than those with a high proportion of saturated acids. Since, as demonstrated in this study, phospholipid compositions of three major membrane fractions; mitochondria, pellicles and microsomes were constant during first 10 h after the temperature shift, it is suggested that the fatty acid composition would play a major part in controlling the physical state of membrane lipids. Indeed, time-dependent alterations ob-

TABLE IV

EFFECT OF CARDIOLIPIN ON FLUIDITY OF PHOSPHOLIPIDS OF *TETRAHYMENA* WHOLE CELLS

Total lipids were extracted from *Tetrahymena* cells grown at 39°C or cells acclimated to 15°C for 10 h. Neutral lipids and cardiolipins were separated by silica gel H TLC using the same solvent system as described in Table I. Values are order parameters, S, calculated from ESR spectra measured at 15°C with 5-nitroxide stearate spin probe. '%' refers to content (%) of added cardiolipin.

Sources of cardiolipin	Cardiolipin-free phospholipids from:							
	39°C-grown cells				15°C-acclimated cells			
%:	0	5	15	30	0	5	15	30
39°C-grown cells	0.667	0.667	0.668	0.663	0.654	0.651	0.661	0.674
15°C-acclimated cells	0.667	0.668	0.664	0.672	0 654	0.646	0 663	0 670
Bovine heart	0 667	0 659	0 662	0.664	0.654	0.655	0.656	0.662

served in fatty acid composition and fluidity of both pellicular and microsomal lipids are consistent with the notion reported in previous papers [6,8,14]. However, such correlation was not seen in mitochondrial lipids. Based on the results obtained here, cardiolipin, which is localized almost exclusively in the inner mitochondrial membranes [22], would act as a membrane stabilizer to keep the fluidity constant during temperature acclimation. Since solely the fluidity of cardiolipin-free phospholipids of 15°C-acclimated cells was affected by re-addition of cardiolipin, irrespective of its source, it is presumed that cardiolipin would interact preferentially with phospholipids containing a high content of unsaturated fatty acids and counterbalance the increased fluidity due to enhanced unsaturation during cold acclimation. However, at the present time, no plausible explanation is available for physiological significance of consistent fluidity observed in mitochondrial lipids. It is also uncertain whether this proposed theory of cardiolipin as a fluidity stabilizer can be extrapolated to mitochondria of other types of cell. Apart from the actual role of cardiolipin in the regulation of membrane fluidity, this acidic phospholipid is known to have specific functions associated with energy transduction Cardiolipin was co-purified with several components involving the oxidative phosphorylation [23] including cytochrome *c* oxidase [24]. Recently, Wodtke [25,26] reported that the activity of cytochrome *c* oxidase in mitochondrial membranes of carp during thermal adaptation is controlled by viscotropic regulation. *Tetrahymena* mitochondria also should be examined for activity of cytochrome *c* oxidase and other enzymes in the course of thermal adaptation. In the intact mitochondrion, cardiolipin may be localized in a limited region, for example, as the annular lipids for cytochrome *c* oxidase [27]. And the measurement of

TABLE V

FATTY ACID COMPOSITION OF CARDIOLIPIN

Lipids were extracted from mitochondria of *Tetrahymena* grown at 39°C or acclimated to 15°C for 10 h Cardiolipin was separated by silica gel H TLC using the same solvent system as described in Table I. Bovine heart cardiolipin was purchased from Sigma. Fatty acids were methylated and then analysed by GLC. Values indicate relative percentage of major fatty acids

Fatty acid	*Tetrahymena* mitochondria		Bovine heart
	39°C-grown cells	Acclimated cells (15°C for 10 h)	
14 0	0 5	0.5	0.2
16 0	1.2	0.8	1.1
16 · 1	4.2	2.6	1 2
16 2 [a]	2.3	2.6	–
18 1	4.4	4.0	7.4
18 . 2	35.2	38.2	86.7
18 3	44.4	44 4	–

[a] Contains also small amounts of 17 0 which was not separable from 16 . 2 under the conditions used.

fluidity of mitochondrial membranes during temperature acclimation will reveal more clearly the role of cardiolipin in the regulation of membrane fluidity. But the spin labeling technique cannot be applied to intact mitochondria because of the reduction of nitroxide radical by electron transport system [28], and fluorescent probes or other methods should be utilized for determining the membrane fluidity of mitochondria.

Recent studies using ^{31}P-NMR and freeze-fracture electron microscopy have provided much information regarding physical properties of cardiolipin. One of them demonstrated Ca^{2+}-induced formation of hexagonal (H_{II}) phase in artificial lipid membranes containing cardiolipin [29,30]. More recently, De Kruijff and Cullis reported that addition of cytochrome *c* resulted in an emergence of the hexagonal (H_{II}) phase in cardiolipin-containing model membranes [31], and proposed some functional roles of the hexagonal (H_{II}) phase, such as flip-flop [32], fusion [33] and channel [34]. The ^{31}P-NMR study of total extracted lipids obtained from rat liver inner mitochondrial membrane suggested that cardiolipin may have the ability to form a hexagonal (H_{II}) phase in mitochondrial membranes in the presence of Ca^{2+} [35]. Consequently, it would not be unreasonable to consider that specific domains of H_{II} phase may be formed in the cold-acclimated mitochondria and be implicated in adjusting the fluidity to maintain the functions normal. *Tetrahymena* is thought to be a useful model system for studying functions of cardiolipin in mitochondria because of the effective modification of lipid composition and ease of isolation of subcellular organelles [3,36]. Further extensive studies, including physicochemical analysis of cardiolipin, will yield useful information regarding the role of this lipid in the regulation of mitochondrial membrane fluidity.

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