*Biochimica et Biophysica Acta*, 731 (1983) 251–260
Elsevier



BBA 71688

# COLD STRESS INDUCES IN SITU PHOSPHOLIPID MOLECULAR SPECIES CHANGES IN CELL SURFACE MEMBRANES

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(Received December 15th, 1982)

*Key words: Ciliary membrane; Cold acclimation; Phospholipid modification; (T. pyriformis)*

**The structural organization of *Tetrahymena pyriformis* is such that its cilia are remote from the main centers of lipid metabolism. As a result, the ciliary membrane lipid composition of cells exposed to low-temperature stress is initially unaffected by the significant metabolic changes induced in microsomal membranes. Nevertheless, changes in the ciliary membrane lipid composition can be detected during the first 4 h of cold exposure. A combination of in vivo and in vitro experiments has provided strong evidence for a substantial retailoring of ciliary phospholipid molecular species in situ in the absence of any importation of lipids from the cell interior or change in overall ciliary fatty acid composition. The mechanism responsible for the ciliary lipid changes is independent of the one(s) triggering internal acclimation responses. Our observations establish for the first time that chilling stress can simultaneously induce separate and distinctive lipid modification responses in different parts of a cell. This finding could be important in identifying the molecular 'sensor' capable of actuating stress-induced lipid changes.**

## Introduction

The fluidity of a biological membrane is widely acknowledged to be of great importance in controlling the metabolic functions of that membrane and is primarily a function of (1) the lipid composition of the membrane, and (2) the environmental factors, e.g., temperature, pH, ionic strength, affecting the membrane at any given time.

It appears that many organisms strive to maintain the fluidity of their membranes within rather narrow limits. These organisms can reverse the destabilizing effects of environmental stress on fluidity by enzymatically altering the composition of their membrane lipids.

The ciliate *Tetrahymena* has been used extensively as a model system to study the response of membranes to temperature changes [1–3]. Several molecular mechanisms, ranging from fatty acid desaturation to alterations in sterol/phospholipid ratios, were demonstrated to contribute at one stage or another to the overall temperature-acclimation process. Recent reports [4,5] have revealed a hitherto unrecognized mechanism causing a very rapid alteration of microsomal membrane fluidity in response to chilling. This alteration consisted of an intramolecular redistribution of phospholipid-bound fatty acids so as to yield new combinations of molecular species.

In the present report we describe the capacity of the metabolically remote ciliary membranes of low-temperature-stressed *Tetrahymena* cells to alter their phospholipid molecular species by a

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; AEPL, 2-aminoethylphosphonolipid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

mechanism entirely self-contained within these organelles. Thus, it becomes clear that sensing low temperature and responding via membrane lipid alterations is not a phenomenon confined to one locus within the cell.

## Materials and Methods

*Culture conditions. Tetrahymena pyriformis*, strain NT-1, was grown in 2% proteose peptone medium as described by Fukushima et al. [2]. Cells were routinely grown with shaking at 39°C to their mid-logarithmic phase of growth ($(2–3) \cdot 10^5$ cells/ml). They were harvested at this stage or chilled rapidly to 15°C as described by Dickens and Thompson [4] and maintained on a 15°C shaker for specified periods of time before harvest.

*Deciliation.* Cells were deciliated by the $Ca^{2+}$-shock procedure described earlier [6]. The treated suspension was centrifuged at $480 \times g$ for 5 min to pellet out deciliated cells. The supernatant was centrifuged twice at $800 \times g$ for 10 min each, and the final supernatant was centrifuged at $18\,000 \times g$ for 20 min to pellet the cilia. The purity of cilia was routinely checked by phase microscopy. Lipid analysis of cilia labeled with radioactive fatty acids showed no evidence of degradation during isolation.

*Isolation of microsomes.* The deciliated cell bodies obtained in the above procedure were washed twice with 0.25 M phosphate buffer (pH 7.2) containing 0.2 M NaCl and 0.01 M EDTA. Microsomes were isolated from the deciliated cells by the procedure described earlier [4].

*Isolation and separation of phospholipids.* Lipids from cilia and microsomes were extracted by the procedure of Bligh and Dyer [7]. Individual phospholipid fractions were separated by TLC on silica gel H plates as described elsewhere [4], and the phospholipid composition was quantified by the method of Rouser et al. [8].

*Analysis of phospholipid molecular species.* Individual phospholipid bands separated by TLC were eluted from the silica gel using chloroform/methanol/water (1 : 2 : 0.4, v/v). After evaporating the solvents under nitrogen, the lipids were dissolved in 1.0 ml of peroxide-free diethyl ether and digested for 2 h with 30–60 units of phospholipase C from *Bacillus cereus* (Calbiochem, La Jolla, CA). The resulting diacylglycerols were converted to *tert*-butyldimethylsilyl derivatives by heating at 110°C for 15 min in sealed ampules as described earlier [4].

The *tert*-butyldimethylsilyl derivatives were analyzed by a Varian model 3700 gas chromatograph with a 3% OV 101 packed column (0.25 inch diameter, 6 ft length) at 280°C using a flame-ionization detector. The resolution of the eluted derivatives into peaks containing derivatives with the same carbon number was much better than that achieved previously on 3% OV 1 columns [4,5]. The identity of the carbon numbers of the GC peaks was confirmed by a Finnigan model 4000 gas-chromatograph-mass spectrometer as described earlier [4].

*Analysis of fatty acid composition.* Fatty acid methyl esters derived from ciliary phospholipids were analyzed using a Varian model 3700 gas chromatograph with a 10% Silar 10C column (0.25 inch diameter, 6 ft. length) at 170°C using a flame-ionization detector. The radioactivity in different fatty acid fractions of microsomal and ciliary phospholipids was analyzed after trapping a fixed proportion of the column effluent using a stream splitter.

*[1-$^{14}$C]Palmitate incorporation.* Incorporation of [1-$^{14}$C]palmitic acid by *Tetrahymena* was carried out as follows: $(2–3) \cdot 10^6$ dpm of [1-$^{14}$C]palmitic acid (specific radioactivity, 56.3 mci/mmol; New England Nuclear, Boston, MA) in 100 $\mu$l of ethanol were added to a 200 ml logarithmic phase culture at 39°C. 10 min after the addition of the radioisotope, the cells were chilled to 15°C and incubated at 15°C for specified periods of time, after which they were harvested and fractionated for the extraction of ciliary and microsomal lipids. Different phospholipid fractions were separated by TLC for the determination of specific radioactivity [6]. Control experiments showed no significant changes in the labeling pattern during the chilling step.

*[1-$^{14}$C]Linoleate incorporation.* Incorporation of [1-$^{14}$C]linoleate by *Tetrahymena* was carried out as follows: $0.5 \cdot 10^6$ dpm of [1-$^{14}$C]linoleic acid (specific radioactivity, 56.0 mci/mmol; New England Nuclear, Boston, MA) in 100 $\mu$l of ethanol were added to 200 ml of culture medium at 39°C 4 h after inoculating the medium with cells. After allowing the cells to grow overnight, reaching their

midlogarithmic phase, they were either harvested directly or chilled to 15°C and incubated at that temperature for specified periods of time before harvest (by 6–8 h the added radioactivity had equilibrated between different phospholipid classes). Analysis of radioactivity in phospholipid-derived fatty acids trapped after gas chromatography revealed that 20–25% of the radioactivity remained in linoleic acid (18 : 2(6,9) while the rest of the radioactivity was in γ-linolenic acid (18 : 3(6,9,12)). Radioactivity was never observed in other fatty acids.

*Hydrolysis of phospholipids with phospholipase $A_2$.* $^{14}$C-labeled phosphatidylcholine (PC), phosphatidylethanolamine (PE) and 2-aminoethylphosphonolipid (AEPL) isolated from cilia were each hydrolyzed with phospholipase $A_2$ from *Crotalus adamanteus* venom (Sigma Chem. Co., St. Louis, MO). The lipids were dissolved in 1 ml of peroxide-free diethyl ether; then, 50 μl of sodium borate buffer (0.1 M, pH 7.6) and 0.2–1.0 mg of snake venom in 50 μl of 0.1 M Tris-HCl buffer containing 0.4 mM $CaCl_2$ were added. Each vial was capped air-tight and incubated in a 337°C water bath with vigorous shaking. The samples were incubated for 2 h in the case of PC and PE, and 3 h for AEPL. After incubation the diethyl ether was evapoarated, and the lipids were extracted from the residual buffer. They were then separated into free fatty acids and lysophospholipids by TLC and assayed for radioactivity.

*Incubation of cilia in vitro.* For different in vitro studies, described in Results, incubations were carried out essentially as follows: cilia were isolated from cells grown at 39°C in the presence or absence of radioactive fatty acids. The ciliary pellet was suspended in 2.0–3.0 ml of cold 10 mM Hepes buffer (pH 7.2) containing NaCl (50 mM), $MgCl_2$ (3 mM), $K_2HPO_4$ (5 mM), sucrose (40 mM), ATP (3 mM) and CoA (40 μM). The suspension was mixed vigorously for 2 min in a Vortex mixer to form membrane vesicles from the intact cilia. The vortexed suspension was transferred into reaction vials, capped under $N_2$ and incubated at 15°C for 8 h. At the end of the incubation, the reaction was stopped by adding 2.0 ml of methanol. The lipids were then extracted and separated by TLC. No significant levels of radioactivity were found in free fatty acids or lysophospholipids when cilia isolated from cells grown in the presence of radioactive fatty acids were incubated at 15°C for 8 h. The overall ciliary fatty acid compositions were the same before and after the incubations, thus eliminating the possibility of artifactual changes in the phospholipids due to lipid breakdown during the in vitro incubation. Radioactive phospholipids obtained from cilia isolated from cells grown in the presence of radioactive fatty acids were used either for positional analysis of fatty acids by phospholipase $A_2$ digestion or for determining the distribution of radioactivity, while phospholipids obtained from non-labeled cilia were used for molecular species analysis by gas chromatography.

*Incorporation of [1-$^{14}$C]palmitic acid into ciliary phospholipids in vitro.* Incorporation of exogenously added labeled fatty acids into ciliary phospholipids in vitro was carried out as follows: cilia isolated from cells grown at 39°C were suspended in 1–2 ml of 10 mM Hepes buffer (pH 7.2) containing 50 mM NaCl, 40 mM sucrose, 5 mM $K_2HPO_4$ and 4 mM $MgCl_2$. ATP (3 mM) and CoA (40 μM) were added as indicated. The suspension was vortexed vigorously for 30 s, after which $(0.8–1.5)\cdot 10^6$ dpm of [1-$^{14}$C]palmitic acid freshly complexed with fatty acid-free bovine serium albumin (Sigma Chem. Co., St. Louis, MO) were added (the final concentration of bovine serum albumin in this reaction mixture was between 0.1 and 0.2%), and the sample was vortexed vigorously for an additonal 90 s. The sample was then transferred to a reaction vial, capped, and incubated at 37°C for 90 min. At the end of incubation, the reaction was stopped by adding 2.0 ml methanol and the lipids were extracted. Phospholipid classes were separated by TLC, and their radioactivity was determined. In some experiments microsomal preparations were also incubated with [1$^{14}$C]palmitic acid with or without ATP and CoA.

## Results

The *Tetrahymena* surface membrane is physically separated from the cell's main cytoplasmic compartment by an extensive system of alveolar membranes [9,10]. Reports from, our laboratory [6,11,12] have indicated that these cortical structures restrict the dissemination of membrane lipids from microsomal biosynthetic sites to the cell

surface. This limited exchange of lipids between the two compartments is exemplified by the finding that sudden exposure of *Tetrahymena* to low temperature, which is known to cause a prompt rise in microsomal lipid unsaturation, led to similar changes in the surface membrane only after many hours [6]. Because of its relative isolation, the *Tetrahymena* surface membrane is particularly well suited for studying possible in situ changes in lipid composition occurring independently of those taking place in more centrally located cellular organelles.

We have now gathered additional data illustrating the extent to which the surface membrane is metabolically remote from other regions of the cell. *Tetrahymena* grown at 39°C were exposed to tracer amounts of [$^{14}$C]palmitic acid. After a period of 10 min, during which virtually all radioactivity was incorporated into phospholipids, the cells were chilled to 15°C over a 5 min interval and then maintained at that temperature. At selected times, aliquots of the cells were harvested and fractionated in such a way as to yield microsomes and a highly purified preparation of cilia, whose enclosing membranes are direct extensions of the adjacent plasma membrane.

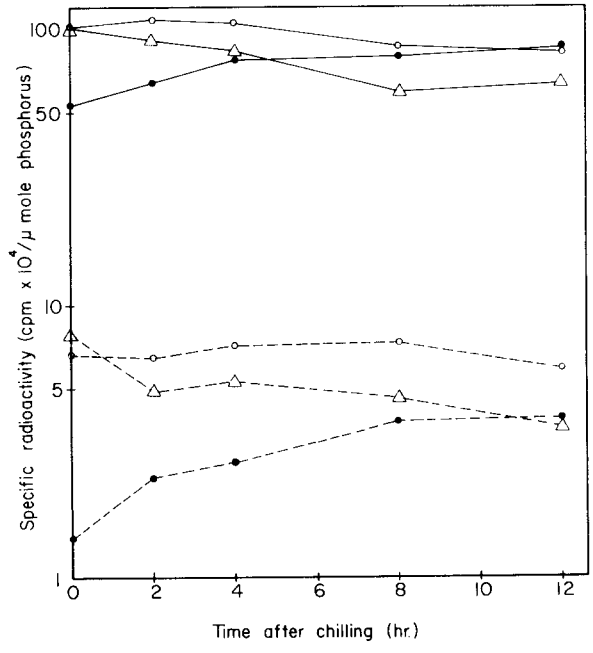


Fig. 1. Incorporation of [1-$^{14}$C]palmitic acid into microsomal (———) and ciliary (- - - - -) individual phospholipid classes in chilled cells. ●, AEPL; ○, PE; △, PC. The radioisotope was added 10 min before chilling.

As depicted on Fig. 1, the specific radioactivities of all three major phospholipid classes of microsomal membranes were more than 10 times as high as the equivalent values for ciliary phospholipids. The fact that these differences were maintained for at least 12 h in vivo rules out any sizeable movement of microsomal phospholipids into the ciliary membrane during that time.

The radioactivity found in ciliary phospholipids was low, but not negligible. Analysis of individual fatty acids of the ciliary phospholipids 4 h after chilling revealed no significant radioactivity in $C_{18}$ or polyunsaturated fatty acids, despite the fact that substantial radioactivity was present in these fatty acid species of microsomal lipids isolated from the same cells. It appears likely that the radioactivity associated with ciliary lipids under these conditions arose through a limited amount of acylation at the surface membrane during the time when most of the [$^{14}$C]palmitate was being transported across the surface membrane and into the cell interior.

*Evidence for ciliary phospholipid molecular species retailoring in vivo.* Cilia from *Tetrahymena* grown at 39 and 15°C are much more different in their lipid compositions than are the internal membranes from the same cells [6]. No systematic effort has been made to determine what is responsible for the striking low-temperature-induced changes in ciliary lipid composition. The suprisingly slow dissemination of lipids from the cell interior made it seem feasible to test the capacity of attached cilia to modify phospholipids in situ.

Cilia were isolated from cells that had been grown at 39°C and then chilled to 15°C for 4 h. There was no significant change in fatty acid composition following chilling, in agreement with earlier findings [6]. However, analysis of the molecular species of the glycerophospholipids revealed sizable and reproducible differences from the 39°C pattern. Typical GC patterns showing PE molecular species of cilia from cells grown at 39°C and from cells shifted from 39 to 15°C for 4 h are presented in Fig. 2. The differences in some of the molecular species of PE are very obvious from this figure. Table I quantifies these and other

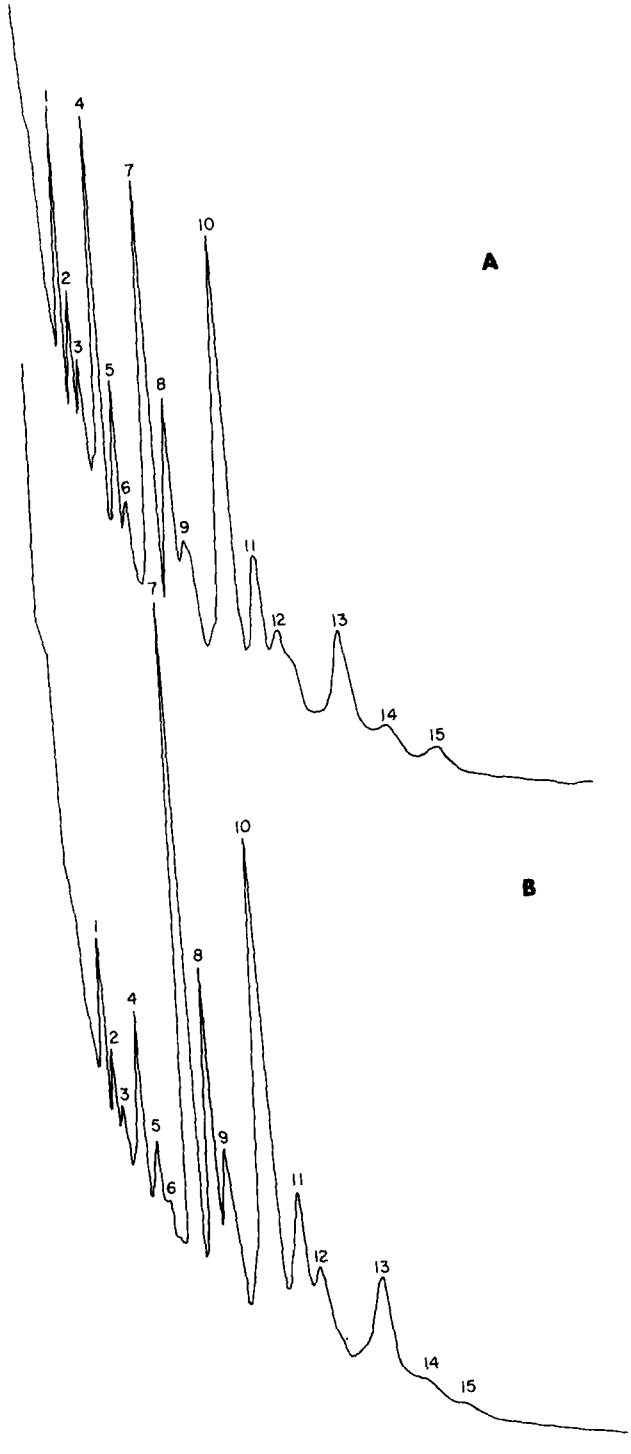


Fig. 2. Gas chromatographic elution pattern of *tert*-butyldimethylsilyl-diacylglycerol ethers prepared from ciliary phosphatidylethanolamine. A, Cells grown at 39°C. B, Cells grown at 39 and shifted to 15°C for 4 h. Peak codes are identified in Table I. The specific fatty acids paired in each peak are identified in an earlier communication [5]. Experimental details and GC operation conditions are given in Materials and Methods.

major differences observed in the PE and PC molecular species compositions following the temperature shift. PE and PC did not exhibit the same pattern of molecular species change. Thus, $C_{26}$, $C_{27}$, $C_{28}$ and $C_{29}$ of PE decreased, while $C_{30}$, $C_{31}$, and $C_{32}$ increased significantly following the shift. In contrast, $C_{28}$, $C_{29}$, $C_{30}$ and $C_{32}$ of PC increased slightly while $C_{34}$ decreased significantly. The relative amounts of the ether-containing peaks ($C_{e32}$ and $C_{e34}$) of both AEPL and PC were unaffected by the temperature shift. However, changes in the unsaturated fatty acid content of the ether peaks cannot be ruled out, since the analytical technique measures only chain length and not degree of unsaturation. It is interesting to note that the changes observed here in cells chilled for 4 h were both qualitatively and quantitatively different from those observed in ciliary PE and PC of cells grown for several generations at 15°C [5]. Quantitative comparisons with this earlier published work are not entirely valid because the more recently used GC columns gave a superior resolution of the various molecular species.

Because of the highly restricted importation of lipids from the internal membranes of cells into cilia under these conditions, the observed lipid changes were suspected to originate locally and arise through redistribution of preexisting fatty acids among the ciliary phospholipid molecules. More evidence for such a reshuffling of fatty acid moieties among ciliary phospholipids was obtained by the following experiments.

*Redistribution of [$^{14}$C]18:2 and [$^{14}$C]18:3 among ciliary phospholipids in vivo.* Cells grown overnight at 39°C after administering trace amounts of [1-$^{14}$C]linoleic acid contained lipids labeled exclusively in phospholipid-bound linolenic (18:2) and γ-linolenic (18:3) acid in a very stable distribution among the major lipid classes (data not shown). The partition of this radioactivity among the major phospholipid classes was compared in cilia of the cells grown at 39°C and cilia of cells exposed to 15°C for 4 h. Table II shows the distribution of radioactivity in different

TABLE I

EFFECT OF TEMPERATURE SHIFT ON THE PHOSPHOLIPID MOLECULAR SPECIES COMPOSITION OF THE CILIARY MEMBRANE

All values are expressed as weight percentages. Peak numbers correspond to those shown in Fig. 2. Values for AEPL and PE are mean ± S.D. of a total of three or more determinations from two or more separate pooled samples. Those for PC are average of two determinations from separate pooled samples. Trace represents under 0.5%. Each odd-numbered $C_n$ component is resolved into two peaks, one of which (not yet identified) contains a branched-chain, odd-numbered fatty acid.

| Peak No. | Carbon No. | AEPL | | PE | | PC | |
|---|---|---|---|---|---|---|---|
| | | 39°C | 39 → 15°C (4 h) | 39°C | 39 → 15°C (4 h) | 39°C | 39 → 15°C (4 h) |
| 1 | 26 | – | – | 8.5 ± 2.0 | 2.2 ± 0.5 | – | – |
| 2 | 27 | – | – | 4.0 ± 1.3 | 0.6 ± 0.1 | – | – |
| 3 | 27 | – | – | 4.1 ± 1.8 | trace | – | – |
| 4 | 28 | – | – | 11.0 ± 2.5 | 3.9 ± 0.7 | 6.4 | 9.7 |
| 5 | 29 | – | – | 4.2 ± 1.6 | 1.2 ± 0.6 | 4.0 | 5.5 |
| 6 | 29 | – | – | 1.4 ± 0.9 | trace | 3.5 | 5.9 |
| 7 | 30 | trace | 1.0 ± 0.1 | 16.8 ± 2.7 | 24.9 ± 2.3 | 23.5 | 21.6 |
| 8 | 31 | trace | 0.5 ± 0.1 | 7.8 ± 1.4 | 11.6 ± 1.1 | 12.9 | 4.0 |
| 9 | 31 | – | – | 2.7 ± 0.9 | 6.8 ± 1.7 | 1.0 | 4.4 |
| | e32 [a] | 7.2 ± 0.1 | 7.9 ± 0.3 | – | – | 11.9 | 11.7 |
| 10 | 32 | 4.7 ± 0.4 | 6.9 ± 0.2 | 24.4 ± 3.9 | 32.0 ± 0.2 | 14.1 | 20.7 |
| 11 | 33 | – | – | 5.3 ± 1.3 | 7.5 ± 0.2 | – | 1.3 |
| 12 | 33 | – | – | 1.5 ± 1.1 | 2.1 ± 0.7 | – | – |
| | e34 [a] | 83.0 ± 0.2 | 78.5 ± 0.1 | – | – | 11.7 | 11.5 |
| 13 | 34 | 4.9 ± 0.4 | 5.2 ± 0.5 | 6.9 ± 1.5 | 6.3 ± 2.1 | 10.7 | 2.0 |
| 14 | 35 | – | – | 0.5 ± 0.1 | – | – | 1.3 |
| 15 | 35 | – | – | 0.6 ± 0.1 | – | – | – |
| | 36 | – | – | trace | trace | – | trace |

[a] Ether linkage at the *sn*-1 position.

phospholipids of cilia isolated from cells grown at 39°C and temperature-shifted cells. Increased radioactivity in PE and PC of the 4 h-shifted cells was accompanied by decreases in the radioactivity of AEPL and ceramide lipids, thus indicating a movement of 18:2 and 18:3 fatty acids from AEPL and ceramide lipids to PE and PC. Since there were no lysophospholipids observed under these conditions, the transfer of 18:2 and 18:3 fatty acids should be accompanied by a corresponding reverse transfer of some other fatty acids from PE and PC to AEPL and ceramide lipids. In an effort to show this, experiments were performed using a different fatty acid, [$^{14}$C]palmitic acid. In this case, the tracer was administered only 5 min prior to chilling from 39 and 15°C, so as to minimize elongation and desaturation of the added [$^{14}$C]palmitate. This resulted in more than

TABLE II

REDISTRIBUTION OF $^{14}$C RADIOACTIVITY AMONG CILIARY PHOSPHOLIPIDS FOLLOWING CHILLING

Cells were grown overnight in the presence of [$^{14}$C]18:2. They were then shifted to 15°C and incubated as described in Materials and Methods. Analysis of the ciliary fatty acids revealed that the radioactivity was present almost exclusively in 18:2 and 18:3 fatty acids. Therefore, the redistribution of radioactivity represents the redistribution of 18:2 and 18:3 fatty acids (see text for details). Values are mean ± S.D. of 3–4 determinations.

| | 39°C | % of total dpm | 39 → 15°C (4 h) | % of total dpm |
|---|---|---|---|---|
| AEPL | 11 152 ± 230 | 65.9 | 9684 ± 468 | 57.5 |
| PE | 1696 ± 315 | 10.0 | 3995 ± 698 | 23.7 |
| Sphingolipids | 3360 ± 522 | 14.9 | 1690 ± 576 | 10.0 |
| PC | 710 ± 95 | 4.2 | 1483 ± 518 | 8.9 |

80% of the radioactivity in cilia being in 16:0. AEPL sustained a redistribution of radioactivity at 15°C opposite that observed with [$^{14}$C]linoleic acid as the tracer. However, the other phospholipids did not show any consistent and significant changes in their radioactivities (data not shown). This is, however, not unexpected in light of other findings (unpublished data) that administered [$^{14}$C]16:0 requires more time than allowed here to equilibrate among the different ciliary phospholipid classes. Furthermore, comparisons of changes involving 18:2 and 18:3 with those of 16:0 leave unresolved any contribution of other fatty acids also present,

*Positional distribution of fatty acids in phospholipids of cells grown at 39°C or shifted from 39 to 15°C.* Yet another possible manifestation of phospholipid molecular species modification is the interchange of certain fatty acid species between the phospholipid *sn*-1 and the *sn*-2 positions. Changes in the positional specificity of fatty acids in phospholipids are known to influence physical properties of the phospholipids [13,14]. Such positional changes are known to occur in *Tetrahymena* whole cell lipids and in the lipids of individual *Tetrahymena* organelles, including pellicles (cell-surface structures containing the plasma membrane along with the more metabolically accessible alveolar membranes) [15–17].

Cells were grown overnight at 39°C in the presence of [$^{14}$C]linoleic acid. Cilia were isolated from one batch of cells, and the other batch was chilled to 15°C and incubated at 15°C for 4 h before cilia were isolated. Lipids were extracted from both the ciliary preparations, and the radioactivity present in *sn*-1 and *sn*-2 positions of PC, PE and AEPL was determined by phospholipase $A_2$ digestion. Table III shows that chilling significantly alters the positional distribution of fatty acids in the ciliary phospholipids. The changes in PC were more marked than were those in PE or AEPL.

Because these experiments were carried out in vivo, it seemed possible that the observed changes were distorted to some extent by importation of small amounts of lipids from elsewhere in the cell. In order to resolve this uncertainty, experiments were done in vitro using freshly isolated and purified cilia.

TABLE III

POSITIONAL DISTRIBUTION OF THE $^{14}$C RADIOACTIVITY IN CILIARY PHOSPHOLIPIDS BEFORE AND AFTER THE TEMPERATURE SHIFT

Experimental details are the same as in Table II. Since the radioactivity was present only in 18:2 and 18:3 fatty acids, the changes in the positional distribution of the radioactivity represent the changes in the positional distribution of these fatty acids. Values are expressed as mean ± S.D. of more than three determinations.

| | 39°C | 39 → 15°C (4 h) |
|---|---|---|
| PC | | |
| *sn*-2 | 65.9 ± 3.3 | 86.4 ± 4.5 |
| *sn*-1 | 34.1 ± 3.3 | 13.1 ± 4.5 |
| PE | | |
| *sn*-2 | 69.5 ± 4.2 | 61.5 ± 5.2 |
| *sn*-1 | 30.5 ± 4.2 | 37.8 ± 5.8 |
| AEPL | | |
| *sn*-2 | 68.7 ± 2.0 | 76.0 ± 3.8 |
| *sn*-1 | 31.3 ± 2.0 | 24.0 ± 3.8 |

*Ciliary phospholipid molecular species retailoring in vitro.* Cilia isolated from cells grown at 39°C were incubated at 15°C for 8 h, as described in Materials and Methods. Table IV summarizes the phospholipid molecular species compositions after the in vitro incubation. While the changes observed in the PE and PC molecular species compositions (compare Table IV with data for 39°C cilia in Table I) were not as marked as observed in the in vivo chilling studies (see Table I), it is interesting to note that they followed the same general pattern observed in the in vivo experiments. Thus, following 8 h of in vitro incubation of cilia at 15°C, there was a slight decrease in $C_{26}$, $C_{27}$ and $C_{28}$ of PE while $C_{30}$ increased slightly. During the same time, $C_{25}$, $C_{31}$ and $C_{32}$ of PC increased while $C_{34}$ decreased significantly during the incubation.

It should also be pointed out here that incubation of [1-$^{14}$C]linoleate-labeled cilia for 8 h in vitro resulted in slight but consistent changes in the distribution of radioactivity as well as positional distribution of the fatty acyl chains in the ciliary phospholipids (data not shown). These changes also followed the same pattern observed in the earlier in vivo experiments.

TABLE IV

PHOSPHOLIPID MOLECULAR SPECIES COMPOSITION OF CILIA AFTER 8 h OF IN VITRO INCUBATION

All values are expressed as weight percentages. Values for AEPL and PE are Mean ± S.D. of a total of three or more determinations from two or more separate pooled samples. Those for PC are averages of two determinations from separate pooled samples. Trace represents under 0.5%. Each odd-numbered $C_n$ component is resolved into two peaks, one of which (not yet identified) contains a branched-chain, odd-numbered fatty acid.

| Carbon No. | AEPL | PE | PC |
|---|---|---|---|
| 26 | – | 5.3 ± 1.9 | – |
| 27 | – | 3.0 ± 1.1 | – |
| 27 | – | 2.8 ± 1.5 | – |
| 28 | – | 7.3 ± 0.8 | 7.0 |
| 29 | – – | 3.7 ± 0.7 | 4.4 |
| 29 | – | 1.5 ± 0.9 | 3.9 |
| 30 | 0.7 ± 0.1 | 13.0 ± 1.1 | 26.2 |
| 31 | trace | 7.2 ± 0.01 | 7.5 |
| 31 | – | 4.3 ± 0.3 | 5.4 |
| e32 [a] | 7.4 ± 0.9 | – | 13.5 |
| 32 | 8.3 ± 1.9 | 27.5 ± 4.1 | 26.4 |
| 33 | – | 7.3 ± 1.0 | – |
| 33 | – | 4.5 ± 0.5 | – |
| e34 [a] | 77.9 ± 1.6 | – | 9.6 |
| 34 | 5.1 ± 0.8 | 8.4 ± 0.8 | 1.3 |
| 35 | trace | 3.0 ± 0.3 | – |
| 35 | – | – | – |
| 36 | – | trace | 1.0 |

[a] Ether linkage at the *sn*-1 position.

*Demonstration of acylation activity in isolated cilia.* The results of the in vivo and in vitro experiments described above indicate that *Tetrahymena* cilia can independently alter their phospholipid molecular species composition by rearranging the fatty acyl chains. Such rearrangements would be expected to involve deacylation and reacylation reactions. Therefore, experiments were conducted to measure directly the ability of isolated cilia to catalyze the incorporation of fatty acids into phospholipids.

The results presented in Table V clearly show that purified cilia, when incubated with [$^{14}$C]-palmitic acid in the presence of ATP and CoA, incorporated significant amounts of exogenous fatty acids into these phospholipids. Although the amount of radioactivity incorporated was very small (under 1% of the added radioactivity), the absence of any incorporation of the label ([1-$^{14}$C]palmitic acid) into phospholipids in control samples (zero h incubation) and in samples incubated without the added cofactors indicated the authenticity of the acylation activity. Contamination of the preparations by microsomal membranes was ruled out because (1) the pattern of incorporation of [1-$^{14}$C]palmitic acid into microsomal phospholipids, as measured in separate experiments (data not shown), was different from that observed in cilia, (2) the radioactivity incorporated into ciliary phospholipids was essentially in palmitic acid alone, while that incorporated into microsomal phospholipids was present not only in 16 : 0 and 16 : 1 but also (in small amounts) in 18 : 1 and 18 : 2, as expected in a system with the potential for fatty acid elongation and desaturation.

TABLE V

INCORPORATION OF [$^{14}$C]16:0 INTO CILIARY PHOSPHOLIPIDS IN VITRO

90-min values are mean ± S.D. of three or more determinations. 0-min values are average of two determinations. Incubation conditions and other experimental details are given in the text.

| Phospholipid | dpm/fraction | | |
|---|---|---|---|
| | Without cofactors (90 min) | With cofactors | |
| | | 0 min | 90 min |
| AEPL | 59 ± 38 | 35 | 273 ± 66 |
| PE | 82 ± 53 | 23 | 1336 ± 8 |
| Sphingolipid | 46 ± 22 | 0 | 514 ± 44 |
| PC | 15 ± 11 | 0 | 386 ± 80 |

## Discussion

Earlier resports from this laboratory have indicated that the surface membranes of *Tetrahymena* are metabolically isolated from the microsomal enzymes [6,11,12]. Further studies of ciliary membranes presented in this communication confirm the relative isolation of these organelles. Although chilling induces rapid changes in microsomal phospholipids, the dissemination of the altered

microsomal lipids into the ciliary membranes is slow and much delayed.

Accordingly, any short-term metabolic response of ciliary membrane fluidity to low-temperature stress must of necessity occur in situ. The evidence described above shows clearly that lipid compositional changes do take place and that the enzymes reposible for the changes are indeed present in the cilia.

Gas chromatographic analysis of *tert*-butyldimethylsilyl-diacylglycerols derived from the ciliary phospholipids showed reproducible differences in carbon number ($C_n$) distribution within 4 h after chilling the cells to 15°C. Each of the principal phospholipids showed a characteristic pattern of change, and pronounced alterations during this time period were apparent in two of them, PE and PC (Fig. 2 and Table I). On average, the changes in $C_n$ percentages sustained by these two ciliary phospholipids were not only greater than those noted earlier in the equivalent microsomal lipids of cells chilled for 1 h, but were greater even than the differences between microsomal lipids of cells fully acclimated to 39 and 15°C [4].

The detailed composition of each $C_n$ peak has been reported by us earlier [5]. For example, the $C_{30}$ peak of ciliary PE is composed mainly of the 14:0/16:0, 14:0/16:1 and 18:1/12:0 combinations, as determined by coupled gas chromatography-mass spectrometry. Although this type of detailed characterization was not routinely performed in the present study, GC-MS is expected to reveal in greater detail the nature of specific modifications leading to our observations made using [$^{14}$C]linoleic acid-labeling and phospholipase $A_2$ digestion.

The observed phospholipid molecular species changes in ciliary membranes over the first 4 h at 15°C took place in the absence of any significant changes in fatty acid composition or polar head group distribution [6]. In this respect the ciliary response resembled only the early behavior of microsomal membranes. Microsomes of these same cells showed sizable phospholipid molecular species changes within the 1st h after chilling, following by a steady rise in fatty acid unsaturation during the subsequent 10–15 h, and finally ending with major polar head group changes continuing over a much longer period of 36–48 h [18].

A variety of experiments was designed to establish whether the molecular species modifications taking place in ciliary membranes were indeed occurring in situ. This question could be posed with some assurance of obtaining a clear answer because the ciliary preparations were so homogeneous. Several observations support in situ modifications. (1) The changes happened more rapidly than the kinetics of lipid dissemination from microsomes would allow (Fig. 1). (2) The patterns of change were qualitatively distinct from and quantitatively more pronounced than those taking place simultaneously in microsomes (compare the present results with those obtained by Dickens and Thompson [4]). (3) Similar, although somewhat less conspicuous, changes appeared in preparations of detached cilia incubated for several h at 15°C (Table IV). (4) Detached cilia, incubated in the presence of suitable cofactors, were capable of incorporating exogenous fatty aids into phospholipids (Table V).

Taken together, the findings confirm the ability of ciliary membranes to retailor phospholipid molecular species in response to chilling. The exact mechanism by which this is achieved cannot yet be verified. Deacylation and reacylation reactions of some type must be involved. No firm evidence is at hand to show that this occurs via the action of phospholipase $A_1$ or $A_2$ followed by a fatty acyltransferase-catalyzed reacylation of the lysophospholipid. Measurable levels of free fatty acids or lysophospholipids have not been observed, despite efforts to find them. An alternative mechanism would be the recently described direct transfer of a phospholipid fatty acyl group to CoA, bypassing the free fatty acid step [19,20]. Experiments are presently underway to investigate this point.

The ability of a cell to initiate stress-induced changes in membrane lipid composition simultaneously and independently in two (or more) locations has not, to our knowledge, been recognized before. This finding calls for a reevaluation of theories devised to explain the molecular mechanism regulating low-temperature acclimation. Although there are plainly several different processes affecting lipid composition during the acclimation period, the sensor triggering the initial response, namely, phospholipid molecular species changes, must be present at least in both the microsomes and the ciliary membranes. Short-term lipid

changes in cilia and perhaps other portions of the plasma membrane following exposure to low temperature may permit the cell to withstand more easily chilling stress until the slow importation of modified phospholipids from the cell's interior brings about more appropriate compositional changes.

In order to identify and describe this putative stress-sensing device, relatively simple experimental systems must be available. *Tetrahymena* cilia provide a homogeneous system capable of responding to stress via molecular species changes, yet apparently lacking other key enzymatic mechanism, such as those catalyzing fatty acid desaturation and polar head-group alterations, that have been implicated in lipid fluidity alteration. Using cilia, we intend to study the effects of various factors, including calcium-mediated membrane depolarization and changes in the fluidity of the ciliary membrane itself, which might trigger the first steps in low-temperature-induced acclimation.

## Acknowledgments

This work was supported in part by grants from the National Science Foundation (PCM-8200289) and the Robert A. Welch Foundation (F-350). C.S.R. is a Robert A. Welch Postdoctoral Fellow.

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