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Restructuring of plasma membrane phospholipids in isolated hepatocytes of rainbow trout during brief in vitro cold exposure

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Abstract. Adaptive changes in membrane physical properties in response to changing environmental temperature (e.g., increased fluidity at low growth temperatures) are well known in poikilotherms; however, the time-course of this response has received little attention. In this study the plasma membrane lipids of hepatocytes prepared from 20 °C-acclimated trout were analyzed for phospholipid class and molecular species composition and metabolism after the cells were exposed to 5 °C for 6 hours. Proportions of phosphatidylethanolamine and phosphatidylcholine were not altered by in vitro incubation at either 5 or 20 °C. Molecular species analysis revealed that proportions of 18:1/20:5-phosphatidylcholine were significantly lower in plasma membranes of 5 °C incubated cells, while decreases in 16:0/20:4-phosphatidylcholine, an unidentified phosphatidylcholine species, and 16:0/16:0-phosphatidylethanolamine as well as increases in 16:0/16:1-phosphatidylcholine bordered on significance. Exogenous radiolabeled molecular species of phosphatidylcholine (16:0/16:0-phosphatidylcholine and 16:0/18:1-phosphatidylcholine) were converted into other species at both temperatures, and the formation of some was influenced by incubation temperature. For example, cells exposed to 5 °C convert significantly more 16:0/16:0-phosphatidylcholine into 16:0/20:4-phosphatidylcholine and 18:0/16:1-phosphatidylcholine and less into 18:1/18:1-phosphatidylcholine and 16:0/22:6-phosphatidylcholine than cells incubated at 20 °C. In addition, cells at 5 °C metabolized 16:0/18:1-phosphatidylcholine to a lesser extent than those at 20 °C. The profile of conversion products indicates that deacylation/reacylation, elongation and desaturation reactions all participate in this early membrane restructuring. It is concluded that the plasma membrane of trout hepatocytes is a highly dynamic structure characterized by continuous lipid restructuring/turnover which can be rapidly altered upon acute cold exposure to adjust membrane phospholipid molecular species composition to the prevailing thermal environment.

Key words: Temperature acclimation/adaptation – Membrane lipid metabolism – Deacylation/reacylation cycle – Lipid desaturation/elongation – Trout, *Oncorhynchus mykiss*

Introduction

Many poikilotherms adjust the physical properties of their cellular membranes in response to a change in temperature in such a way as to retain a particular phase state or fluidity despite T_a (Sinensky 1974). It is well known that membranes from cold-acclimated organisms are more fluid than membranes from warm-acclimated conspecifics; however, when measured at the acclimation temperature, fluidities of membranes from cold- and warm-acclimated individuals are frequently similar (Hazel and Williams 1990). The time-course of this HVA (Sinensky 1974) has received little attention. Recently, we have reported that after only 6 h of incubation at 5 °C, plasma membranes isolated from hepatocytes of 20 °C-acclimated trout exhibit increased membrane fluidity (Williams and Hazel 1994). This increase in fluidity is sufficient to compensate for approximately 50% of the cold-induced reduction in fluidity, and accounts for half of the compensation observed in these membranes in fully acclimated organisms (Hazel et al. 1992).

Such adaptive alterations in membrane fluidity are most likely based on changes in membrane lipid composition. Indeed, differences in membrane lipid composi-

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; HEPES, N-(2-hydroxyethyl)piperazine-*N*'-(-2-ethanesulphonic acid); HPLC, high-performance liquid chromatography; HVA, homeoviscous adaptation; MS, molecular species; MS-222, 3-aminobenzoic acid ethyl ester (methanesulphonate salt); RRT, relative retention time; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; TRIS, tris(hydroxymethyl)aminoethane; T_a ambient temperature

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tion between cold- and warm-acclimated individuals are a hallmark (if not a definition) of temperature acclimation (Hazel and Williams 1990), and there is increasing evidence that some changes in membrane phospholipid composition occur very early in the process of temperature acclimation. Surprisingly, perhaps, these early responses appear to be supplanted by other membrane alterations as acclimation proceeds. For example, the weight percentage of PE in total phospholipids of 20 °C-acclimated trout gill increased dramatically within 24 h of transfer from 20 to 5 °C but returned to nearly pre-transfer levels after a month at 5 °C (Hazel and Carpenter 1985). Similarly, in plasma membranes of trout kidney the percentage of monoenoic molecular species peaked just 24 h after transfer from 20 to 5 °C (Hazel and Landrey 1988). In the ciliate *Tetrahymena* grown at 39 °C, ciliary membrane fluidity was completely compensated after 1 h at 15 °C (Dickens and Thompson 1981; Ramesha and Thompson 1982; Martin and Thompson 1978) due to a redistribution of acyl chains among the phospholipids without any overall change in membrane acyl chain composition (Dickens and Thompson 1982; Ramesha and Thompson 1983; Ramesha et al. 1982; Ramesha and Thompson 1984).

Temperature acclimation thus appears to advance through a hierarchy of steps. Early alterations constitute emergency measures to contend with rapid temperature changes while more permanent, and perhaps more effective, changes are introduced as the stress continues (Hazel and Williams 1990; Hazel and Landrey 1988; Lynch and Thompson 1984). In order to examine the compositional and metabolic basis for previously documented rapid adjustments in the fluidity of trout hepatocyte plasma membrane (Williams and Hazel 1994), we examine plasma membrane phospholipid composition, and patterns of phospholipid metabolism in isolated hepatocytes from 20 °C-acclimated fish during brief in vitro incubation at 20 and 5 °C.

Materials and methods

Materials. Phospholipase C (type I from *C. perfringes*, and type V from *B. cereus*), direct blue 15 (trypan blue), HEPES, MS-222, Percoll, 3,5-dinitrobenzoyl chloride, BSA, methylene blue, BHT, heparin (Na salt, grade I from porcine intestinal mucosa), TRIS and silica gel type G were from Sigma. Collagenase A was from Boehringer Mannheim. Lipid standards were from Sigma and Avanti Polar Lipids Inc. (Alabaster, Ala., USA). Protein was assayed by the method of Smith et al. (1985) using a kit purchased from Pierce (Rockford, Ill., USA). All other chemicals were analytical reagent grade or better. Organic solvents were redistilled just before use; chloroform was routinely washed, dried and redistilled before use (Kates 1972). HPLC-grade solvents were supplied from Fisher Scientific (Fair Lawn, N.J., USA) and J. T. Baker (Phillipsburg, N.J., USA).

L-α-Dipalmitoyl, [dipalmitoyl-1-^{14}C]-phosphatidylcholine (16:0/16:0-PC, 114 Ci · mol^{-1}, see footnote b in Table 1 for an explanation of this terminology) and L-α-1-palmitoyl-2-oleoyl, [oleoyl-1-^{14}C]-phosphatidylcholine (16:0/18:1-PC, 58 Ci · mol^{-1}) were from New England Nuclear (Boston, Mass., USA). Radiolabeled phospholipids were routinely purified (to remove minor but significant quantities of radiolabel present in other MS) before cell labeling by preparative chromatography on a C_{18} reversed-phase column eluted with the solvent described by Patton et al. (1982).

Animals. Rainbow trout *(Oncorhynchus mykiss)* were obtained from the Alcehsay National Fish Hatchery, Whiteriver, Ariz., USA, and were held in a 2300 l cylindrical aquarium continuously supplied with fresh dechlorinated water. The total aquarium volume was exchanged every 24 h and was maintained at 20 °C under a 12:12 light:dark cycle. Fish were fed once daily with trout chow (Glencoe Mills). The lipid composition of this chow has been reported previously (Hazel 1979). All trout were maintained under these conditions for at least 1 month prior to the experiments.

Hepatocyte isolation and incubation. Hepatocytes were isolated (Williams and Hazel 1994) from anesthetized (MS-222, 0.005%, pH 8.0 with $NaHCO_3$) 20 °C-acclimated fish (311.4±130.5 g). Livers were perfused through a cannula in the hepatic portal vein, first (3–5 min) using medium A (mmol · l^{-1}: glucose 10, HEPES 20, NaCl 176, KCl 5.4, $NaHCO_3$ 8.0, $MgSO_4$ 8.1, KH_2PO_4 4.4, Na_2HPO_4 3.5 including 36.2 USP units of heparin · ml^{-1}, continuously gassed with 2% CO_2/98% O_2) followed by medium B (30–40 min, prepared as medium A, but including 0.4 mg collagenase · ml^{-1} instead of heparin). The resulting disrupted liver was carefully removed from the fish and passed sequentially through 250 and 74 µm nylon mesh. Hepatocytes were collected and washed by three centrifugations at 100 *g* for 5 min. Cell viability was determined by trypan blue exclusion and was always better than 98%. For metabolic studies, hepatocyte preparations from two fish were routinely pooled.

Hepatocyte Labeling and Incubation. 10 µCi of 16:0/16:0-PC or 16:0/18:1-PC in $CHCl_3/CH_3OH$ (2:1 v/v) were added to a glass tube and dried under a stream of N_2 before addition of 20 ml of medium C (medium A excluding heparin and containing 1 mmol · l^{-1} $CaCl_2$ and 2% BSA). This mixture was sonicated for 5 min at 5 °C in a bath-type sonicator resulting in the complete suspension of the radiolabels. Washed hepatocytes were resuspended in labeling medium at cell densities below 50×10^6 cells · ml^{-1} in silanized 25-ml Erlenmeyer flasks and incubated at 20 °C for 1 h or 5 °C for 6 h (because of reduced phospholipid metabolism at 5 °C this large disparity in incubation time was necessary to ensure comparable degrees of metabolism, i.e., product formation of the parent molecular species; see Results). During incubation the hepatocytes were continuously shaken under prehumidified 98% O_2/2% CO_2. For membrane compositional studies, cells were incubated as described above but in the absence of radiolabel.

Plasma membrane purification procedure. After incubation at the test temperature, cells were thoroughly washed by three centrifugations (as above) in Medium D (Medium C free of BSA) and their plasma membranes isolated as described previously (Williams and Hazel 1994). Control cells were sampled immediately after isolation from the fish. Hepatocytes were homogenized in three liver volumes (1 ml per g liver) of medium E (0.32 mmol · l^{-1} sucrose, 20 mmol · l^{-1} TRIS, pH 7.45) until >85% were disrupted, and subsequently diluted with four liver volumes of medium F (0.25 mmol · l^{-1} sucrose, 20 mmol · l^{-1} TRIS, ph 7.45). A crude plasma membrane/mitochondrial preparation (Mt) was isolated by differential centrifugation (the supernatant over a 10-min, 600-*g* pellet was centrifuged at 7000 *g* for 20 min (Williams and Hazel 1994)). This pellet was resuspended in 0.5 liver volumes of medium F and 1-ml portions were layered onto 20 ml of a solution of 0.25 mmol · l^{-1} sucrose, 20 mmol · l^{-1} TRIS (pH 7.45) and 18% (v/v) Percoll (Anonymous 1993). Following centrifugation for 18 min at 33 000 *g* (50.2Ti rotor) on this density gradient medium the plasma membrane fragment (top of tube, mitochondria settled to the bottom), were collected and washed free of Percoll by centrif-

ugation for 2 h at 185 000 *g* (Anonymous 1993). The cleaned membranes were resuspended in 0.5 liver volumes of 20 mmol · l^{-1} TRIS (pH 7.45) and stored at −80 °C until lipid extraction. These plasma membranes have been shown to be enriched 116-fold over the cellular homogenate based on the activity of the plasma membrane marker enzyme 5′-nucleotidase (Williams and Hazel 1994).

Lipid extraction and analysis. Total plasma membrane lipids were extracted using the method of Bligh and Dyer (1959) and subsequently stored in $CHCl_3/CH_3OH$ (2:1, v/v) containing 0.01% (w/v) BHT under N_2 at −80 °C until analysis. Phospholipid classes were separated from each other and from neutral lipids by normal-phase silica HPLC (10 mm ID×25 cm, 5 µm packing, Rainin Instruments, Woburn, Mass., USA) according to the method of Patton et al. (1982). Hexane/2-propanol (1:1 v:v, flow rate 2 ml · min^{-1}, detection 205 nm) was used to elute neutral lipids, and was followed by hexane/2-propanol/25 mmol · l^{-1} potassium phosphate buffer (pH 7.0)/ethanol/acetic acid (356:490:62:100:0.6 v:v) previously filtered through 0.5-µm Teflon filters (Nucleopore Corp., Pleasanton, Calif., USA). PC was eluted at a flow rate of 3 ml · min^{-1}. Peak identities were verified by co-chromatography with authentic standards on both HPLC and TLC (Christie 1982). Since PC and PE together make up 80% of the total membrane phospholipid (Hazel 1979; Malak et al. 1989) further analysis focused on these lipids. PE and PC fractions were collected from the column, taken to dryness in a rotary evaporator, dissolved in $CHCl_3/CH_3OH$ (2:1 v/v, 0.05% w/v BHT) and stored at −80 °C.

Quantification of MS was accomplished by first treating the lipids with phospholipid-specific phospholipase C to form diacylglycerols (Kito et al. 1985) and subsequently converting these into dinitrobenzoyl derivatives (Kito et al. 1985; Takamura et al. 1986). Just prior to phospholipase C digestion, known quantities of distearoyl PE or PC (which are not normal constituents of these membranes) were added as internal standards. For PC, phospholipase C (type I) and a solution of 10 mmol · l^{-1} TRIS (pH 7.5), 30 mmol · l^{-1} H_3BO_3 and 0.6 mmol · l^{-1} $CaCl_2$ was added to dried lipid. For PE, the reaction was performed in a similar medium containing 0.4 mmol · l^{-1} $ZnCl_2$ instead of $CaCl_2$ and the type V enzyme. Diacylglycerols were extracted with diethyl ether.

Dinitrobenzoyl derivatives were formed by reaction with 3,5-dinitrobenzoyl chloride in pyridine (Kito et al. 1985; Takamura et al. 1986), taken up in hexane containing 0.05% BHT, gassed with N_2, and stored at −80 °C. Derivatives were dried, redissolved in 75 µl acetonitrile and individual MS separated on a 4.6 mm I.D.×25 cm Microsorb C-18 HPLC column (5 µm packing, Rainin Instrument Co., Woburn, Mass., USA) eluted with acetonitrile/2-propanol (90:15 v:v, flow rate 1 ml · min^{-1}; detection at 254 nm). Peak identities were confirmed by comparison of observed retention times with those of authentic standards as well as to published retention times (Bell and Dick 1991) and also by gas chromatography of methyl esters prepared by acid catalyzed transesterification (Christie 1982) of the separated dinitrobenzoyl-diacylglycerols.

Peak areas were determined with Chromatochart PC software (Interactive Microware, State College, Pa., USA) and converted to mass by comparison with the internal standard. Total PE and PC masses were calculated by summation of the MS masses. Derivatized MS from experiments using radiolabeled lipids were collected from the column in scintillation vials and allowed to dry under a stream of air before determination of radioactivity using a Beckman LS-7000 liquid scintillation counter. Quenched and unquenched standards were used and all samples were corrected for quenching using the H# method of Horrocks (1976).

Statistics. Fractional data were arcsine transformed for statistical analyses (Sokal and Rohlf 1981) performed using ABstat (Anderson Bell, Parker, Colo., USA), and the level of significance was based on the transformed data before reconversion into percentages as recommended (Sokal and Rohlf 1981). *P* values below 0.05 were considered significant.

Results

Phospholipid analysis

Hepatocytes isolated from 20 °C-acclimated rainbow trout remained >98% viable (i.e., excluded trypan blue) after 6 h of in vitro incubation at both 5 and 20 °C, and previous data indicate that these cells remain metabolically active throughout the periods employed here (Williams and Hazel 1994; Hazel 1990; Sellner and Hazel 1982). Lipid analysis of the isolated plasma membrane fraction revealed no statistically significant changes in the total PE [83.0±16.0 (mean±SE) µg lipid per mg protein] and PC (901±215.0 µg lipid per mg protein) content between membranes isolated from control cells, from cells maintained at the acclimation temperature of 20 °C for 6 h, and from cells maintained at 5 °C for 6 h. The ratio of PE to PC (0.12±0.03), a more sensitive indicator of PE/PC changes, was also unaffected by cold exposure for this amount of time.

Phospholipid molecular species analysis

The PE and PC fractions were further resolved into their individual MS by HPLC. Thirty-five separate MS of PC and 38 of PE were detected and quantified (Table 1), representing the first such analysis of these highly purified membranes (Williams and Hazel 1994). Consistent with observations made on similar membranes (Hazel and Williams 1990), PC MS of plasma membrane from trout hepatocytes were dominated by molecules containing 16:0 at the *sn*-1 position while the PE pool was characterized by having 18:0 as the major contributor in that position. Both the PC and PE pools contained predominantly long chain (18–22 carbon) polyunsaturated acyl groups at the *sn*-2 position with 18:2, 20:4 and 22:6 particularly abundant.

In only a few cases (given in Table 2) were significant differences in the mole percentage of MS observed between the temperature treatments. Plasma membranes from 5 °C-incubated cells contained significantly lower (by 47%, Table 2) proportions of 18:1/20:5-PC compared to 20 °C-incubated cells. Similarly, proportions of 16:0/20:4-PC, as well as an unidentified MS of PC and 16:0/16:0-PE were also lower in cells incubated at 5 °C, though these differences only border on significance. In contrast, proportions of 16:0/16:1-PC increased. While these particular MS comprise a relatively small proportion of the total membrane phospholipid, the percentage change between membranes of 5 °C- and 20 °C-incubated cells is substantial (Table 2).

Incorporation and metabolism of radiolabeled PCs

Parallel experiments focused on changes in phospholipid metabolism during cold exposure, particularly the metabolism of saturated (i.e., monoene precursor) and monoenoic species of PC (the membrane content of which was higher upon cold incubation, Table 2). When

Table 1. The molecular species composition of phosphatidylcholine and phosphatidylethanolamine from plasma membranes prepared from isolated hepatocytes of 20 °C-acclimated trout

Combined chain length	10 × log RRT[a]	MS[b]	Percent composition	
			PC	PE
32	1.36	14:0/18:0	n.d.[c]	0.76±0.06[d]
	1.37	16:0/16:0	4.74±1.00	1.60±0.36
	1.07	16:0/16:1	0.67±0.07	0.60±0.20
34	1.34	16:0/18:1	4.68±0.90	0.90±0.14
	1.28	18:0/16:1	3.23±0.57	10.47±1.33
	1.10	16:0/18:2	13.23±2.45	5.38±0.83
	1.09	16:1/18:1	1.18±0.21	0.86±0.40
	0.97	16:1/18:2	2.92±0.85	4.87±2.45
36	1.64	16:0/20:1	n.d.	0.63±0.63
	1.67	18:0/18:1	2.27±0.80	4.62±0.12
	1.32	18:0/18:2	1.75±0.18	1.82±0.21
	1.33	18:1/18:1	1.37±0.34	n.d.
	1.20	16:0/20:2	5.24±1.05	0.62±0.10
	1.06	16:0/20:4	0.78±0.07	1.03±0.15
	0.93	16:0/20:5	5.18±0.64	3.60±0.38
	1.30	16:1/20:1	1.41±0.46	1.47±0.27
	0.86	16:1/20:4	3.61±0.67	1.25±0.31
	0.83	18:2/18:3	1.51±0.28	1.61±0.10
	0.81	16:1/20:5	0.41±0.41	2.16±0.17
38	1.12	16:0/22:4	1.85±0.14	5.63±1.03
	1.05	16:0/22:5	1.01±0.20	0.72±0.17
	1.00	16:0/22:6	12.12±4.66	5.43±3.14
	0.82	16:1/22:6	2.43±0.32	3.54±1.32
	1.63	18:1/20:1	3.73±0.93	1.01±0.23
	1.16	18:0/20:4	5.86±0.18	11.94±0.89
	0.90	18:1/20:5	1.53±0.06	1.06±0.28
	0.89	18:2/20:4	0.52±0.27	2.27±0.24
	1.57	18:0/22:4	n.d.	0.71±0.71
40	1.14	18:0/22:5	2.00±0.14	0.92±0.35
	1.02	18:1/22:5	4.54±0.93	1.35±0.37
	0.96	18:1/22:6	2.44±1.26	1.02±0.51
	1.18	20:1/20:4	1.24±0.09	n.d.
42	1.15	20:1/22:5	0.51±0.51	n.d.
	1.59	24:1/20:5	n.d.	3.23±0.28
Unk[e]	1.17		2.13±0.50	1.07±0.20
	1.22		1.11±0.23	2.78±0.32
	1.24		1.28±0.18	1.64±0.31
	1.26		0.81±0.21	1.01±0.46
	1.29		0.70±0.06	n.d.
	1.44		n.d.	2.34±1.15
	1.49		n.d.	3.92±0.52
	1.55		n.d.	3.62±0.13

[a] RRT, retention time relative to 16:0/22:6
[b] MS, molecular species
##:#/##:# terminology: the numbers before and after the first colon represent the number of carbon atoms and the number of double bonds, respectively, present in the acyl chain esterified to the *sn*-1 position of the glycerol backbone.The corresponding numbers following the virgule refer to the acyl chain esterified to the *sn*-2 position
[c] n.d., not detected
[d] Data are means±standard errors of three separate preparations
[e] Unidentified molecular species

Table 2. Differences in PC molecular species composition of plasma membranes isolated from hepatocytes of 20 °C-acclimated trout incubated at 20 and 5 °C

MS[a]	Mole%		Δ%[b]	*P*
	5 °C	20 °C		
PC molecular species				
16:0/16:1	0.94±0.08[c]	0.67±0.07	+40.3	0.072
16:0/20:4	0.57±0.06	0.78±0.07	−26.9	0.081
18:1/20:5	0.81±0.21	1.53±0.06	−47.0	0.047
RRT 1.22[d]	0.54±0.10	1.11±0.23	−51.3	0.077
PE molecular species				
16:0/16:0	0.75±0.17	1.60±0.36	−53.1	0.068

[a] MS, molecular species
[b] Percent change in cells incubated at 5 °C versus those at 20 °C, calculated as [(value at 5 °C–value at 20 °C)/(value at 20 °C)] × 100, negative values indicate that 5 °C cells contain less of a given species
[c] Data are means±standard errors of three separate preparations
[d] Unidentified molecular species with a relative retention time of 1.22

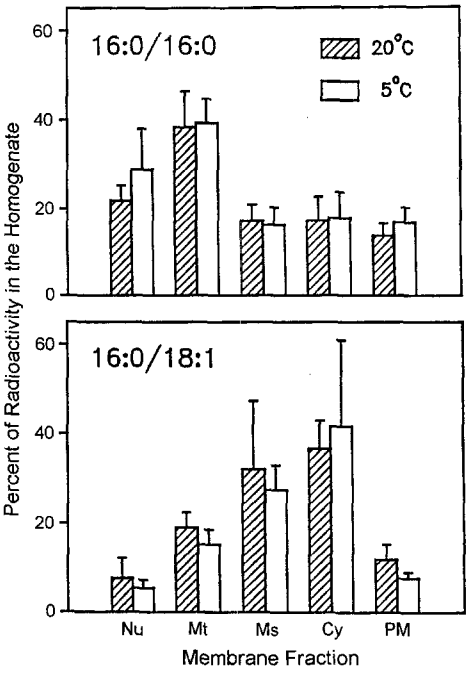

Fig. 1. The distribution of radioactivity among the membrane and cytosolic fractions of rainbow trout hepatocytes. *Upper panel:* the distribution of exogenously added 16:0/16:0-PC. *Lower panel:* the distribution of exogenously added 16:0/18:1-PC. Cellular fractions: *Nu,* nuclear; *Mt,* plasma membrane/mitochondrial; *Ms,* microsomal; *Cy,* cytosolic; *PM,* purified plasma membranes

incubated with medium containing radiolabeled 16:0/16:0-PC or 16:0/18:1-PC, the cells took up 5% and 11% of the phospholipids, respectively, and incorporated them into their cellular membranes. The distribution of radioactivity among the membrane and cytosolic fractions produced upon hepatocyte fractionation (Fig. 1) revealed that 16:0/16:0-PC was associated mainly with the mixed plasma membrane/mitochondrial fraction (Mt) while radioactivity from 16:0/18:1-PC appeared chiefly in the microsomal fraction (Ms). The distribution of the added radiolabels among the cellular membrane fractions was unaffected by incubation temperature. For both labeled phospholipids, considerable radioactivity was as-

sociated with the purified plasma membrane fraction (Fig. 1, PM).

Both labeled 16:0/16:0-PC and 16:0/18:1-PC were metabolized into products which were extracted from the isolated plasma membrane; only background counts were obtained from the aqueous phase of the PM extraction. The rate of formation of these conversion products was such that the percentage of total radioactivity recovered as something other than the added molecular species (i.e., the total extent of conversion) was equivalent in the membranes after 1 h of incubation at 20 °C or 6 h incubation at 5 °C [16:0/16:0-PC: 9.4±1.5% of total radioactivity versus 10.1±1.7%; 16:0/18:1-PC: 13.7±0.7% versus 16.1±1.8% (means±SE), respectively]. Therefore, these incubation times were used for all metabolic experiments. The total radioactivity recovered in the plasma membrane fraction of cells exposed to 20 and 5 °C was also not statistically different and represented $2 \cdot 10^5$–$1.3 \cdot 10^6$ cpm/membrane preparation. Generally, this membrane fraction contained $5–50 \cdot 10^3$ cpm of conversion products of the added label which were present in MS exhibiting up to several thousand counts each. Because of variation in the absolute radioactivity of different membrane preparations, results are presented as mole percentages of total conversion products for the particular preparation.

Temperature-induced changes in MS metabolism

After the appropriate incubation period at each temperature the distribution of radioactivity among the newly synthesized conversion products of PC in isolated plasma membranes was compared between cells held at 20 and 5 °C. Regardless of incubation temperature or radiolabeled precursor molecule, all conversion products were found to be other MS of PC; no radioactivity was found in any of the other phospholipid classes. Also regardless of temperature, both of the precursor species of PC, although converted into many different MS (with the radioactivity associated with most of these species representing less than 2% of the total radioactivity recovered as conversion products), were metabolized into a few dominant MS. The conversion into some of these products was independent of incubation temperature, while for others incubation temperature had a definite impact on species formation (Table 3). Table 3 includes those MS (i.e., conversion products) which represented more than 2% of the total conversion product at either temperature or whose formation was significantly influenced by incubation temperature.

At both test temperatures, radioactivity initially incorporated as 16:0/16:0-PC was converted mainly into 18:1/18:1-PC, and to a substantial degree into 16:0/18:1-PC, 16:0/22:6-PC and 18:1/20:1-PC. Small percentages of radioactivity were also found in 18:0/16:1-PC, 18:0/20:4-PC and 16:0/20:4-PC. Conversion of 16:0/16:0-PC into 18:1/18:1-PC, 18:0/16:1-PC, 16:0/22:6-PC and 16:0/20:4-PC was dependent on incubation temperature (Table 3): recovery of radiolabel in 18:1/18:1-PC was significantly lower in cold- compared to warm-incubated cells, whereas conversion into the other MS was higher.

16:0/18:1-PC was converted into eight other MS of PC at both temperatures (Table 3). Radioactivity originating from 16:0/18:1-PC appeared mostly in 18:1/18:1-

Table 3. Distribution of radioactivity among molecular species of PC formed from the indicated source molecule in the plasma membranes of trout hepatocytes during incubation at 20 °C (1 h) or 5 °C (6 h)

Source	n[a]	MS[b]	% of total non-substrate counts formed (mean±SE)[d]				mean±SE[e]
			Mol% of[c] total PC	5 °C	20 °C	*P*	
16:0/16:0	34	16:0/18:1	5.6	14.4±9.8	6.7±3.5	ns[f]	10.6±2.7
$n = 5$		18:0/16:1	2.7	5.9±2.5	1.0±0.4	<0.05	–
	36	16:0/20:4	0.8	0.2±0.2	0.1±0.1	<0.10	···
		18:1/18:1	0.8	24.6±11.1	33.6±9.9	<0.05	···
	38	16:0/22:6	17.6	9.7±2.6	10.3±4.8	<0.10	···
		18:0/20:4	6.1	20.0±17.1	3.2±1.3	ns	11.6±5.9
		18:1/20:1	2.7	17.5±7.8	20.7±4.2	ns	19.1±1.1
16:0/18:1	34	16:0/18:2	11.9	4.3±0.5	5.0±0.3	ns	4.6±0.2
$n = 4$		16:1/18:1	1.4	3.1±0.6	7.3±0.5	<0.10	···
		16:1/18:2	3.9	6.5±0.8	10.0±1.6	ns	8.2±1.2
	36	16:1/20:1	0.1	11.1±2.7	7.2±1.2	ns	9.2±1.4
		18:0/18:1	1.4	6.3±0.7	11.7±0.7	<0.05	···
		18:1/18:1	0.8	11.3±1.2	15.4±0.4	ns	13.4±1.4
	38	18:1/20:1	2.7	1.3±0.1	2.2±0.2	<0.10	···
		18:1/20:2	3.4	8.9±0.7	7.5±0.4	ns	8.2±0.5

[a] *n*, Total number of carbons in the acyl chains of the phospholipid
[b] MS, molecular species
[c] of 20 °C-acclimated trout
[d] The difference between the sum of the listed species and 100% reflects the conversion of the source molecules into numerous other MS, each contributing less than 2% of the total
[e] The means of values not significantly different from each other
[f] ns, Not significant

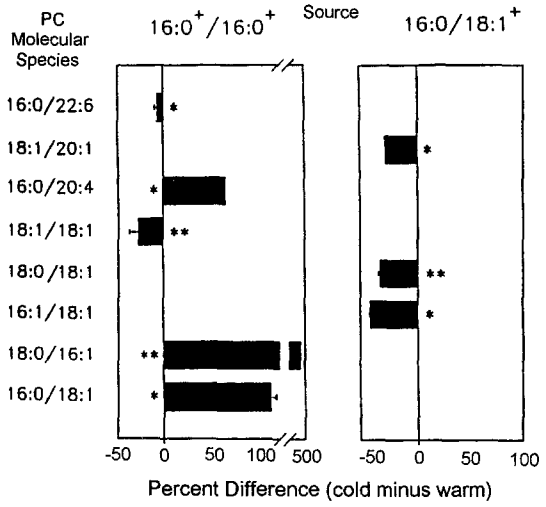

Fig. 2. The temperature dependent difference in the metabolism of the identified molecular species upon incorporation of the source molecule into the plasma membranes of rainbow trout. The *positive* signs indicate the radiolabeled acyl group; *asterisks* indicate level of significance of difference from 0: *, $P \leq 0.1$; **, $P \leq 0.05$

PC, 16:1/20:1-PC, 16:1/18:2-PC, 18:0/18:1-PC and 18:1/20:2-PC. All significant temperature-dependent conversions of this species were lower in the cold-incubated cells and included the formation of 18:0/18:1-PC, 16:1/18:1-PC, and 18:1/20:1-PC.

Relative specific radioactivities were also calculated (radioactivity of MS per mol% of MS; calculated as count per area); no significant differences were observed between temperature groups in the experiments using radiolabeled 16:0/16:0-PC. However, in the experiments using 16:0/18:1-PC, the specific activity of 18:0/18:1-PC decreased significantly from 6.7±1.0 (mean±SE) at 20 °C to 3.6±1.0 at 5 °C, and that of 18:1/20:1-PC decreased from 1.3±0.2 at 20 °C to 0.5±0.3 (means±SE) at 5 °C, though for the latter species the difference only bordered on significance ($P<0.1$).

The effect of temperature upon the conversion of radiolabeled MS of PC into metabolites can be expressed most clearly in terms of the percent change in conversion product formation found in the plasma membranes of 5 versus 20 °C-exposed hepatocytes (Fig. 2). It is noteworthy that the percent conversion of 16:0/16:0-PC into monoenoic species increases dramatically at 5 °C, while all significantly temperature-dependant conversions 16:0/18:1-PC decrease at low temperature.

Discussion

It is well established that after a sufficient period in the cold, selected membranes of 20 °C-acclimated rainbow trout possess increased proportions of PE, elevated PE/PC ratios and altered MS compositions of both PE and PC (Hazel and Williams 1990; Hazel 1979; Hazel and Zerba 1986). These findings suggest that the change in plasma membrane fluidity previously observed after a 6-h exposure to 5 °C in hepatocytes of 20 °C-acclimated trout (Williams and Hazel 1994) may have its basis in alterations in membrane lipid composition. For this reason, we examined alterations in phospholipid and MS composition in addition to the metabolism of exogenously added phospholipids in the plasma membrane of hepatocytes from 20 °C-acclimated trout during this brief period of cold exposure.

Although changes in phospholipid head group composition are common in thermally-acclimating organisms, they cannot explain the fluidity adjustments evident in trout hepatocyte plasma membranes after 6 h of exposure to 5 °C, since no changes in PE or PC content were observed. However, regardless of the incorporation temperature, exogenous PC was incorporated into the cell's various membranes (Fig. 1) and subsequently converted to a variety of other MS then found in the plasma membrane. Though the specific cellular location of these conversion reactions was not determined, they clearly influence the composition of purified plasma membrane. Three of the conversion products of 16:0/16:0-PC, namely 16:0/18:1-PC, 18:0/20:4-PC and 16:0/22:6-PC are among the most abundant MS of PC in these membranes [Table 1; Hazel and Zerba (1986); Hazel et al. (1992)]. Thus, when incorporated into trout hepatocytes, 16:0/16:0-PC, which itself constitutes 4.7% of the total plasma membrane PC, is rapidly converted into species which together make up 32 mol% of the total PC. Similarly, radioactivity derived from 16:0/18:1-PC appeared as 18:1/18:1-PC, 16:1/20:1-PC, 16:1/18:2-PC and 18:1/20:2-PC. Thus, at both 20 and 5 °C, 10–30% of the radioactivity originating from 16:0/16:0-PC and 16:0/18:1-PC is converted into plasma membrane-associated 18:1/18:1-PC. This is remarkable in view of the fact that 18:1/18:1-PC makes up only 1.4% of the total plasma membrane PC composition.

The influence of temperature on phospholipid conversion product formation may be of particular significance to the overall process of temperature acclimation. In 5 °C-incubated cells, 16:0/16:0-PC was converted only into products more unsaturated than itself, with the formation of monounsaturated species being particularly large (Fig. 2). In addition, cells incubated at 5 °C convert significantly less 16:0/18:1 into other species. Thus, cold exposed cells tend to replace saturated species of PC with monounsaturated MS which are then retained without much subsequent modification. This cold-induced acyl chain remodeling could constitute an effective restructuring strategy. Monoenoic species have lower melting points than the corresponding saturated species and the increase in fluidity gained by introducing the „first" double bond is maximal, i.e., subsequent double bonds increase fluidity to a lesser extent (Coolbear et al. 1983). In addition, double bond placement in the center (as opposed to the termini) of the acyl chain (i.e., as in 18:1) is maximally effective in increasing membrane fluidity (Barton and Gunstone 1975). Thus, the increased conversion of 16:0/16:0-PC into 16:0/18:1-PC in cold-exposed cells can be rationalized based on HVA. However, according to HVA theory, some of the observed membrane modifications are paradoxical. For example, the conversion of 16:0/16:0-PC into the more unsaturated

18:1/18:1-PC and 16:0/22:6-PC actually decreases significantly in the cold, as does the conversion of 16:0/18:1-PC into 16:1/18:1-PC and 18:1/20:1-PC. Furthermore, one of the most conspicuous changes known to occur in these membranes upon long-term temperature acclimation is a rise in the content of 16:0/22:6-PC (Hazel and Williams 1990; Hazel and Zerba 1986). However, after 6 h of exposure to 5 °C the total 16:0/22:6-PC content of these membranes is unchanged and the conversion of 16:0/16:0-PC into this species is reduced. Combined, these data support the idea that the initial membrane restructuring in response to temperature change is not necessarily the same as that observed in fully acclimated organisms.

The mechanism(s) responsible for phospholipid restructuring can be inferred from examination of the products formed (Table 3). Species containing unaltered acyl chains (16:0 or 18:1) were abundant in the conversion products of both PCs, indicating the involvement of the deacylation/reacylation (D/R) pathway (i.e., 16:0/22:6-PC can only be formed from 16:0/16:0-PC by removing one 16:0 chain and replacing it with 22:6). It is perhaps not surprising that all of the radiolabelled conversion products were derivatives of PC since PC is present in a higher proportion than other phospholipids in this membrane and in general is thought to turn over more rapidly than PE. However, it is interesting that this finding is also entirely consistent with a recently proposed mechanism of phospholipase A_2 that involves head group recognition (Scott et al. 1990). Since the precursor molecules were labeled in the acyl groups, the involvement of D/R enzymes would likely result in the loss of some label and the observed extent of remodeling may represent only a fraction of the total. Similar rapid, and apparently continuous, phospholipid restructuring via the D/R pathway has been observed in rat hepatocytes, and although individual phospholipid MS turned over at vastly different rates, some turned over as rapidly as $15\% \cdot h^{-1}$ (Schmid et al. 1991). Phospholipid turnover/restructuring may thus be extensive in the plasma membrane of trout hepatocytes and this organelle may represent a dynamic structure that can respond rapidly to temperature stress and other stimuli. In fact, the D/R pathway has been implicated in the preferential removal at low temperature of saturated acyl chains from PC (Neas and Hazel 1984), which is consistent with the extensive conversion of 16:0/16:0-PC at low temperature observed in the present experiments. Thus, in poikilotherms one function of this pathway may be to vary lipid composition in an adaptive fashion during the early stages of temperature acclimation.

Other conversion products, however, can not be explained by D/R reactions alone. For example, the recovery of relatively large amounts of radioactivity derived from 16:0/16:0-PC in 18:1/18:1-PC and 18:1/20:1-PC and in 16:1/20:1 and 16:0/18:2-PC derived from 16:0/18:1-PC implicates the involvement of desaturation and elongation pathways. Although activity of the Δ9 desaturase (involved in the formation of 18:1 and 20:1 from 16:0) rises dramatically almost immediately upon cold exposure of carp (Schünke and Wodtke 1983), this activity required 3 or more days to increase significantly in trout liver. The observation of desaturation products in 6 h in trout hepatocyte plasma membrane suggests that the observed reactions may be occurring in situ in the plasma membrane, possibly utilizing intact 16:0/16:0-PC as a substrate (Kates and Paradis 1973). In situ modification of membrane lipids has been shown to occur in response to T_a change in *Candida* (Kates and Paradis 1973) and in the ciliate *Tetrahymena* (Ramesha and Thompson 1984; Ramesha and Thompson 1983). Desaturation and elongation events appear to play significant roles in the initial stages of temperature acclimation.

The metabolic conversions observed resulted in significant differences in radioactivities of species present in the plasma membrane in only small quantities and did not significantly alter the overall proportion of any individual species, even though such changes are known to occur with prolonged cold exposure. In fact, although the proportion of radioactivity derived from 16:0/16:0-PC recovered in 16:0/20:4-PC was elevated at 5 compared to 20 °C (Table 3), the overall mole% of this MS actually declined (Table 2). These data indicate that metabolic measurements are more sensitive to early adaptational responses than determination of composition. Also, since only a few MS form the majority of membrane phospholipid mass, each of the remaining large number of MS consequently comprise a proportionally small percentage of the total. Small changes in overall membrane phospholipid content can represent relatively large changes in the proportions of these minor species.

Whether changes in MS metabolism and composition of the magnitude found in this study can account for the rapid fluidization of hepatocyte plasma membranes (Williams and Hazel 1994) or for findings like those showing large changes in membrane fluidity in the absence of any change in the overall membrane acyl chain composition (Bly and Clem 1988) is not known and deserves further study. The potential significance of small changes in phospholipid architecture is indicated by the observation that the formation of 16:0/18:1-PC by substituting 18:1 for 16:0 at the *sn*-2 position of 16:0/16:0-PC, as occurred in trout hepatocytes exposed to 5 °C more so than in cells at 20 °C, reduces the gel-to-liquid crystalline phase transition temperature by 50 °C (Hazel and Williams 1990). In addition, even very subtle modifications of phospholipid MS can have surprising effects; for example, bilayers composed of an equal mixture of 18:0/18:0-PC and 18:1/18:1-PC exhibit a phase transition temperature that is 20 °C different from bilayers composed solely of 18:0/18:1-PC (Lynch and Thompson 1984).

In the light of an absence of large-scale changes in MS composition, it is important to point out that mechanisms other than, or in addition to, alterations in membrane phospholipid MS might be responsible for the previously observed compensation of membrane fluidity (Dey et al. 1993). For instance, modification of membrane cholesterol content may be involved. Although cholesterol levels in trout liver plasma membranes do change with long-term temperature acclimation (Robertson and Hazel 1993), the time-course of cholesterol re-

moval from these membranes at low temperature is unknown.

In conclusion, we have observed significant modifications in acyl chain metabolism which involve deacylation, reacylation, desaturation and elongation reactions in the plasma membrane of trout hepatocytes during relatively brief cold exposure. The rapidity of apparently adaptive metabolic adjustments may reflect a relatively high basal rate of membrane turnover/restructuring. The significance of substantial changes in the proportions of minor MS to fluidity and functional modification of biological membranes remains to be established.

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References

Anonymous (1993) Percoll methodology and applications and density marker beads for calibration of gradients of Percoll. Pharmacia Laboratory, Separation Division, Uppsala, Sweden

Barton PG, Gunstone FD (1975) Hydrocarbon chain packing and molecular motion in phospholipid bilayers formed from unsaturated lecithins. J Biol Chem 250:4470–4476

Bell MV, Dick JR (1991) Molecular species composition of the major diacyl glycerophospholipids from muscle, liver, retina and brain of cod *(Gadus morhua)*. Lipids 26:565–573

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Bly JE, Clem LW (1988) Temperature-mediated processes in teleost immunity: homeoviscous adaptation by channel catfish peripheral blood cells. Comp Biochem Physiol 91A:481–485

Christie WW (1982) Lipid analysis, 2nd edn. Pergamon Press, New York

Coolbear KP, Berde CB, Keough KMW (1983) Gel to liquid-crystalline phase transitions of aqueous dispersions of polyunsaturated mixed-acid phosphatidylcholines. Biochemistry 22: 1466–1473

Dey I, Szegletes T, Buda C., Nemcsók J, Farkas T (1993) Fish erythrocytes as a tool to study temperature-induced responses in plasma membranes. Lipids 28:743–746

Dickens BF, Thompson GA (1981) Rapid membrane response during low-temperature acclimation. Correlation of early changes in the physical properties and lipid composition of *Tetrahymena* microsomal membranes. Biochim Biophys Acta 664:211–218

Dickens BF, Thompson GA (1982) Phospholipid molecular species alterations in microsomal membranes as an initial key step during cellular acclimation to low temperature. Biochemistry 21:3604–3611

Hazel JR (1979) Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am J Physiol 236:R91–R101

Hazel JR (1990) Adaptation to temperature: phospholipid synthesis in hepatocytes of rainbow trout. Am J Physiol 258: R1495–R1501

Hazel JR, McKinley SJ, Williams EE (1992) Thermal adaptation in biological membranes: interacting effects of temperature and pH. J Comp Physiol B162:593–601

Hazel JR, Carpenter R (1985) Rapid changes in the phospholipid composition of gill membranes during thermal acclimation of the rainbow trout, *Salmo gairdneri*. J Comp Physiol B155:597–602

Hazel JR, Landrey SR (1988) Time course of thermal adaptation in plasma membranes of trout kidney. II. Molecular species composition. Am J Physiol 255:R628–R634

Hazel JR, Williams EE (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog Lipid Res 29:167–227

Hazel JR, Zerba E (1986) Adaptation of biological membranes to temperature: molecular species compositions of phosphatidylcholine and phosphatidylethanolamine in mitochondrial and microsomal membranes of liver from thermally-acclimated rainbow trout. J Comp Physiol B156:665–674

Horrocks DL (1976) Absolute disintegration rate determination of beta-emitting radionuclides by the pulse height shift-extrapolation method. In: Noujaim AA et al (eds) Liquid scintillation science and technology. Academic Press, New York, pp 185–198

Kates M (1972) Techniques of lipidology. Isolation, analysis and identification of lipids. In: Work TS, Work E (eds) Laboratory techniques in biochemistry and molecular biology, vol 3. North-Holland, Amsterdam

Kates M, Paradis M (1973) Phospholipid desaturation in *Candida lypolytica* as a function of temperature and growth. Can J Biochem 51:184–197

Kito M, Takamura H, Narita H, Urade R (1985) A sensitive method for quantitative analysis of phospholipid molecular species by high-performance liquid chromatography. J Biochem 98: 327–331

Lynch DV, Thompson GA (1984) Retailored molecular species: a tactical mechanism for modulating membrane properties. TIBS 9:442–445

Malak NA, Birchon G, Meister R, Zwingelstein G (1989) Environmental temperature and metabolism of the molecular species of phosphatidylcholine in the tissues of the rainbow trout. Lipids 24:318:324

Martin CE, Thompson GA (1978) Use of fluorescence polarization to monitor intracellular membrane changes during temperature acclimation. Correlation with lipid compositional and ultrastructural changes. Biochemistry 17:3581–3586

Neas NP, Hazel JR (1984) Temperature-dependent deacylation of molecular species of phosphatidylcholine by microsomal phospholipase A_2 of thermally acclimated rainbow trout, *Salmo gairdneri*. Lipids 19:258–263

Patton GM, Fasulo JM, Robins SJ (1982) Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. J Lipid Res 23: 190–196

Ramesha CS, Dickens BF, Thompson GA (1982) Phospholipid molecular species alterations in *Tetrahymena* ciliary membranes following low-temperature acclimation. Biochemistry 21:3618–3622

Ramesha CS, Thompson GA (1982) Changes in the lipid composition and physical properties of *Tetrahymena* ciliary membranes following low-temperature acclimation. Biochemistry 21:3612–3617

Ramesha CS, Thompson GA (1983) Cold stress induces in situ phospholipid molecular species changes in cell surface membranes. Biochim Biophys Acta 731:251–260

Ramesha CS, Thompson GA (1984) The mechanism of membrane response to chilling – the effect of temperature on phospholipid deacylation and reacylation reactions in the cell surface membrane. J Biol Chem 259:8706–8712

Robertson J, Hazel JR (1993) Alteration in plasma membrane cholesterol content upon long term temperature acclimation (Abstract). The Physiologist 37:A89

Schmid PC, Johnson SB, Schmid HHO (1991) Remodeling of rat hepatocyte phospholipids by selective acyl turnover. J Biol Chem 266:13690–13697

Schünke M, Wodtke E (1983) Cold-induced increase of delta-9 and delta-6 desaturase activities in endoplasmic membranes of carp liver. Biochim Biophys Acta 734:70–75

Scott DL, White SP, Otwinowski Z, Yuan W, Gelb MH, Sigler PB (1990) Interfacial catalysis: the mechanism of phospholipase A_2. Science 250:1541–1546

Sellner PA, Hazel JR (1982) Desaturation and elongation of unsaturated fatty acids in hepatocytes from thermally acclimated rainbow trout. Arch Biochem Biophys 213:58–66

Sinensky M (1974) Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli.* Proc Nat Acad Sci USA 71:522–525

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Sokal RR, Rohlf FJ (1981) Biometry, 2nd edn. Freeman, New York, pp 427–428

Takamura H, Narita H, Urade R, Kito M (1986) Quantitative analysis of polyenoic phospholipid molecular species by high performance liquid chromatography. Lipids 5:356–361

Williams EE, Hazel JR (1994) Membrane fluidity and hemilayer temperature sensitivity in trout hepatocytes during brief in vitro cold exposure. Am J Physiol 226:R773–R780