Membrane Lipid Modification of Chinese Hamster Ovary Cells

THERMAL PROPERTIES OF MEMBRANE PHOSPHOLIPIDS*

(Received for publication, March 28, 1978)

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A simple and reproducible method for altering the acyl chain composition of Chinese hamster ovary cell phospholipids is described. By supplementation of the medium with 5% delipidized serum and 12 μg/ml of various fatty acids, extensive incorporation of the added fatty acid into membrane phospholipids was obtained. No significant changes in the phospholipid head group composition or the sterol/phospholipid ratio were detected during the experimental time course. Analysis of the thermal properties of these supplemented phospholipids is also described. The onset of formation of a "solid" lipid phase was detected using the fluorescent fatty acid probe, trans-parinaric acid. The temperature of onset of the solid lipid phase in extracted phospholipids was correlated with the percentage of unsaturated fatty acids esterified to the phospholipids. Fluorescence polarization measurements with cis- and trans-parinaric acid in the phospholipids indicated that the amount of solid phase lipid was significantly greater in palmitate-supplemented cells than in unsupplemented or oleate-supplemented cells. The polarization analysis also indicated that the amount of solid phase lipid was approximately equal in unsupplemented and oleate-supplemented cells. This analysis also indicated that the onset of solid formation in these phospholipids occurred at approximately the same temperature as the onset in the total phospholipid extract.

Previous investigators have used several approaches to modification of the acyl composition of animal cell membranes. Alteration of the acyl chains of membrane phospholipids has been achieved by blocking endogenous fatty acid synthesis with avidin or desethylbiotin, while supplementing the medium with bovine serum albumin complexes of fatty acids (1) or with Tween esters of fatty acids (2). Modification of the phospholipid head group composition of cultured cells has also been reported (3-5). Fusion of phospholipid vesicles with isolated membranes (6) and maintenance of laboratory rodents on defined lipid supplements (7, 8) have also been successful in altering the composition of animal cell membranes. These techniques have enabled previous investigators to detect lipid modulation of lectin binding (9, 10), Newcastle disease virus infectivity (11), (Na⁺K⁺)-ATPase activity (7), and adenylyl cyclase activity and stimulation (12, 13). It is therefore apparent that in vivo or in vitro modifications of animal cell membranes can have significant effects on the functions of these membranes. The application of these techniques to the elucidation of the structural and functional relationships of lipids and membrane proteins has yet to be fully explored. We present here our observations on the effects of acyl chain modification on the membrane phospholipids of the Chinese hamster ovary cell line.

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary K1 cells, obtained from the American Type Culture Collection (ATCC CCL-61), were routinely maintained in either Ham's F12 (Gibco) or minimal Eagle's medium (Flow Laboratories) supplemented with nonessential amino acids and 5% fetal calf serum (Gibco). Delipidized serum protein was prepared according to the method described by Sklar et al. (16) or the method described by Horwitz et al. (9). These methods routinely removed 90 to 95% of the serum acyl rhizos, as assayed by gas-liquid chromatography. After delipidation, the serum protein was reconstituted in glass-distilled water at 4 g/100 ml. For the supplementation experiments, cells were detached with 0.25% trypsin (Gibco), washed once with serum-containing medium, and inoculated into Wheaton roller bottles (680 cm²) in F12 + 5% fetal calf serum. The cells were incubated in this medium for 24 h at 37°C and 2 to 4 rpm. The medium was decanted and replaced with F12 + 5% DLT containing 12 μg/ml of fatty acid, and grown for 12 to 24 h at 37°C and 2 to 4 rpm. Stock fatty acids, dissolved in ethanol at 5 mg/ml, were added directly to the medium. The cells were harvested by scraping with a rubber policeman, washed twice in Dulbecco's phosphate-buffered saline, and the pellets were extracted as described previously (15).

Fluorescence Analysis—cis- and trans-Parinaric acid were prepared and stored as described by Sklar et al. (16). Lipid samples were dried under N₂ and dispersed with a Varian mixer in 0.05 M potassium phosphate buffer (pH = 7.2) at a phospholipid concentration of 30 to 50 μg/ml. Butylated hydroxytoluene was added as an antioxidant at a mole ratio of 1:200 to 400 (butylated hydroxytoluene:phospholipid phosphates), as was the parinaric acid. The samples were bubbled with argon before analysis. Fluorescence intensity was monitored continuously while heating and cooling the phospholipid dispersions at approximately 1°C/min in a Hitachi Perkin-Elmer MFP-44 spectrofluorimeter equipped with a water-jacketed cuvette holder essentially as described before (15). Excitation was 320 nm and 325 nm for trans- and cis-PnA, respectively, and emission was monitored at 420 nm.

The abbreviations used are: cis-PnA, 9,11,13,15-cis-trans-parinaric acid; trans-PnA, 9,11,13,15-trans-cis-parinaric acid; CHO, Chinese hamster ovary; DLT, delipidized serum protein.
Lipid Phospholipids were prepared from the total lipid extract by chromatography on silicic acid columns as described previously (18). Preparative two-dimensional thin layer chromatography was performed on Analtech 2-mm silica gel plates, activated overnight at 120°C, using the solvent systems described by Kimmelberg and Papahadjopoulos (18). Lipids were visualized by spraying the plates with distilled water; spots were scraped and eluted with 25 ml of chloroform:methanol:water (5:5:1). Known lipid standards were spotted on replicate plates to ensure proper identification. Gas-liquid chromatography was done on a Hewlett-Packard 7610A GLC, equipped with a 6-foot column containing 15% SP-2340 on 100/120 Chromosorb P AW DMCS (Supelco). Methyl esters were prepared by transesterification of anhydrous lipids in 2% H2SO4 in methanol.

Analytical Methods—Phospholipid phosphate was determined using the method of Rabheja et al. (19), and sterol was assayed according to the method of Solow and Freeman (20). Cell growth experiments were performed as described previously, and protein was assayed according to a modification of the method of Lowry et al. (21). All reagents were analytical grade.

RESULTS AND DISCUSSION

Lipid Modification—Study of the effects of acyl chain modifications on bacterial membrane-associated processes (22) was facilitated by the isolation of fatty acid auxotrophs (23), which enabled these investigators to greatly simplify the fatty acid composition of these organisms. The lack of such mutants in animal cells has necessitated slightly more sophisticated experimental manipulations. One successful approach involved inhibition of endogenous fatty acid synthesis with avidin or dethiocholin, and supplementation of the medium with either bovine serum albumin complexes of fatty acids or Tween esters of fatty acids (1, 2). The convenient method described in this report results in substantial alteration of the phospholipid acyl composition without the use of inhibitors. Cell growth is relatively rapid; the cell population doubles in approximately 27 h (Fig. 1). The acyl composition of the unsupplemented cells is relatively simple, and two fatty acids, olate and palmitate, make up over 70% of the acyl chains (Table I). No differences in the sterol/ phospholipid ratio could be detected in cells supplemented with various fatty acids in delipidized serum, although the ratio was altered in cells grown in fetal calf serum (Table II). This is in contrast to previous reports that the sterol/phospholipid ratio is altered in cells undergoing phospholipid head group modifications (24). In our studies, phospholipid head group composition also did not change appreciably during the experimental period, as shown in Table II. Since changes in the phospholipid head group composition or in the sterol concentration can drastically alter the properties of membranes (25), these observations suggest that these CHO cells constitute an internally consistent and simplified experimental system with which to further study the physical properties of biological membranes. The remainder of this report will deal with our preliminary characterization of these supplemented cells.

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1 L. A. Sklar, G. Miljanich, and E. Dratz, manuscript in preparation.


3 D. A. Rentoul, J. Ryan, and R. D. Simoni, manuscript in preparation.
assay for phospholipid phospho-

also resume proliferation after 3 days in medium containing 12 μg/ml of palmitate, if the medium was decanted and replaced with fresh medium containing fetal calf serum (data not shown).

Analyses of the thermal properties of the phospholipids isolated from these supplemented cells are shown in Fig. 5. The onset of a sharp increase in the fluorescence intensity, detected using trans-PnA, was at approximately 20–24°C in all cases but one. Palmitate supplementation resulted in cellular phospholipids whose onset of fluorescence increase was 10–15°C higher. It is interesting that elaidate supplementation did not alter the thermal properties of these lipids. This is in contrast to the observations of Horwitz et al. (9) that radical changes can be seen in temperature dependence of lectin agglutination in elaidate-supplemented cells. The explanation can perhaps be found in the fact that elaidate, although it is a trans-unsaturated fatty acid, seemed to replace primarily saturated fatty acids in the CHO cell phospholipids (Table I). The melting point of elaidate-containing phospholipids is significantly higher than the melting point of the corresponding oleate-containing phospholipids; the elaidate thus behaves as if it were a saturated fatty acid (25).

The correlation of the unsaturated acyl chain percentage with the high temperature slope change, or onset of fluorescence increase, of the phospholipids from cells grown in various media, various supplements, and various combinations of supplements is shown in Fig. 6. These data imply that the thermal properties of the lipids from this fairly simple biological system are highly sensitive to the unsaturated fatty acid content. This relationship also holds true if the oleate/palmitate ratio is plotted versus the change in slope, as expected from the observation that these two fatty acids are the major unsaturated and saturated species in the phospholipid acyl chains. Interpretation of this result depends upon the identification of the physical process represented by the change in slope of fluorescence intensity of trans-PnA in these phospholipids.

trans-PnA preferentially partitions into ordered or solid lipid phases (27). The quantum yield of the probe also increases in the ordered lipid phase as demonstrated using model systems (17, 27). The most likely explanation for the increased fluorescence intensity below characteristic temperatures in these lipids, then, is the appearance of some solid phase lipid below that temperature. The amount of solid phase lipid in these extracts may be estimated from fluorescence polarization determinations which consist of measuring the ratio of fluorescence intensity emitted parallel (I_||) and perpendicular (I_⊥) to a polarized exciting beam. The estimate is based upon a comparison of observed polarization ratios (I_|| / I_⊥) versus temperature for these extracted CHO phospholipids to standard curves generated with various percentages of a solid phospholipid, dipalmitoylphosphatidylcholine, and a fluid phospholipid, 1-palmitoyl,2-docosahexaenoylphosphatidylcholine. In pure solid lipid phases, both cis-PnA and trans-PnA exhibit polarization ratios (I_|| / I_⊥) near 2.7 (which approaches the theoretical maximum of 3.0 for these molecules embedded in a rigid matrix). Due to the preference of trans-PnA for solid phase lipids and its increased quantum yield, the polarization ratio of trans-PnA is very sensitive to small amounts of solid phase. Whereas it exhibits a polarization ratio of 1.5 to 1.6 in fluid phase lipids, the polarization is 2.0 or greater in the presence of as little as 5% solid and 2.5 or greater in the presence of 25% solid. In contrast, cis-PnA has a relative preference for fluid phase lipids. It exhibits a polarization ratio of 1.3 to 1.4 in pure fluid phases, ~1.7 with 20% solid, ~2.1 with 50% solid, and ~2.4 with 75% solid.

Fluorescence polarization ratios for cis- and trans-PnA were measured as a function of temperature in phospholipids extracted from CHO cells supplemented with palmitate (Fig. 7A), CHO cells supplemented with oleate (Fig. 7B), and unsupplemented cells (Fig. 7C). Comparison of the results of Fig. 7 with the standard curves described above indicate that about 10 to 20% of the lipids are solid at 5°C in oleate-supplemented and unsupplemented CHO cell phospholipids. The amount of solid phase lipid in the palmitate-supple-

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<th>Acyl composition of CHO cell phospholipids</th>
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Cells were grown for 24 h in the indicated medium. After extraction and silicic acid chromatography, the phospholipids were transesterified and analyzed by gas-liquid chromatography. The data shown are representative of many separate analyses.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidyl ethanolamine</th>
<th>Sphingomyelin</th>
<th>Sterol/phospholipid mol/mol</th>
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<tbody>
<tr>
<td>F12 + 5% fetal calf serum</td>
<td>0.14 ± 0.04</td>
<td>0.68 ± 0.04</td>
<td>0.18 ± 0.02</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>F12 + 5% DLP</td>
<td>0.19 ± 0.02</td>
<td>0.68 ± 0.05</td>
<td>0.14 ± 0.02</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>F12 + 5% DLP + 12 μg/ml 18:1</td>
<td>0.22 ± 0.02</td>
<td>0.65 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.26 ± 0.02</td>
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* c = cis isomer; t = trans isomer.
Thermal Properties of CHO Cell Phospholipids

FIG. 2. Log of trans-PnA fluorescence intensity versus reciprocal temperature in total lipids (lower curve) and phospholipids (upper curve) extracted from CHO cells grown for 24 h in F12 + 5% DLP. Phospholipid concentrations were 40 µg/ml in 0.05 M potassium phosphate, pH = 7.2. The probe/lipid ratio was 1/200. The curves are displaced vertically by an arbitrary amount.

Phospholipid Fractionation—Thin layer chromatography of phospholipids from oleate- and palmitate-enriched cells was undertaken in order to identify the phospholipid species involved in this solid phase formation. These data are shown in Table III. Phosphatidylethanolamine and phosphatidylcholine from oleate-supplemented cells had changes in slope of trans-PnA fluorescence intensity at 18°C and 21°C, respectively. The slope change for the total phospholipids was at 18°C. Phosphatidylethanolamine and phosphatidylcholine from cells supplemented with palmitate for 12 h had slope changes at 32°C and 28°C, respectively. The total phospholipids had a slope change at 29°C. Phosphatidylethanolamine and phosphatidylcholine from cells supplemented with palmitate for 24 h had slope changes at 39°C and 36°C, respectively, while the slope change for the total phospholipids was at 35°C. The other major phospholipid, sphingomyelin, had a major slope change at approximately 50°C in all cases, and...
probably did not contribute appreciably to the onset of solid phase formation detected in the bulk phospholipids. These data indicate that the onset of solid lipid formation in the total phospholipids was due to both the phosphatidylethanolamine and the phosphatidylcholine. It is also apparent that both phospholipid species are undergoing palmitate supplementation with somewhat similar rates, as indicated by the drop in unsaturated fatty acid percentage listed in Table III. The sphingomyelin also becomes more highly saturated, but even in the oleate-supplemented cells this species is already considerably more saturated than the other two species.

In summary, we have demonstrated that substantial modification of the acyl chains of animal cell phospholipids can be obtained in 24 h, in medium supplemented with fatty acids, and without the presence of inhibitors of endogenous fatty acid synthesis. Cell growth was fairly rapid in the supplementation medium under most conditions. The acyl composition of the phospholipids isolated from these cells was relatively simple; the phospholipid head group composition and the sterol/phospholipid ratio did not change appreciably during the experimental period. Using lipids from cells grown under these conditions, we observed a significant change in slope in the fluorescence intensity of trans-PnA versus temperature and a substantial temperature dependence of the fluorescence polarization ratio ($I_1/I_0$) of both cis- and trans-PnA. The temperature of this slope change was correlated with the percentage of unsaturated fatty acids esterified to the phospholipids. The amount of solid phase lipid below the temperature of this slope change was estimated and found to be significantly higher in phospholipids from palmitate-supplemented cells as compared to oleate-supplemented or unsupplemented cells. This estimate was qualitatively corroborated using freeze fracture electron microscopy of the cell membranes, indicating that the effects were not unique to the phospholipid extracts (data not shown). The difference be-

![Image of Figure 6](https://via.placeholder.com/150)

**Fig. 6.** Unsaturated acyl chains/total acyl chains of CHO cell phospholipids versus the temperature of the change in slope observed using trans-PnA as described under “Materials and Methods.” Cells were grown for 24 h in the indicated media, acyl chains were calculated after transesterification and gas-liquid chromatography of the phospholipids. $\Delta = F12 + 5\%$ fetal calf serum; $\Box = F12 + 5\%$ DLP; $\bullet = F12 + 5\%$ DLP supplemented with 12 $\mu$g/ml of 16:O; $\diamond = F12 + 5\%$ DLP supplemented with 12 $\mu$g/ml of 18:1c; $\circ = F12 + 5\%$ DLP supplemented with 12 $\mu$g/ml of 18:1c; $\bigcirc = F12 + 5\%$ DLP supplemented with 12 $\mu$g/ml of 18:2; $\blacksquare = F12 + 5\%$ delipidized serum supplemented with 12 $\mu$g/ml of 18:2. The line was calculated using a least squares linear regression analysis, $r^2 = 0.80$.

![Image of Figure 7](https://via.placeholder.com/150)

**Fig. 7.** Fluorescence polarization ratios ($I_1/I_0$) of cis- and trans-PnA versus temperature. Samples were prepared as described under “Materials and Methods” and scanned in both directions (heating and cooling), while $I_1$ and $I_0$ were monitored. After subtraction of background fluorescence, the ratios were calculated and multiplied by a factor to correct for machine bias. The PnA/phospholipid ratio was 1:100, and the probe did not decay appreciably during the heating and cooling cycle, as measured by absorbance. A, phospholipids from cells grown in F12 + 5% DLP supplemented with 12 $\mu$g/ml of 16:O; upper curve is obtained from trans-PnA, lower curve is obtained from cis-PnA. B, phospholipids from cells grown in F12 + 5% DLP supplemented with 12 $\mu$g/ml of 18:1c; upper curve is obtained from trans-PnA, lower curve is obtained from cis-PnA. C, phospholipids from cells grown in F12 + 5% DLP; upper curve is obtained from trans-PnA, lower curve is obtained from cis-PnA.

### Table III

<table>
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<th>Thermal discontinuities in isolated phospholipid fractions</th>
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<td>Growth conditions</td>
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<td>-------------------</td>
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<tr>
<td>Oleate, 12 $\mu$g/ml for 24 h</td>
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<tr>
<td>Palmitate, 12 $\mu$g/ml for 12 h</td>
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<tr>
<td>Palmitate, 12 $\mu$g/ml for 24 h</td>
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* Numbers in parentheses are unsaturated fatty acid/total fatty acids.
between our results and the observations of Schroeder et al. (26), that four separate characteristic temperatures could be detected in isolated LM cell membranes or total lipid extracts, using trans-PnA as a probe, remains to be explained. Multiple characteristic temperatures could be due to the more rapid rate of temperature variation (2°C versus 1°C/min) used by Schroeder et al. (26), or they could be due to differences in the cell type or lipid composition of the cells, especially since the LM cells were grown in suspension in a serum-free medium, and the CHO cells were grown in monolayers in a medium containing serum protein. A more likely explanation may lie in the methods of plotting the data. If our corrected fluorescence intensity data are plotted versus temperature according to the method used by Schroeder et al. (26), straight lines drawn through segments of the curve will yield what appear to be multiple discontinuities (data not shown). These do not appear if the data are plotted as the log of the fluorescence intensity versus the reciprocal of the absolute temperature, as in Figs. 2 and 5 of this report. Since the fluorescence intensity of this probe varies exponentially with temperature in a given phase (17), we feel that deviations from this exponential relationship, i.e. slope changes, will be most apparent if the data are plotted in this manner. Thus, the multiple characteristic temperatures described by Schroeder et al. (26) do not normally appear in the membranes or total lipid extracts of the unsupplemented or supplemented CHO cells described herein. This is probably due to the sterol in the membrane and total lipid extracts. Previous investigators have observed abolition or diminution of phospholipid phase transitions, as detected by PnA fluorescence in the presence of cholesterol (27). An interesting exception is found in the total lipid extract of palmitate-supplemented cells, where the break point at 34-37°C although reduced in magnitude, can still be detected. This observation is not particularly surprising in view of the fact that an estimated 50% of the phospholipids were undergoing a phase change in these extracts. Usually, however, no slope changes could be observed in the total lipid extracts, and only one change in slope is observed in the phospholipid extracts. This result is in agreement with many previous observations in prokaryotic systems (22, 28). We are presently engaged in experiments designed to determine if the observed lipid phase change can be correlated with changes in several membrane functions.

Acknowledgments—We would like to thank Cathy Rintoul for excellent technical assistance and Drs. Bruce Hudson, Evelyn Tecoma, and Charles Berde for many helpful discussions. We would also like to thank Dr. Charles Yanofsky for the use of his gas-liquid chromatography apparatus, and Dr. Ed Dratz for the use of his fluorimeter.
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