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Preparation of inside-out vesicles from erythrocyte membranes inactivates the pathway for oleic acid incorporation into phospholipid

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The pathway for membrane phospholipid fatty acid turnover in situ may be important in the regulation of the composition and turnover of the lipid microenvironment of membrane proteins. This pathway has been characterized further by studying the activation and incorporation of [9,10(n)-^{3}H]oleic acid and transesterification of [1-^{14}C]oleoyl-CoA into membrane phospholipids by isolated erythrocyte membrane ghosts and inside-out vesicles derived from these ghosts. Erythrocyte ghosts and sealed vesicles of defined orientation prepared from them have been widely employed in studies of the function of membrane proteins, particularly those which mediate the transport of ions and sugars. Preparation of inside-out vesicles from ghosts by exposure to alkaline hypotonic conditions results in elution of some membrane proteins but no loss of membrane phospholipid. Compared to ghosts, the ability of inside-out vesicles to activate and incorporate [9,10(n)-^{3}H]oleic acid into phospholipid is diminished by over 90% and the ability of inside-out vesicles to transesterify [1-^{14}C]oleoyl-CoA to phospholipid is diminished by over 50%. These findings indicate that exposure of erythrocyte membranes to the alkaline hypotonic conditions required for inside-out vesicle preparation results in loss or inactivation of both acyl-CoA ligase and acyl-CoA-lysophospholipid acyltransferase activities. This lability of the enzymes for in situ phospholipid fatty acid turnover should be considered in the design and interpretation of studies concerned with elucidation of the relationship between phospholipid fatty acid turnover and the regulation of membrane protein function in this membrane preparation.

Introduction

The alteration of membrane phospholipid fatty acid composition by deacylation and reacylation of existing membrane phosphoglycerides in situ may be an important mechanism for regulating membrane function [1–5]. The deacylation-reacylation pathway may also be a mechanism for replacing fatty acids damaged by oxidant injury [6,7], and for acylating membrane lysophospholipids, which are potent detergents capable of disrupting membrane structure [8,9]. Study of phospholipid fatty acid turnover in situ in eukaryotic cells has been a complex problem because these cells contain membrane-bound organelles and can alter membrane phospholipid fatty acid composition by either fatty acid turnover or de novo

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine.

phospholipid synthesis. The erythrocyte provides an attractive system for examining in situ phospholipid fatty acid turnover because: (i) erythrocytes are easily isolated and purified; (ii) erythrocytes contain only one membrane so that changes in membrane function and phospholipid fatty acid composition must occur in the same membrane; and, most importantly, (iii) erythrocytes cannot synthesize phospholipid or fatty acid de novo, or alter fatty acid chain-length or degree of unsaturation [10,11]. Turnover of phospholipid fatty acids in situ thus occurs primarily by the deacylation-reacylation pathway in intact human erythrocytes [12–14]. This pathway consists of three steps: (i) the formation of monoacylglycerophospholipids is catalyzed by an endogenous phospholipase; (ii) fatty acids taken up by the cell or released from endogenous phospholipid are activated by thioesterification to CoA in an ATP-dependent reaction catalyzed by acyl-CoA ligase; (iii) transesterification of the acyl group from acyl-CoA thioester to lysophospholipid, catalyzed by one or more acyl-CoA-lysophospholipid acyltransferases, then completes the cycle, regenerating a diacyl phospholipid. The enzymes in this pathway are membrane-bound, since incorporation of fatty acid into phospholipid can be demonstrated using isolated erythrocyte membranes obtained after hypotonic hemolysis [12,13,16]. The incorporation of fatty acids into phospholipid by isolated membranes is dependent on addition of ATP and is enhanced by exogenous CoA. Acylation of both endogenous and exogenous lysophospholipid also occurs when acyl-CoA thioesters are employed as substrate in the absence of ATP and CoA [12,17]. In both intact cells and unsealed ghosts, exogenous fatty acids are incorporated primarily into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [12–17].

Erythrocyte ghosts and sealed vesicles of defined orientation prepared from them have been widely employed to characterize membrane transport mechanisms and the function of other membrane proteins [18–21]. Many of these membrane protein-mediated processes are influenced by the composition of the lipid microenvironment surrounding membrane proteins. Because the acyl group composition of phospholipids in this lipid microenvironment is regulated at least in part by the pathway for phospholipid fatty acid turnover in situ, we have characterized further this pathway in unsealed ghosts and inside-out vesicles derived from them. By comparing the activity of the pathway for in situ phospholipid fatty acid turnover in unsealed ghosts and inside-out vesicles, it is possible to distinguish direct effects of various perturbants on membrane-bound enzymes from effects due to the generation of ion or transmembrane potential gradients, since the latter exist only in inside-out vesicles. We have observed differences between ghosts and inside-out-vesicles in the incorporation of exogenous fatty acids into phospholipid. A marked decrease in acyl-CoA ligase and acyl-CoA-lysophospholipid acyltransferase activity occurs during inside-out vesicle preparation. These findings contribute to the understanding of the mechanism of phospholipid fatty acid turnover in erythrocytes and should be considered in studies dealing with the relationship between phospholipid fatty acid turnover and the regulation of membrane function, especially where these specific membrane preparations are utilized.

Materials and Methods

Membrane preparations. Erythrocytes were separated from heparinized venous human blood by centrifugation at room temperature and washed four times with 5 vol. of buffer comprising 150 mM NaCl/5 mM Na_2HPO_4 (pH 8.0). Unsealed ghosts and inside-out vesicles were prepared according to the methods of Steck and Kant [22,23], as modified by Waisman et al. [18]. Briefly, washed erythrocytes were lysed in 35 vol. of ice-cold 5 mM Na_2HPO_4 (pH 8.0) (buffer 1). After centrifugation at 35 000 × *g* at 4°C for 10 min, the membrane pellets were washed two times with 35 vol. of buffer 1 to yield hemoglobin-free membranes (unsealed ghosts). To prepare inside-out vesicles, ghosts were then incubated for 60 min in ice-cold 0.5 mM Na_2NPO_4 (pH 8.0) to induce vesiculation. The membranes were collected by centrifugation at 43 700 × *g* at 4°C for 10 min and the resulting membrane pellet was passed five times through a 27-gauge needle into a 5-ml syringe, to complete shearing and sealing of vesicles. Sealed and unsealed vesicles were sep-

arated over a 1.03 g/ml Dextran barrier by centrifugation at 110000 × *g* at 4°C for 60 min. The orientation and degree of sealing of inside-out vesicles were determined by assessing acetylcholinesterase activity in the presence and absence of Triton X-100 [22,23]. Routinely, inside-out vesicle preparations showed 80–90% of acetylcholinesterase inaccessible to substrate in the absence of Triton X-100, indicating that the vesicles were both sealed and of inside-out orientation. No further purification was performed. Protein content of ghosts and inside-out vesicles was measured by the assay of Lowry et al. [24].

Incubation and lipid extraction. Suspension of ghosts or inside-out vesicles (10%, v/v) were incubated with [1-^{14}C]oleoyl-CoA, 1 mM ATP and 1 mM EGTA at 37°C in buffer comprising 20 mM glycylglycine/3 mM magnesium gluconate (pH 7.1). For incubations utilizing free fatty acid as substrate [9,10(n)-^{3}H]oleic acid complexed to fatty-acid-free bovine serum albumin, 4 μM CoA and 15 μM dithiothreitol were added in place of [1-^{14}C]oleoyl-CoA. Duplicate aliquots of the suspension were added to 5 vol. of methanol followed by 5 vol. of chloroform with vigorous mixing to extract neutral lipids and phospholipids. The extracts were washed with 3.5 vol. of 50 mM KCl to produce a biphasic mixture. To measure activation of fatty acid to acyl thioester, selective derivatization of acyl thioesters to *N*-acylhydroxamic acids was carried out by substituting neutral methanolic hydroxylamine for methanol as described [12].

Isolation of phospholipids. The lower phase of lipid extracts was concentrated under nitrogen. After removal of aliquots for determination of total lipid phosphorus and radioactivity [12], the extract was applied to a thin layer of Silica H, prepared with 1 mM Na_2CO_3, and developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$, (50 : 20 : 5 : 2, v/v) as described [12]. Samples containing *N*-acylhydroxamic acids derived from acyl thioesters were first developed in $CH_3COOC_2H_5/CH_3COOH$ (98 : 2, v/v) to separate neutral lipids (particularly free fatty acids) from *N*-acylhydroxamic acids and phospholipids. After air-drying for 10 min, these plate were then developed in $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50 : 2 : 5 : 2, v/v) to separate *N*-acylhydroxamic acids from individual phospholipid classes. Lipids were visualized by brief exposure of the plates to iodine and identified by comparison with standards. Radioactivity present in each lipid fraction was then determined by liquid scintillation counting after scraping gel fractions into scintillation vials and adding ACS II (Amersham Corp.). This counting solution elutes neutral lipids, phospholipids, and *N*-acylhydroxamic acids quantitatively from Silica H. Calculations are based on the specific activity of the exogenous fatty acid. All data presented are representative of duplicate experiments with identical results.

[1-^{14}C]Oleoyl-CoA (spec. act. 56.7 mCi/mmol) and [9,10(n)-^{3}H]oleic acid (spec. act. 5.7 mCi/mol) were obtained from Amersham-Searle Co. (Arlington Heights, IL). [1-^{14}C]Oleoyl-CoA was diluted with unlabeled oleoyl-CoA in 10 mM sodium acetate (pH 6.0) to give a specific activity of 2.3 mCi/mmol. [9,10(n)-^{3}H]Oleic acid was diluted with unlabeled oleic acid and complexed to fatty-acid-free bovine serum albumin as described previously [12] to give a final specific activity of 1.1 Ci/mmol.

Statistical analysis was performed using Student's *t*-ratio and the paired *t*-test. Statistical significance was defined as $p < 0.05$.

Results

Phospholipid and protein content of ghosts and inside-out vesicles

Preparation of inside-out vesicles from ghosts by incubation in ice-cold 0.5 mM Na_2HPO_4 (pH 8.0) results in a decrease in membrane protein due primarily to elution of membrane cytoskeletal protein spectrin [23,25]. Acetylcholinesterase activity is not eluted from the membrane during inside-out vesicle preparation [22,23] and can be employed to assess protein and phospholipid recovery. When recovery of phospholipid and protein is assessed following preparation of inside-out vesicles from ghosts, the ratio of acetylcholinesterase activity to total protein and the ratio of phospholipid to protein both increase by 55–60% in inside-out vesicles compared to ghosts, while no difference in the ratio of acetylcholinesterase activity to phospholipid is detected between inside-out vesicles and ghosts (Table I). Thus, inside-out vesicle preparation does not significantly alter the phos-

TABLE I

RATIO OF PHOSPHOLIPID AND ACETYLCHOLINESTERASE TO PROTEIN IN GHOST AND INSIDE-OUT VESICLES

Phospholipid and protein content and acetylcholinesterase activity of ghosts and inside-out vesicles were measured in duplicate aliquots of ghosts and inside-out vesicles prepared from the same erythrocytes. Lipid phosphorus was measured following lipid extraction as described [12]. Protein was measured by the method of Lowry et al. [24]. Acetylcholinesterase activity was measured as described [18,22]. Values in parentheses represent the number of different membrane preparations examined.

	Ghosts	Inside-out vesicles	*p* value
Phospholipid/protein (μmol P/mg protein)	0.73 ± 0.08(7)	1.14 ± 0.15(7)	< 0.005 [a]
Acetylcholinesterase/protein (mol/acetylcholinesterase/min per mg protein)	1.99 ± 0.06(3)	3.14 ± 0.36(7)	< 0.05 [b]
Acetylcholinesterase/phospholipid (mol/acetylcholinesterase/min per μmol P)	2.60 ± 0.45(3)	2.80 ± 0.09(7)	> 0.005 [b]

[a] Student's *t*-ratio.
[b] Student's *t*-test.

pholipid content of the membrane. In addition, no significant difference in the distribution of the major phospholipid classes was detected between ghosts and inside-out vesicles (Table II). The phospholipid composition of these isolated membranes is similar to that of intact cells [26,27], indicating that membrane phospholipid distribution is unaltered by either ghost or inside-out vesicle preparation.

Incorporation of [9,10(n)-^{3}H]oleic acid and [1-^{14}C]oleoyl-CoA into phospholipid by ghosts

The dependence of [9,10(n)-^{3}H]oleic acid incorporation into phospholipid by ghosts on substrate and cofactor concentration was examined by incubating ghosts with varying concentrations of ATP, CoA, or [9,10(n)-^{3}H]oleic acid for 15 min at 37°C. Under these conditions, [9,10(n)-^{3}H]oleic acid is incorporated into PC, PE and *N*-acylhydroxamic acid derived from acyl thioesters (Fig. 1) [12]. No incorporation of [9,10(n)-^{3}H]oleic acid into phospholipids occurs in the absence of ATP, while a small amount of [9,10(n)-^{3}H]oleic

TABLE II

PHOSPHOLIPID COMPOSITION OF GHOSTS AND INSIDE-OUT VESICLES

Phospholipid composition of ghosts and inside-out vesicles prepared from the same erythrocytes was determined by assaying the phosphorus content of individual phospholipid classes after lipid extraction and separation by thin-layer chromatography [12]. Phosphatidylserine and phosphatidylinositol comigrate in this system. Data are expressed as the percentage of total lipid phosphorus recovered and represent the mean ± S.E. of four different membrane preparations. There was no statistically significant difference between ghosts and inside-out vesicles in the content of individual phospholipid classes (Student's *t*-ratio).

	Ghosts	Inside-out vesicles
Phosphatidylcholine	28.93 ± 0.85	30.73 ± 1.16
Phosphatidylethanolamine	30.53 ± 2.73	21.25 ± 1.70
Phosphatidylserine-phosphatidylinositol	14.55 ± 3.12	12.90 ± 2.51
Sphingomyelin	25.10 ± 0.57	25.13 ± 0.39

acid is detected in *N*-acylhydroxamic acids (Fig. 1A). This non-ATP-dependent *N*-acylhydroxamic acid may represent other types of acyl linkages sensitive to NH_2OH, possibly acylated proteins [28]. Incorporation of [9,10(n)-^{3}H]oleic acid into PC, PE and *N*-acylhydroxamic acid shows little dependence on ATP concentration over the range of 0.1–1 mM ATP. In the absence of added CoA, [9,10(n)-^{3}H]oleic acid is incorporated into both phospholipids and *N*-acylhydroxamic acids at 25–30% of the maximal rate achieved in the presence of added CoA (Fig. 1B). This may reflect the retention of endogenous CoA by ghosts although the presence of a non-CoA-dependent pathway for [9,10(n)-^{3}H]oleic acid incorporation cannot be excluded. Incorporation of [9,10(n)-^{3}H]oleic acid into both PC and PE demonstrates no dependence on CoA concentration over the range of 1–10 μM, while acyl thioester formation increases steadily over this range. Similarly, when the concentration of [9,10(n)-^{3}H]oleic acid is varied, the rate of [9,10(n)-^{3}H]oleic acid incorporation into PC and PE changes little over the range of 5–20 μM, while the rate of acyl thioester formation continues to increase up to 20 μM (Fig. 1C). These results suggest that formation of the acyl thioester intermediate is not the rate-limiting step in the path-

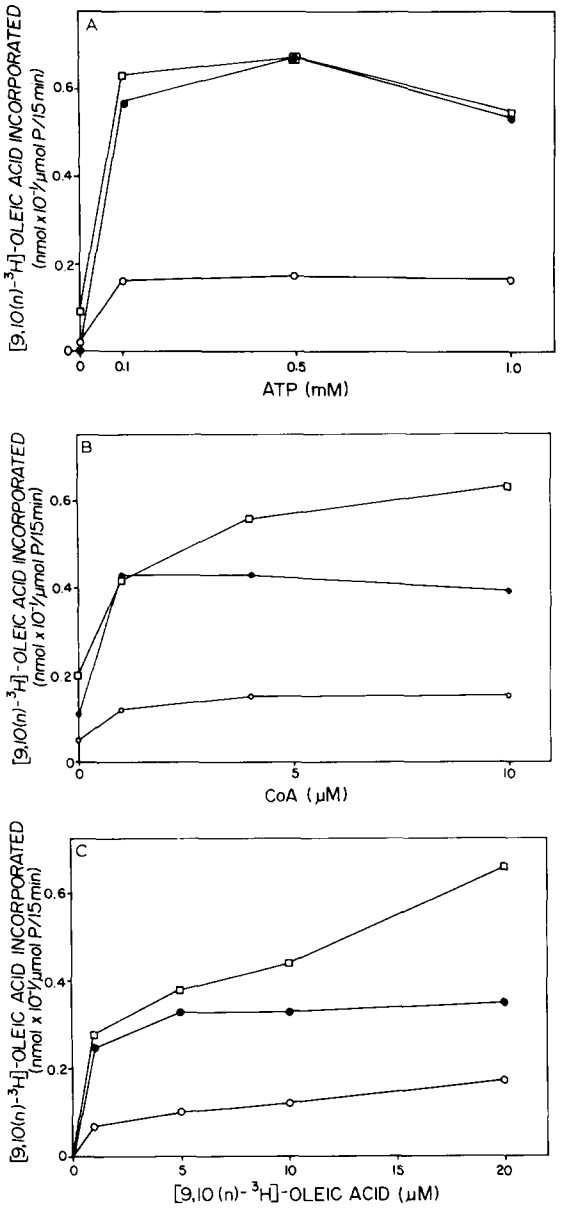

Fig. 1. Dependence of [9,10(n)-^{3}H]oleic acid incorporation into phospholipid by erythrocyte ghosts on (A) ATP, (B) CoA and (C) [9,10(n)-^{3}H]oleic acid concentration. Erythrocyte ghosts (10%, v/v) in 20 mM glycylglycine/3 mM magnesium gluconate (pH 7.1) were incubated for 15 min at 37°C with varying concentrations of either (A) ATP, (B) CoA or (C) [9,10(n)-^{3}H]oleic acid. As one component was varied, the other two were held constant. (ATP, 1 mM; CoA, 4 μM; [9,10(n)-^{3}H]oleic acid, 10 μM). Lipid extraction, acyl thioester derivatization and separation of phospholipids and *N*-acylhydroxamic acids derived from acyl thioesters were performed as described [12]. Data represent the mean of duplicate determinations. PC (●); PE (○); *N*-acylhydroxamic acid (□).

way and that the acylation of lysophospholipid is dependent on the activities of the acyl-CoA-lysophospholipid acyltransferase and/or endogenous phospholipase.

Transesterification of oleic acid into PC and PE by ghosts also occurs when ghosts are incubated with [1-^{14}C]oleoyl-CoA (Fig. 2) [12,17]. Incorporation of [1-^{14}C]oleic acid into PC by ghosts is maximal at 2 μM [1-^{14}C]oleoyl-CoA, while incorporation into PE does not saturate up to 4 μM [1-^{14}C]oleoyl-CoA. On a molar basis, oleic acid incorporation into phospholipid by ghosts is greater with [1-^{14}C]oleoyl-CoA as substrate than with [9,10(n)-^{3}H]oleic acid. Oleic acid incorporation into PC from [1-^{14}C]oleoyl-CoA is approx. 30-fold greater than that from [9,10(n)-^{3}H]oleic acid, while incorporation into PE is increased 6-fold (Figs. 1C, 2). These results suggest either that separate acyltransferases exist for PC and PE or that there are differences in the availability of lysoPE or lysoPC. The differences between incorporation of oleic acid from [9,10(n)-^{3}H]oleic acid and [1-^{14}C]oleoyl-CoA also suggest that there is

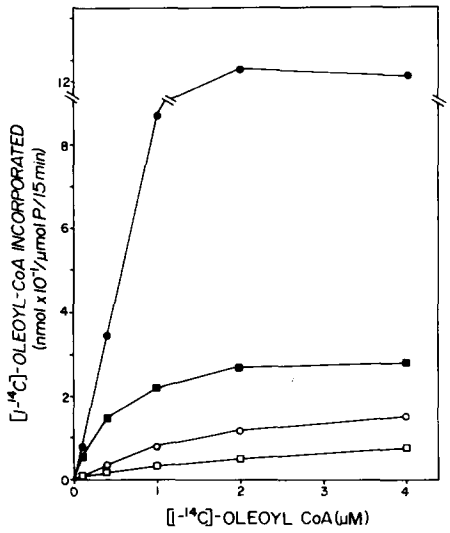

Fig. 2. Dependence of transesterification of [1-^{14}C]oleoyl-CoA into phospholipid by ghosts and inside-out vesicles on [1-^{14}C]oleoyl-CoA concentrations. Ghosts and inside-out vesicles were incubated in 20 mM glycylglycine/3 mM magnesium gluconate (pH 7.1) with varying concentrations of [1-^{14}C]oleoyl-CoA for 15 min. Lipid extraction and separation were carried out as described [12]. Ghosts: PC (●), PE (○); inside-out vesicles: PC (■), PE (□).

coupling between acyl-CoA ligase and acyl-CoA-lysophospholipid acyltransferase activities within the membrane.

Direct comparison of oleic acid incorporation into phospholipid by ghosts and inside-out vesicles

When ghosts are incubated in the presence of concentrations of ATP, CoA and [9,10(n)-^{3}H]oleic acid found to give maximal incorporation into phospholipid, incorporation of [9,10(n)-^{3}H]oleic acid into PC and PE is linear for 60 min, with incorporation into PC about 3-times as great as that into PE (Fig. 3). Parallel incubation of inside-out vesicles prepared from the same ghost preparation results in negligible incorporation into either phospholipid. The activation of [9,10(n)-^{3}H]oleic acid to acyl thioester by inside-out vesicles is also reduced compared to that by ghosts (Fig. 4). Incubation of ghosts and inside-out vesicles under identical conditions with [1-^{14}C]oleoyl-CoA results in incorporation of [1-^{14}C]oleic acid into phospholipids by both ghosts and inside-out vesicles, but the rate of incorporation into PC and PE by

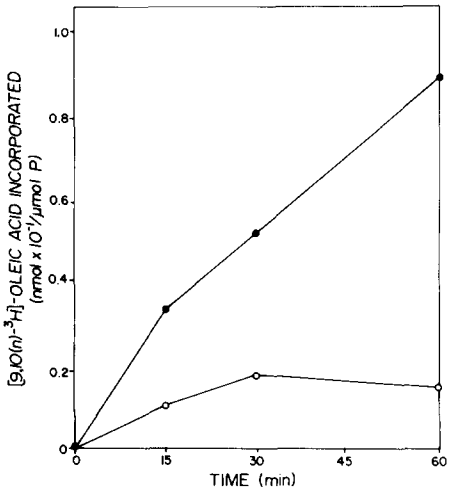

Fig. 4. Activation of [9,10(n)-^{3}H]oleic acid to acyl thioester by ghosts and inside-out vesicles. Ghosts and inside-out vesicles were incubated in buffer as described in Fig. 3. Derivatization of acyl thioester to *N*-acylhydroxamic acids, lipid extraction and separation of phospholipids and *N*-acylhydroxamic acids were carried out as described [12]. Ghosts (●); inside-out vesicles (○).

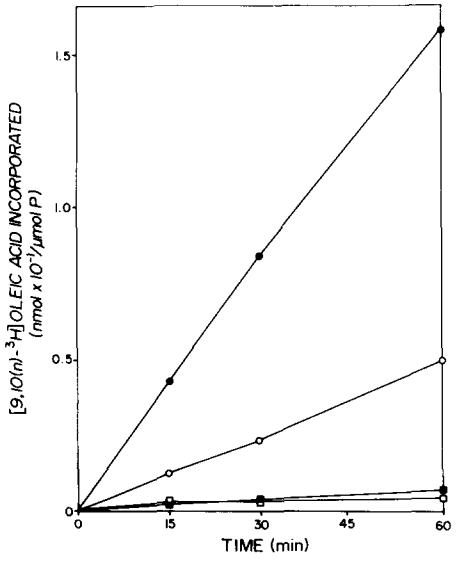

Fig. 3. Incorporation of [9,10(n)-^{3}H]oleic acid into PC and PE by ghosts and inside-out vesicles. Ghosts or inside-out vesicles were incubated as described in Fig. 1 in buffer comprising 1 mM ATP/4 μM CoA/10 mM [9,10(n)-^{3}H]oleic acid. Lipid extraction and separation were performed on duplicate aliquots at the times indicated as described [12]. Ghosts: PC (●), PE (○); inside-out vesicles: PC (■), PE (□).

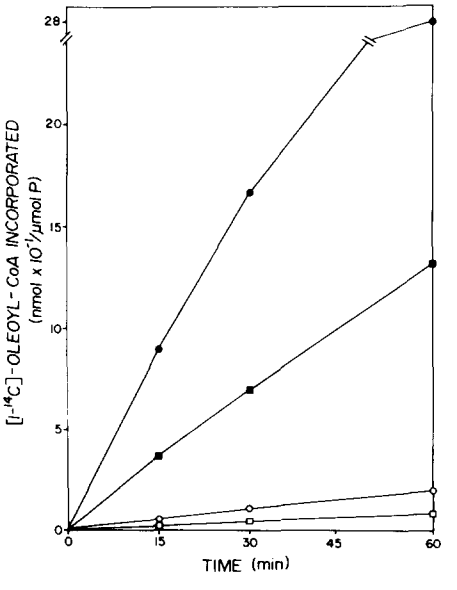

Fig. 5. Transesterification of [1-^{14}C]oleoyl-CoA into phospholipid by ghosts and inside-out vesicles. Ghosts and inside-out vesicles were incubated as described in Fig. 3 but with 4 μM [1-^{14}C]oleoyl-CoA substituted for CoA and [9,10(n)-^{3}H]oleic acid. Lipid extraction and separation were carried out on duplicate aliquots as described [12]. Symbols are the same as in Fig. 3.

inside-out vesicles is 50% of that found in ghosts (Fig. 5). Ghosts show greater incorporation of [1-^{14}C]oleic acid into both PC and PE than inside-out vesicles when the concentration of [1-^{14}C]oleoyl-CoA is varied from 0.4 to 4.0 μM, with no difference at the lowest substrate concentration examined (0.1 μM) (Fig. 2).

Discussion

The modification of membrane phospholipid fatty acid composition by deacylation and reacylation of plasma membrane phosphoglycerides in situ may play a role in regulating the function of membrane proteins, particularly those involved in cation transport [1]. The activity of membrane transport mechanisms has been extensively studied in a variety of plasma membrane derivatives including erythrocyte ghosts and inside-out vesicles, but the effects of preparation of these isolated membranes on the enzymatic pathway for phospholipid fatty acid turnover in situ has not been examined. We have compared the activity of the pathway for phospholipid fatty acid turnover in unsealed ghosts and inside-out vesicles because the influence of transmembrane ion and potential gradients and ion transport (which occur only in inside-out vesicles) can be separated from direct effects of perturbants on the enzymes for phospholipid fatty acid turnover, which would affect both ghosts and inside-out vesicles. The pathway for phospholipid fatty acid turnover has not been examined previously in inside-out vesicles. The present study extends earlier studies of the pathway for phospholipid fatty acid turnover in unsealed ghosts [12,16,17] and reports the first direct comparison of phospholipid fatty acid turnover in this membrane preparation to that in the inside-out vesicle preparation widely employed in transport studies.

Comparison of the protein and phospholipid composition of ghosts and inside-out vesicles derived from then reveals an increase in the phospholipid/protein ratio in inside-out vesicles (Table I), confirming previous findings that peripheral membrane proteins including the membrane skeleton protein spectrin are eluted from the membrane during inside-out vesicle preparation [22,23]. The ratio of acetylcholinesterase activity to protein also increases in inside-out vesicles compared to ghosts, since acetylcholinesterase is quantitatively retained in inside-out vesicles [23]. The ratio of acetylcholinesterase activity to phospholipid and phospholipid distribution are the same in ghost and inside-out vesicles, indicating that no alteration of membrane phospholipid composition occurs as a result of inside-out vesicle preparation (Table II). However, when the incorporation of [9,10(n)-^{3}H]oleic acid by ghosts and inside-out vesicles is compared, the incorporation of [9,10(n)-^{3}H]oleic acid into phospholipid is markedly reduced in inside-out vesicles compared to ghosts (Fig. 3). Since acylation of lysophospholipid occurs on the inner surface of the membrane [15], this difference in acylation does not simply reflect reduced uptake or transport of substrates or cofactors. This loss of activity appears to be related to the hypoosmotic conditions used in inside-out vesicle preparation, since identical erythrocyte ghosts kept at 4°C for a period equivalent to that required for inside-out vesicle preparation (approx. 5 h) readily incorporate [9,10(n)-^{3}H]oleic acid.

To determine whether the decrease in the acylation of endogenous lysophospholipid by inside-out vesicles reflects a decrease in enzyme activity, and to delineate the steps in the pathway which are altered during inside-out vesicle preparation, two approaches were employed: (i) the ability of membrane preparations to activate fatty acids to acyl thioester was assayed using a procedure involving selective derivatization of acyl thioesters to *N*-acylhydroxamic acids [12] (Fig. 4); and (ii) the ability of each membrane preparation to acylate endogenous lysophospholipids directly was assayed by using [1-^{14}C]oleoyl-CoA as substrate (Fig. 2, 5). These two approaches permit evaluation of acyl-CoA ligase and acyl-CoA-lysophospholipid acyltransferase activity separately. Activation of [9,10(n)-^{3}H]oleic acid to acyl thioester was markedly reduced in inside-out vesicles compared to ghosts in the presence of sautrating concentrations of substrate and cofactors (Fig. 4), indicating that there has been loss of enzyme activity either via denaturation or elution from the membrane. Incorporation of [1-^{14}C]oleic acid into phospholipid from [1-^{14}C]oleoyl-CoA was also reduced, though to a lesser extent, indicating that reduction in acyl-CoA ligase activity is not the only reason

for decrease in [9,10(n)-^{3}H]oleic acid incorporation into PC and PE by inside-out vesicles (Figs. 2, 5) and suggesting that denaturation or elution of acyl-CoA-lysophospholipid acyltransferase activity may also occur during inside-out vesicle preparation. The decreased acylation of lysophospholipid by [1-^{14}C]oleoyl-CoA in inside-out vesicles was not enhanced by raising [1-^{14}C]oleoyl-CoA concentration (Fig. 2), indicating that an alteration in substrate affinity is not responsible for the difference in rates of acylation. The reduced incorporation of oleic acid into phospholipid by inside-out vesicles could also reflect reduced availability of lysophospholipid. However, since the amounts of lysoPC and lysoPE present and any differences between ghost and inside-out vesicle would be very small (1–10 pmol/μmol P) and undetectable [12], it is not possible to completely exclude lysophospholipid availability as the basis for the difference in oleic acid incorporation.

The effects of membrane isolation on the function of membrane proteins, particularly the enzymes for phospholipid turnover, has not been extensively examined. Some membrane components such as acetylcholinesterase are fully recovered in ghosts and inside-out vesicles, while spectrin, the major membrane skeleton protein, and adenylate cyclase are lost as a consequence of exposure to the hypotonic conditions used during ghost and inside-out vesicle preparation [23,29]. Freeze-etch electron microscopic studies have further shown that the distribution of intramembranous particles is different in inside-out vesicles than in ghosts, suggesting that structural rearrangement of membrane protein occurs during inside-out vesicle preparation [30]. In the present study, loss of acyl-CoA ligase and acyl-CoA-lysophospholipid acyltransferase activity from the erythrocyte membrane has been detected as a consequence of the procedures employed for preparation of inside-out vesicles. It is possible that these enzymes could display similar behavior in other membranes. Therefore, the effect of procedures employed for membrane preparaton on the pathway for phospholipid fatty acid turnover in situ should be considered in studies concerned with elucidation of the relationship between phospholipid fatty acid turnover and the regulation of membrane function in both erythrocytes and more complex cells.

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