The Mechanism of Membrane Response to Chilling

EFFECT OF TEMPERATURE ON PHOSPHOLIPID DEACYLATION AND REACYLATION REACTIONS IN THE CELL SURFACE MEMBRANE*

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The ciliary membrane of Tetrahymena pyriformis is physically and metabolically remote from the main centers of lipid metabolism. Nevertheless, it possesses an independent capacity to modify its phospholipid molecular species composition rapidly under stress. The role of ciliary phospholipid deacylating and reacylating enzymes in this phenomenon has been evaluated.

Isolated cilia showed substantial phospholipase A (combined A₁ and A₂), acyl-CoA synthetase and acyltransferase activities. Activities of all the three enzymes of cilia from 39 °C-grown cells were greatly reduced when the cilia were incubated at 15 °C. In contrast, the phospholipase A and acyltransferase activities in cilia from 15 °C-grown cells were surprisingly high at 15 °C and twice as high at 37 °C as were the equivalent activities in preparations from 39 °C-grown cells. While the in vitro substrate specificity of phospholipase A could not be meaningfully assessed, the acyltransferases exhibited a temperature-dependent substrate specificity in vivo. Growth temperature also affected the positional distribution of fatty acids incorporated into ciliary phospholipids in vivo.

The ability of acyltransferases to utilize added [¹⁴C]acyl-CoA could be markedly stimulated, and their lipid class specificity could be significantly altered in vitro by supplementing the incubation mixture with exogenous lysophospholipid acceptors. These findings suggest that the rate-limiting factor in acyl chain turnover is not the activity of acyltransferases per se but rather the availability of suitable substrates and acceptors. Therefore, we postulate that temperature alters the rate and specificity of ciliary membrane phospholipid retailoring primarily by controlling the in situ phospholipase A activity.

It is well established that a great many organisms alter their membrane lipid composition in response to changing temperature. Changes in phospholipid acyl chain unsaturation, phospholipid polar head group composition, and sterol content of membranes are a few of the well known responses of cells to altered temperature. These changes, in general, alter the membrane fluidity to a level more compatible with the cell's ability to survive at the new temperature (1–3).

For understanding the molecular mechanisms responsible for thermal adaptation, the protozoan ciliate, Tetrahymena pyriformis, has been extensively used as a model system. Earlier, we demonstrated that the molecular species compositions of the three major ciliary phospholipid classes from 30 °C-grown Tetrahymena differ markedly from those of the same classes isolated from cilia of 15 °C-grown cells (4, 5). Because of the demonstrated metabolic isolation of the cilia from intracellular lipid-metabolizing enzymes, it was possible to show that the low temperature response in this organelle occurs independently of equivalent activities in other parts of the cell and thus involves mainly an intra- and intermolecular rearranging of fatty acids already present in the ciliary membrane (6, 7).

In this communication, we confirm the presence of phospholipid deacylating and reacylating enzymes in ciliary membranes and describe the effect of temperature on the activities and specificities of these enzymes.

EXPERIMENTAL PROCEDURES

RESULTS

Activities of Phospholipid Deacylating and Reacylating Enzymes in Isolated Cilia

Tetrahymena ciliary preparations were examined for the presence of the following enzymes necessary for phospholipid deacylation and reacylation: (a) phospholipase A; (b) acyl-CoA synthetase (EC 6.2.1.3), and (c) acyltransferase (acyl-CoA:lysophospholipid acyltransferase, EC 2.3.1.23).

Phospholipase A

Incorporation of radiolabeled fatty acids into ciliary lipids in vitro and in vivo (see Ref. 7 and data presented below) indicate the availability of lysophospholipid acceptors for reacylation. These acceptor substrates are formed by the

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1 Portions of this paper (including "Experimental Procedures," Figs. 1–3, and Tables I–III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1592, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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action of phospholipase A₁ or A₂. The combined activity of these two phospholipases was measured in cilia isolated from 39 °C-grown cells grown for 12–14 h after the addition of [1,4°C]palmitic acid. Under these conditions elongation and desaturation led to an approximately equal labeling of all major fatty acids (8). About 16 nmol of the ciliary phospholipid fatty acids (equivalent to approximately 15% of the total phospholipids present) were hydrolyzed over a period of 1 h of incubation (Table I). The full extent of phospholipase activity was apparent only in the presence of added sodium cholate and of p-chloromercuribenzoate, which is known to inhibit reutilization of the product fatty acids by the enzyme acyltransferase (17). Omission of CaCl₂ or addition of EDTA (5–6 mM) completely inhibited the enzyme activity.

Acyl-CoA Synthetase

The activity of this enzyme in isolated cilia from 39 °C-grown cells was measured using bovine serum albumin-complexed [1,4°C]palmitic acid (16:0) or [1,4°C]linoleic acid (18:2) as the substrate. The results presented in Table I show significant activity of the enzyme in isolated cilia and establish its ATP and CoA requirement. The rate of formation of palmitoyl-CoA was significantly higher than that of linoleoyl-CoA, thus indicating a preferential utilization of 16:0 fatty acid for the acyl-CoA synthesis at 37 °C (Table II and Fig. 1). The accumulation of both [14C]16:0-CoA and [14C]18:2-CoA reached a peak at 20 min, after which there was a rapid decrease in the amounts of the acyl-CoAs (Fig. 1). This decrease is probably due to a gradual inactivation of the synthetase enzyme coupled with a breakdown of the product into free fatty acids and CoA. The hydrolysis of fatty acyl-CoA, presumably by endogenous thiolase, was confirmed by adding authentic [14C]fatty acyl-CoA to the incubation mixture in the absence of ATP and CoA and observing that the labeled substrate was gradually broken down to radioactive free fatty acid. ATP and CoA at the levels routinely used reduced the net hydrolysis of [14C]fatty acyl-CoA in some undetermined way.

Acyltransferase

The final step of the reacylation process, esterification of acyl-CoA to lysophosphatidate, is catalyzed by an acyltransferase. The activity of this enzyme in isolated cilia was assessed by measuring the incorporation of [1,4°C]palmitoyl-CoA and [1,4°C]linoleoyl-CoA into phospholipids of isolated ciliary membranes. Significant amounts of acyl-CoA were incorporated into ciliary phospholipids, but only in the presence of supplementary ATP and CoA (Table III). Addition of glycerol 3-phosphate had no effect on the extent of acylation. This latter finding eliminated the possibility that contaminating micromolar acyltransferases were responsible for the observed acylation, since the microsomes readily acylate glycerol 3-phosphate. The addition of small amounts of lysophosphatidates stimulated the enzyme activity significantly (Table III).

Since ciliary membrane preparations also exhibited acyl-CoA synthetase activity, the ability of the ciliary membrane preparation to incorporate free fatty acids was examined. Even though small amounts of the added labeled fatty acids were incorporated into ciliary phospholipids, the extent of this acylation was comparatively lower than that observed with the acyl-CoA. When 14C-fatty acid was used as the substrate instead of its CoA derivative, the incorporation of the label into the ciliary phospholipids was linear only up to about 20 to 30 min of incubation, after which there was no further rise in the amounts of the label incorporated, thus indicating a gradual inactivation of the acyl-CoA synthetase enzyme (Fig. 2). Therefore, acyl-CoA was routinely used as the substrate to monitor acyltransferase activity in all the subsequent in vitro experiments. Because the acyl-CoA was added in large excess, any small loss due to thiolase activity could be neglected.

Effect of Temperature on Phospholipid Decacylation-Reacylation Reactions in Vivo

In vitro studies of membrane-bound enzymes are prone to artifacts of measurement and errors of interpretation because of the complex and often unnatural interactions between lipid and aqueous phase components in the cell-free preparations. Because ciliary membranes are metabolically isolated with respect to short term lipid metabolism (6, 7), it is feasible to conduct brief in vitro studies with assurance that the observed changes in lipid metabolism reflect only the isolated action of ciliary enzymes. We have taken advantage of this unique structural feature to determine the labeling patterns of ciliary lipids in the intact cell under conditions where only deacylation-reacylation enzymes residing in the cilia could be involved.

Specificity of the Acylation Reaction in Vivo

39 °C-grown cells were pulse labeled with either [1,4°C]palmitic acid or [1,4°C]linoleic acid for 5 min at 39 °C. This led to the incorporation of more than 80% of the added radioactivity into cellular lipids. The cilia, which are labeled entirely by in situ acylation under these conditions (7), were then quickly isolated, and the specific activities of the ciliary phospholipids and the positional distribution of the incorporated label were determined.

[1,4°C]Palmitic acid was incorporated almost equally into PC and PE, with lower amounts entering AEPL and sphingolipids (Table IV). A similar distribution was found using cells incubated for 10 or 15 min rather than 5 min. A different pattern, featuring heavy labeling of PE, was obtained when [1,4°C]linoleic acid was administered. The particularly low level of [1,4°C]linoleate incorporation into sphingolipids reflects the small amounts of this fatty acid present in these lipids (18).

The specificity of in vivo [1,4°C]palmitate and [1,4°C]linoleate incorporation was also measured in 15 °C-grown Tetrahymena cilia. Following isotope addition, the low temperature-acclimated cells were incubated for 15 min before cilia isolation rather than the 5 min used for 39 °C-grown cells because more time was required at 15 °C to achieve full (>80%) incorporation of radioactivity into the cellular lipid. It was previously determined that during this period of 15 min there was still no importation of lipids from the cell interior into the cilia (6, 7).

The main difference between the incorporation pattern of [1,4°C]palmitate into phospholipid classes of high and low temperature-grown cells lay in the considerably elevated specific radioactivity of PC at 15 °C relative to the other phospholipids (Table IV). In contrast, [1,4°C]linoleate was incorporated almost equally into PC and PE with small amounts entering AEPL.

The ciliary glycerophospholipid classes from the above experiment were hydrolyzed with snake venom phospholipase A₂. Surprisingly, [1,4°C]palmitic acid was incorporated almost equally into the sn-1 and sn-2 positions (Table V). This nearly

2 C. S. Ramesha and G. A. Thompson, Jr., unpublished observation.

3 The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; AEPL, 2-aminoethylphosphonolipid.
that phospholipase A can be modified in some way so as to allow higher levels of unsaturated fatty acids recently discovered to be present in intact phospholipids, thereby eliminating any chance for the enzyme to perform the expected role. Accordingly, cilia from 15 °C-grown cells showed incorporation at 37 and 15 °C, but catalyzed very little lipid hydrolysis at 15 °C. Cilia isolated from 15 °C-grown cells were active at 37 °C but catalyzed very little lipid hydrolysis at 15 °C. This observation suggests that phospholipase A can be modified in some way so as to retain optimum activity at different growth temperatures.

Effect of Temperature on Phospholipid Decarboxylating and Acylating Enzyme Activities in Vitro

Phospholipase A—Ciliary membranes from both 15 °C- and 39 °C-grown cells showed significant phospholipase A activity when assayed by measuring the release of radioactive fatty acids from prelabeled cells. However, the effect of temperature on the enzyme activity of these two preparations was strikingly different (Table VI). Cilia from 39 °C-grown cells were active at 37 °C but catalyzed very little lipid hydrolysis at 15 °C. Cilia isolated from 15 °C-grown cells were much more active at either high or low temperature than were their counterparts from 39 °C-grown cells. It is interesting to note that the absolute enzyme activity of cilia from 39 °C-grown cells at 37 °C was fairly similar to that of cilia from 15 °C-grown cells assayed at 15 °C. This observation suggests that phospholipase A can be modified in some way so as to retain optimum activity at different growth temperatures.

Acyl-CoA Synthetase—Ciliary membranes from 39 °C cilia, when assayed at 37 °C, showed significant acyl-CoA synthetase activity (Tables I and II). Palmitoyl-CoA was preferred over 18:2-CoA by a factor of 2 (Table II). However, when the incubation temperature was lowered to 22 or 15 °C, there was only a minimal accumulation of either 16:0-CoA or 18:2-CoA, even when longer incubation periods were used. Ciliary membrane from 15 °C-grown cells showed no significant acyl-CoA synthetase activity with the standard assay either at 15 or 39 °C under a variety of modified incubation conditions. This prevented a comparison of the temperature effect on acyl-CoA synthetase specificity. It also raised questions as to whether the enzyme was sufficiently active in vivo at low temperature to perform the expected role. Accordingly, cilia that had been detached from the cells but not disrupted with detergents were incubated with [3H]16:0 and [14C]18:2 in the presence of ATP and CoA. Significant and roughly equal amounts of the two tracers were incorporated into cilia from 15 °C-grown cells as well as cilia from 39 °C-grown cells, and both types of cilia showed incorporation at 37 and 15 °C. Unfortunately, the bulk of the radioactivity in all cases was found in intact phospholipids, thereby eliminating any chance to study the specificity of the acyl-CoA synthetase in isolation. The data do, however, provide strong evidence for an active acyl-CoA synthetase at both high and low temperature.

Acyltransferase—Both 15 and 39 °C ciliary membranes incorporated significant amounts of radioactivity into their sn-2 position, relative to the values observed in 39 °C-grown cells. This result is in keeping with the higher levels of unsaturated fatty acids recently discovered to accumulate in the sn-1 position at 15 °C (19, 20).

Effect of Temperature on Phospholipid Decarboxylating and Acylating Enzyme Activities in Vivo

Details of incorporation of the radioactive fatty acids into ciliary phospholipids from 39 °C- and 15 °C-grown cells following a short term in vivo labeling with either [3H]16:0 or [14C]18:2

| Position | Phospholipids of 39 °C cilia | Phospholipids of 15 °C cilia |
|----------|-----------------------------|-----------------------------|
|          | PC  | PE | AEPL | PC  | PE | AEPL |
| sn-1     | 51.3 ± 2.4 | 51.4 ± 3.6 | 64.6 ± 7.9 | 46.6 ± 2.5 | 44.5 ± 1.8 | 46.4 ± 6.6 |
| sn-2     | 48.7 ± 2.4 | 48.6 ± 3.6 | 35.4 ± 7.9 | 53.4 ± 2.5 | 55.5 ± 1.8 | 53.6 ± 6.6 |
| sn-1     | 0   | 13.2 ± 1.4 | 0   | 10.4 ± 3.3 | 13.6 ± 0.8 | 13.9 ± 1.8 |
| sn-2     | 100 | 86.8 ± 1.4 | 100 | 89.6 ± 3.3 | 86.4 ± 0.8 | 86.1 ± 1.8 |

TABLE V

Positional distribution of [14C] fatty acids in ciliary phospholipids from 39 °C- and 15 °C-grown cells following a short term in vivo labeling with either [3H]16:0 or [14C]18:2

Details of incorporation of the radioactive fatty acids into 39 °C and 15 °C cilia, extraction of ciliary lipids, and separation of phospholipids by TLC are as given in Table IV. The phospholipids (PC, PE, or AEPL) were suspended in 1 ml of peroxide-free diethyl ether and digested with phospholipase A from Crotalus adamanteus venom. The released free fatty acids (sn-2) and lysophospholipids (sn-1) were separated by TLC and counted for radioactivity. Values are expressed as per cent of total radioactivity (dpm) and are mean ± S.D. of three independent experiments.
Temperature Effect on Phospholipid Enzymes and Cell Membranes

Effect of incubation temperature on ciliary phospholipase A activity of 39 °C- and 15 °C-grown cells

Details of phospholipase A assay are same as in Table I, except that the incubation was carried out at either 37 or 15 °C as indicated. Values are nanomoles of fatty acid hydrolyzed per mg protein h⁻¹ and are mean ± S.D. of 3-5 experiments. Activity was calculated based on specific radioactivities of the total ciliary phospholipids.

| Source of cilia | Incubation temperature |
|-----------------|------------------------|
|                 | 37 °C | 15 °C |
| 39 °C-grown cells | 15.7 ± 1.0 | 1.3 ± 0.9 |
| 15 °C-grown cells | 49.2 ± 3.2 | 27.0 ± 3.8 |

Effect of temperature on ciliary acyltransferase activity of 39 °C- and 15 °C-grown cells

Details of the acyltransferase assay are same as in Table III. ATP (20 mM) and CoA (500 μM) were routinely included in the incubation mixture. Incubation was carried out at the specified temperatures. 1-Acyl-lyso-PE (0.08 μmol) was added as an aqueous suspension in the incubation buffer. Values are picomoles of acyl group incorporated per mg of protein⁻¹ h⁻¹ and are mean ± S.D. of 4 or more experiments.

| Source of cilia | Acyl-CoA added | Acyltransferase activity |
|-----------------|----------------|--------------------------|
|                 | No lysophospholipid | With 1-acyl-lyso-PE |
|                 | 37 °C | 15 °C | 37 °C | 15 °C |
| 39 °C cells     | [14C]16:0 | 9.96 ± 3.1 | 1.31 ± 1.1 | 15.5 ± 2.3 | 9.96 ± 2.7 |
|                 | [14C]18:2 | 5.91 ± 1.8 | 0.1* | 27.4 ± 1.7 | 6.10 ± 1.9 |
| 15 °C cells     | [14C]16:0 | 16.4* | 4.68 ± 1.8 | 35.7* | 15.9 ± 2.7 |
|                 | [14C]18:2 | 18.5* | 6.62 ± 2.3 | ND* | 21.4 ± 3.1 |

* Average of two experiments.

Acyltransferase activity of 15 °C-grown cells was greatly reduced at 15 °C, while the activity of ciliary acyltransferase from 15 °C-grown cells was greatly stimulated at 27 °C.

The enzyme activity in both 39 °C- and 15 °C-grown ciliary membranes was markedly reduced when lyso-PE was included in the reaction mixture. It is noteworthy that although 39 °C-grown ciliary membranes showed very little acyltransferase activity at 15 °C, they did utilize significant amounts of both the 16:0-CoA and 18:2-CoA at that temperature in the presence of added lyso-PE (Table VII).

Specificity of the Acyltransferase Reaction in Vitro

As shown above (Table VII), detached ciliary membranes from both 15 °C- and 39 °C-grown cells incorporate the acyl moiety of fatty acyl-CoA into their phospholipids, albeit at rates lower than achieved in vivo. The relative incorporation of [14C]palmitate and [14C]linoleate into the different phospholipid classes of detached cilia was found to be similar to that measured in vivo (Table IV). The most notable departure from the in vivo labeling pattern was the slope in the percentage of radioactivity in PC to less than half the value found in vivo.

Since addition of exogenous lysophospholipids stimulated the acyltransferase activity, the ability of different lysophospholipids to stimulate the enzyme activity was tested. Except for 1-acyl-lyso-AP and 2-acyl-lyso-PC, all the lysophospholipids tested stimulated the acyltransferase activity significantly (Table VIII). However, the incorporation of 18:2 into 1-acyl-lyso-PE and 2-acyl-lyso-PC was slightly higher than that of 16:0. Addition of increasing amounts of lysophospholipids (from 10 to 50 nmol) stimulated the enzyme activity consistently, but a linear relationship between the substrate concentration and enzyme activity was not observed. 50, 80, and 100 nmol of the added lysophospholipids stimulated the acyltransferase to the same extent, and concentrations of lysophospholipids 800 nmol and higher inhibited the enzyme activity (data not shown). Distribution of the incorporated radioactivity in different phospholipid classes showed that irrespective of the lysophospholipid added, the maximum incorporation was always clearly into that added lysophosphatidylcholine.

In 39 °C-grown ciliary phospholipids, the positional distribution of [14C]palmitate incorporated in vitro (Table IX) was quite similar to that found in vivo. In contrast, the detached ciliary membranes from 39 °C-grown cells incorporated [14C]linoleate into the sn-1 position of all glycerophosphatides to a considerable extent (Table IX), unlike the striking preference for the sn-2 position (Table V).

Added lysophospholipids strongly influenced the pattern of acylation. Thus, in the presence of 1-acylglycerophosphoryldiacylglycerol, the positional distribution of the incorporated radioactivity was similar to that found in vivo. However, lysophospholipids added in vitro inhibited incorporation of lysophospholipids into membranes. The incorporation of [14C]lysophosphoadenosine 5'-triphosphate into phospholipids was inhibited by 0.1-2.0 mM ATP, but 4 mM ATP restored incorporation. The incorporation of [14C]lysophospholipids into membranes was inhibited by 3-5 mM CoA, but 10 mM CoA restored incorporation.

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Effect of different lysophospholipids on ciliary acyltransferase activity

Details of the assay are same as in Table III. ATP (20 mM) and CoA (500 μM) were also included in the reaction mixtures. The lysophospholipids were obtained by digesting respective phospholipids with either phospholipase A2 from C. adamanteus venom (to prepare 1-acylphospholipid) or lipase from Rhizopus (to prepare 2-acylphospholipid), and the lysophospholipids were added as aqueous suspensions in the incubation buffer. Each assay was done in duplicate. Phospholipase A2 analysis of the products confirmed that incorporation was predominantly into the expected position. Values are mean ± S.D. of 3 or more experiments.

| Lysophospholipids added (0.08 μmol) | [14C]16:0 incorporated | [14C]18:2 incorporated |
|------------------------------------|------------------------|------------------------|
| None                              | 9.96 ± 3.1             | 5.91 ± 1.8              |
| 1-Acyl-PC                          | 16.7 ± 3.6             | 14.7 ± 2.5              |
| 1-Acyl-PE                          | 15.5 ± 2.3             | 27.4 ± 1.7              |
| 1-Acyl-AP                          | 7.48*                  | 6.29*                  |
| 2-Acyl-PC                          | 5.82 ± 2.1             | 4.83 ± 1.1              |
| 2-Acyl-AP                          | 14.7 ± 1.1             | 30.0 ± 1.9              |

* Average of 2 experiments.
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Choline or 1-acylglycerophosphorylethanolamine, the incorporation was mostly into the sn-2 positions of PC and PE, respectively, and in the presence of 2-acylglycerophosphorylcholine or 2-acylglycerophosphorylethanolamine, the incorporation of the radioactivity was mostly into the sn-1 positions (data not shown). No attempt was made to examine the in vitro specificity of the acyltransferase reaction in ciliary membranes from 15 °C-grown cells.

**DISCUSSION**

Recent findings in this laboratory (5–7) have led us to refine our concept of how membrane lipid alterations are brought about during cellular acclimation to low temperature. Our evidence suggests that certain organelles within the cell can sense and react to low temperature stress independently and differently from others. Individual responses of this type, if they occur, would be very difficult to detect in most biological systems because of the tightly integrated metabolic pathways. However, *Tetrahymena*, having a somewhat atypical structural organization, lends itself to a detailed study of simultaneous but independent responses to chilling in two associated compartments.

Our previous work has established unequivocally that 1) the ciliary lipid composition of *Tetrahymena* is, for several hours after chilling, unaffected by the profound lipid modification rapidly induced within the body of the cell by low temperature (5, 6) and 2) despite this metabolic isolation from the centers of lipid metabolism, certain enzymes associated with the ciliary membranes do catalyze significant changes in situ in phospholipid molecular species composition during the early phase of low temperature acclimation (4, 7). These latter changes can be demonstrated both in vivo and in vitro.

As a result of the altered lipid composition at low growth temperatures, the fluidity of the *Tetrahymena* ciliary membrane, as estimated by fluorescence depolarization measurements, is significantly increased (4). Although the specific quantitative contribution of the initial molecular species changes to the increased fluidity has not yet been evaluated in cilia, molecular species retailoring of this very type has been clearly identified as a major cause of increased fluidity in *Tetrahymena* microsomal membranes (21). Thus the decylation-reacylation process has the demonstrated potential to effect large changes in the ciliary membrane physical properties.

The present communication is aimed at confirming the presence in cilia of all the enzymes required for the retailoring of phospholipid molecular species and, more importantly, determining whether action by one or more of these enzymes could lead to physiologically important compositional changes. The findings confirm the presence in cilia of the three enzymes needed for phospholipid decylation-reacylation and show that their specificities are indeed altered by changing temperature.

All of the three enzymatic reactions, namely phospholipid decylation, fatty acid activation, and phospholipid reacylation proceeded much more slowly in a given preparation at 15 °C than at 37 °C. The large increase in both phospholipase activity and acyltransferase activity in cilia following acclimation of cells to growth at 15 °C (Tables VI and VII) was unexpected. The interesting fact that these two activities in the high and low temperature-acclimated cells, when measured at their respective growth temperatures, are similar may be fortuitous, but on the other hand, the possibility of a physiologically meaningful compensatory increase in the capacity of these reactions at 15 °C cannot be dismissed.

The in vitro specificity of the acyltransferase reaction for the major phospholipid classes was similar to that measured in vivo (Table IV), indicating that the factors governing this property were not greatly affected by cell fractionation. Likewise, the positional specificity directing the in vivo incorporation of [14C]palmitic acid nearly equally into the sn-1 and sn-2 locations of phospholipids (Table V) was retained in vitro (Table IX). In sharp contrast, the highly specific insertion of [14C]18:2 into the sn-2 position of phospholipids in vivo (Table V) was not retained in vitro (Table IX). In addition to the change in positional specificity of acylation caused by cell disruption, a more physiologically significant change was induced by growing the cells at low temperature (Table V). Under these conditions too, the most notable difference involved the enhanced insertion of 18:2 into the sn-1 position of phospholipids.

The amount of product formed, the lipid class specificity, and the positional specificity could all be markedly altered in vitro by simply adding an exogenous lysophospholipid acceptor (Table VIII). As a result of the altered lipid composition at low growth temperatures, the fluidity of the *Tetrahymena* ciliary membrane, as estimated by fluorescence depolarization measurements, is significantly increased (4). Although the specific quantitative contribution of the initial molecular species changes to the increased fluidity has not yet been evaluated in cilia, molecular species retailoring of this very type has been clearly identified as a major cause of increased fluidity in *Tetrahymena* microsomal membranes (21). Thus the decylation-reacylation process has the demonstrated potential to effect large changes in the ciliary membrane physical properties.

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The amount of product formed, the lipid class specificity, and the positional specificity could all be markedly altered in vitro by simply adding an exogenous lysophospholipid acceptor (Table VIII). Both 1-acyl- and 2-acylsphospholipids became acylated when exogenously added into the assay system, thus indicating the presence in cilia of both sn-2 and sn-1 specific acyltransferases. The stimulation cannot be due merely to the detergent effects of the added lysophosphatides, since 1-acyl-lyso-AEPL and 2-acyl-lyso-PC did not have any effect on the enzyme activity (Table VIII).

The lower level of acyltransferase activity observed in the absence of added acceptor (Tables III and VII) is probably due to limited availability of the endogenous acceptor lysophosphatides to the enzyme. It appears that acyl chain retailoring in cilia is a tightly coupled cyclic process in which intact phospholipids constitute the only sizable metabolic pool. The absence of other pooled intermediates and the lack of pronounced acyltransferase specificity toward exogenous lysophosphatides indicate that the deacylation of phospholipids rather than their reacylation is the key regulatory step in ciliary phospholipid retailoring. Phospholipase A action produces both free fatty acids and lysophospholipids, the two substrates needed for the retailoring process. These substrates are then rapidly reutilized in the formation of new phospholipids having a molecular species composition determined by the specificity of the enzymes involved. The rapidity with which the overall ciliary phospholipid composition is affected under stress will depend upon 1) the rate of the in situ phospholipase action, 2) the degree of enzyme specificity change brought about by the stress, and 3) the importation of more unsaturated fatty acids from other parts of the cell. Under the conditions studied here, the first two factors clearly predominate.

In the present study the individual activities of phospholipases A₁ and A₂ were not resolved. It will be desirable to examine in detail the effects of stress on each enzyme's specificity and rate. Both these enzymes require Ca²⁺, a cation whose concentration inside cilia fluctuates widely in response to various stimuli. Recent evidence for the involvement of calmodulin in phospholipase A regulation (22) coupled with the finding of calmodulin in *Tetrahymena* cilia (23, 24) suggests a possible mechanism for regulating phospholipid deacylation in this organelle. The obvious advantage of having an alternative means of lipid modification in such a metabolically remote part of the cell should heighten interest in its study.

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SUPPLEMENTARY MATERIAL TO: THE MECHANISM OF MEMBRANE RESPONSE TO CHILLING (C. S. RAMESH AND G. A. THOMPSON, JR.)

EXPERIMENTAL PROCEDURES

Materials

- [1-14C]palmitic acid (specific radioactivity 62.5 mCi/mmol).
- [1-14C]stearic acid (specific radioactivity 50.9 mCi/mmol).
- [1-14C]linoleic acid (specific radioactivity 55.9 mCi/mmol).
- [1-14C]a-linolenic acid (specific radioactivity 50.1 mCi/mmol).
- [1-14C]arachidonic acid (specific radioactivity 51.6 mCi/mmol). These lipids were purchased from New England Nuclear Corporation, Boston, MA. Convective (CHCl3-MeOH-H2O) fractionation of [1-14C]-labeled glycerolipids was done at 50°C using a glass column packed with glass beads. The fractions were eluted from the column with chloroform-methanol-acetic acid-water (20:40:10:10, v/v). The fractions containing the radioactivity were evaporated to dryness, dissolved in 0.1% acetic acid in water, and counted in a liquid scintillation counter.

Isolation of cells: Cells were obtained from 250 ml of harvested cultures. The cells were harvested, washed, and resuspended in buffer A (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) at a concentration of 5 x 10^10 cells/ml. The cells were lysed by sonication for 2 min with a 1.5 mm probe at 20 kHz and 50% power output. After sonication, the lysate was centrifuged at 100,000 x g for 1 h at 4°C, and the supernatant was used for further analysis.

Incubation of labeled fatty acids into cells: A suspension of cells in buffer A containing [1-14C]-labeled fatty acids (100 nM) was incubated for 15 min at 37°C. After incubation, the cells were washed with fresh buffer A and resuspended in buffer A at a concentration of 5 x 10^10 cells/ml. The cells were then incubated with [1-14C]-labeled fatty acids (100 nM) at 37°C for 15 min. The reaction was terminated by the addition of 100 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After incubation, the reaction mixture was centrifuged at 100,000 x g for 1 h at 4°C, and the supernatant was used for further analysis.

Preparation of lipids: Lipids were extracted from isolated cells or isolated membranes by the procedure of Bligh and Dyer (12). Individual phospholipids were separated by TLC and used either for further compositional analysis or for determination of radioactivity as described elsewhere (12). Individual phospholipids were identified by their Rf values and by comparison of their radioactivity with that of known standards. The lipids were quantified by scintillation counting of the radioactivity in the lipid fraction and/or by thin-layer chromatography.

Preparation of tritiated water: Tritiated water was prepared by digesting tritiated phospholipids with phospholipase A2 in a buffer containing 0.1 M KCl, 0.1 M NaCl, and 0.01 M Tris-HCl, pH 7.5. The reaction mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of 100 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After incubation, the reaction mixture was centrifuged at 100,000 x g for 1 h at 4°C, and the supernatant was used for further analysis.

The reaction mixture was then incubated with [1-14C]-labeled fatty acids (100 nM) at 37°C for 15 min. The reaction was terminated by the addition of 100 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After incubation, the reaction mixture was centrifuged at 100,000 x g for 1 h at 4°C, and the supernatant was used for further analysis.

The data reported in this study were typical of at least three separate experiments.
Temperature Effect on Phospholipid Enzymes and Cell Membranes

Table I

| Condition | Phospholipase A activity | Acyl-CoA synthetase activity |
|-----------|--------------------------|-----------------------------|
| without additives | 2.1 ± 0.7 | 60.7 |
| with additives | 15.7 ± 1.7 | 48.8 |

Table II

| Substrate | Acyl-CoA synthetase activity |
|-----------|-------------------------------|
| (1-14C)-palmitic acid | 517.9 ± 69.1 |
| (1-14C)-linoleic acid | 280.3 ± 71.8 |

Table III

| Time (min) | Co-factors | Glucose-3-P synthase | Lysophosphatidylcholine synthase |
|-----------|------------|---------------------|---------------------------------|
| 0         | +/−        | −/−                 | +/−                             |
| 10        | +/−        | +/−                 | −/−                             |
| 30        | +/−        | +/−                 | −/−                             |
| 60        | +/−        | +/−                 | −/−                             |

Fig. 1. Time course of acyl-CoA formation in isolated ciliary preparations. The acyl-CoA synthetase was measured as described under "Experimental Procedures." The reaction was carried out at 37°C for the indicated time points. Each point on the curve represents either the mean ± SD of three or more experiments (a−c) or the average of at least two experiments (a−c).

Fig. 2. Time course of ciliary acyltransferase activity: Effect of free fatty acid and lipoprotein. Measurement of the enzyme activity is described under "Experimental Procedures." The incubation was carried out at 37°C for the indicated time periods, and all experiments involved duplicate samples. Each point on the curve represents mean ± SD of three or more experiments.