Definition of the pathway for membrane phospholipid fatty acid turnover in human erythrocytes

Craig A. Dise,[1] David B. P. Goodman,[2] and Howard Rasmussen

Departments of Internal Medicine and Cell Biology, Yale University School of Medicine, New Haven, CT 06510

Abstract Techniques have been developed to permit detection of acyl thioesters derived from exogenous fatty acids in erythrocytes. These acyl thioesters have been shown to act as intermediates in the acylation of endogenous lysophospholipid. Release of fatty acids from erythrocyte phospholipids has also been detected. Such release may reflect the activity of an endogenous phospholipase that utilizes endogenous phospholipid as substrate. These observations permit further definition of the biochemical pathway for erythrocyte phospholipid fatty acid turnover.—**Dise, C. A., D. B. P. Goodman, and H. Rasmussen.** Definition of the pathway for membrane phospholipid fatty acid turnover in human erythrocytes. *J. Lipid Res.* 1980. **21:** 292–300.

Supplementary key words acyl thioester · endogenous phospholipase · hydroxamic acid.

The mechanisms by which cells regulate membrane phospholipid fatty acid turnover and membrane function are not yet fully understood. Elucidation of the relationship between phospholipid fatty acid composition and plasma membrane function in mammalian cells remains a complex problem because these cells contain membrane-bound organelles and because they have the ability to alter membrane phospholipid fatty acid composition by either fatty acid turnover in situ or phospholipid synthesis de novo (1). However, the mammalian erythrocyte is an attractive system to investigate factors which control plasma membrane function and phospholipid fatty acid turnover in situ. In this system, changes in membrane turnover and function must occur in the same membrane because these cells contain only a single membrane, the plasma membrane. In addition, turnover of erythrocyte phospholipid fatty acids in situ is limited to deacylation of endogenous phospholipid and reacylation of the resulting lysophospholipids (2–6) and/or exchange of intact phospholipid molecules with exogenous phospholipids because these cells cannot alter fatty acid chain length or degree of unsaturation or synthesize phospholipid de novo (3, 6–9).

Previous studies have demonstrated that exogenous fatty acids can be incorporated into erythrocyte membrane phospholipids, but have not clearly defined the pathway by which fatty acid incorporation occurs (2, 3, 6, 8–14). The incorporation of fatty acids into erythrocytes by acylation of endogenous lysophospholipids has been assumed to proceed through an initial activation step in which fatty acids taken up by the cell are esterified to Coenzyme A in an ATP-dependent reaction catalyzed by acyl CoA ligase (2, 10, 11, 13, 15). Transesterification of the acyl group from acyl CoA thioester to lysophospholipid by one or more acyl CoA-lysophospholipid acyltransferases would then follow. The formation of acyl thioesters from exogenous fatty acid by erythrocyte membranes has been observed (15), but the role of such acyl thioesters as intermediates in the acylation of endogenous lysophospholipid by human erythrocytes has not been established. In addition, although a pathway for renewal of erythrocyte membrane phospholipid fatty acids by acylation of endogenous lysophospholipid has been described (2, 3, 6, 8–14), the source of the lysophospholipid which undergoes acylation has not been identified. Previous studies have shown that erythrocyte membranes do not hydrolyze exogenous phospholipids to lysophospholipids (6, 10, 11). The possibility that there is an endogenous phospholipase which acts only upon endogenous phospholipid has not been explored fully.

To investigate the regulation of membrane phos-

Abbreviations: BSA, bovine serum albumin; FFA, free fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FANHOH, *N*-acyl hydroxamic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

[1] Present address: Department of Pathology, Yale University School of Medicine, New Haven, CT 06510.

[2] To whom all correspondence should be addressed.

pholipid fatty acid turnover and its relationship to membrane function, it has been necessary to define the biochemical pathway for erythrocyte phospholipid fatty acid turnover more completely. In the present study we have addressed two unanswered questions with regard to the definition of the pathway by which renewal of membrane phospholipid fatty acids in situ occurs in human erythrocytes: *1*) the role of acyl thioester intermediates and *2*) the existence of an endogenous phospholipase.

MATERIALS AND METHODS

Erythrocyte preparation

Heparinized blood was obtained from normal human donors by venipuncture. Erythrocytes were separated from plasma and leukocytes by centrifugation at 750 *g* for 7 min (International Equipment Co. Model CL) and washed four times with a buffer containing NaCl 140 mM, KCl 5 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, NaH_2PO_4 1 mM, Tris 10 mM, and glucose 5 mM, pH 7.4. This washing procedure resulted in an erythrocyte preparation that contained neither leukocytes, when assessed by Coulter counter (Model S) or microscopic examination, nor platelets, when assessed by microscopic examination. Additionally, when the buffy coat from an equal volume of blood was added to uncentrifuged whole blood and washed as described above, no leukocytes or platelets were found in the erythrocyte preparation.

Fatty acid incorporation

Erythrocytes were resuspended in buffer to 20% (v/v) for subsequent incubation with [1-^{14}C]oleic acid (58 mCi/mmol) (New England Nuclear or Amersham-Searle), [9, 10-^{3}H]oleic acid (2.4 Ci/mmol) (Amersham-Searle), unlabeled fatty acids (Supelco) or 2-bromohexadecanoic acid (Eastman) complexed to fatty acid free bovine serum albumin (BSA) (Sigma) (13) in 140 mM NaCl, 10 mM Tris pH 7.4.

Extraction of lipids from intact erythrocytes

After incubation, cells were washed with 10 volumes of either buffer or buffer containing fatty acid-free BSA to remove excess free fatty acid and lysed with an equal volume of water. Lipids were immediately extracted by a modification of the procedure of Ways and Hanahan (16) in which five volumes of methanol were added to the lysate followed after 30 min by 5 volumes of chloroform. After an additional 20 min the extract was centrifuged and the residue was re-extracted twice with 5 volumes of methanol and 5 volumes of chloroform (17). Pooled extracts were washed with 0.8 their volume of 50 mM KCl to produce a biphasic mixture (18).

Preparation, incubation, and lipid extraction of erythrocyte membrane

Hemoglobin-free erythrocyte membranes were prepared by hypotonic hemolysis (19) at 4°C in 40 volumes 10 mM Tris pH 7.4 and isolated by centrifugation (16,000 *g* × 15 min, Sorval RC-2). Membranes were washed twice with 40 volumes of 10 mM Tris and resuspended in 10 mM Tris, pH 7.4, for incubation with ^{14}C-labeled fatty acids complexed to fatty acid-free BSA, ATP (Sigma) and Coenzyme A (CoA) (PL Biochemicals). Membrane phospholipids were extracted by addition to the incubation mixture of 5 volumes of methanol followed after 30 min by 5 volumes of chloroform (16, 17). After an additional 30 min, the lipid extract was washed with 3 volumes of 50 mM KCl to effect phase separation (18).

Extraction of acyl thioesters from intact erythrocytes and isolated membranes

For detection of acyl thioesters, extraction with neutral methanolic hydroxylamine (0.5 M), prepared by mixing equal volumes of 1 M hydroxylamine hydrochloride and 1 M potassium hydroxide in methanol, was substituted for the initial extraction with methanol (20). Samples were chromatographed with palmitoyl hydroxamate prepared by reaction of palmitoyl chloride (Sigma) with neutral methanolic hydroxylamine (21) as carrier to permit detection of *N*-acyl hydroxamate with iodine after chromatography.

Isolation of phospholipids

The lower phase of lipid extracts was concentrated under nitrogen. After removal of aliquots for determination of lipid phosphorus (22, 23) and radioactivity, the extract was applied to a thin-layer of Silica H (E. Merck) prepared with 1 mM Na_2CO_3. Thin-layer plates were developed in diethyl ether–acetic acid 98:2 (v/v) to separate neutral lipids from hydroxamic acids and phospholipids, dried in air for 10 min, and then developed in chloroform–methanol–acetic acid–water 50:20:5:2 (v/v) to separate hydroxamic acids from individual phospholipid classes (24). Lipids were visualized by brief exposure (1 min) of the plates to iodine.

Measurement of exogenous fatty acid incorporated

Radioactivity present in each fraction was determined by liquid scintillation counting after scraping gel fractions into scintillation vials and adding scintillation fluid consisting of toluene–methyl cellosolve

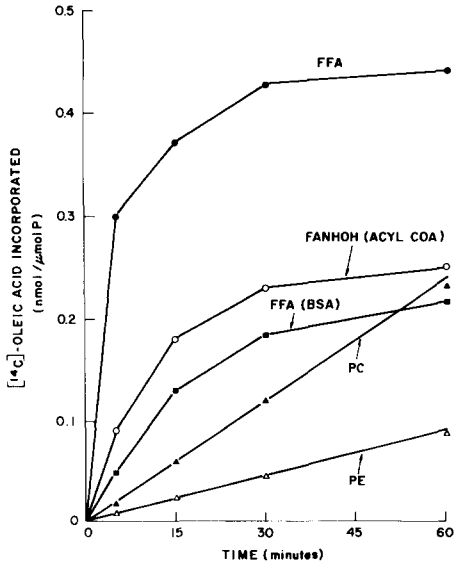

Fig. 1. Uptake and incorporation of [1-^{14}C]oleic acid by human erythrocytes. Erythrocytes were incubated with [1-^{14}C]oleic acid (10 μM), complexed to BSA (1.65 mg/ml) at 37°C. Aliquots were removed and washed with buffer or buffer containing fatty acid-free BSA (1%). Cell lipids were then extracted, using neutral methanolic hydroxylamine, and chromatographed as described in Materials and Methods. Total free fatty acid (FFA) (●), free fatty acid remaining after washing with BSA (FFA (BSA)) (■), *N*-acyl hydroxyamic acid derived from acyl thioester (FANHOH) (○), PC (▲), PE (△). Results are expressed as nmol [1-^{14}C]oleic acid incorporated/μmol total lipid phosphorus.

5:3 with PPO (5 mg/l) and POPOP (62.5 mg/l). This mixture elutes neutral lipids and hydroxamic acids quantitatively from Silica H. Recovery of phospholipids is greater than 95%. Counting efficiency was determined with an external standard (Packard 3385) (25). Calculations are based on the specific activity of the exogenous fatty acid.

RESULTS

Fatty acid incorporation by intact erythrocytes

When human erythrocytes are incubated with ^{14}C-labeled fatty acids, washed, and then extracted with chloroform and methanol, the labeled fatty acid is recovered in the free fatty acid (FFA) and phospholipid fractions (**Fig. 1**). If the cells are washed with a buffer containing fatty acid-free BSA before extraction, a portion of the ^{14}C-labeled FFA is removed. Thus, there are two cellular fractions of FFA which differ in their affinity for the cell. Both fractions are labeled more rapidly than phospholipid. The presence of two cellular pools of FFA has been observed previously (13, 26). However, the relationship between the two pools has not yet been elucidated. ^{14}C-Labeled fatty acids are also incorporated into both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). There is no significant incorporation of exogenous fatty acid into neutral lipids or other phospholipid classes under these conditions. The incorporation of these fatty acids into PC and PE is linear for at least 2.5 hr (**Fig. 2**).

Extraction of acyl thioesters by neutral methanolic hydroxylamine

Acyl thioesters are slightly soluble in aqueous media and would not be completely recovered by chloroform–methanol extraction (15, 27, 28). Thus, derivatization to a lipid-soluble form is required in order to detect and recover fatty acids esterified to thiols. Hydroxylamine has been shown to react selectively with acyl thioesters at neutral pH to form lipid-soluble *N*-acyl hydroxamic acids (15, 27, 28). When an aqueous solution of [1-^{14}C]oleoyl CoA is extracted with chloroform and methanol, 98% appears in the aqueous phase. However, when derivatization with neutral methanolic hydroxylamine is carried out during extraction, 90% of [1-^{14}C]oleoyl CoA is recovered in the chloroform phase (**Table 1**). In order to determine whether acyl thioesters can be recovered from erythrocytes, [1-^{14}C]-palmitoyl CoA was added to erythrocyte lysates that were immediately extracted in the presence and in

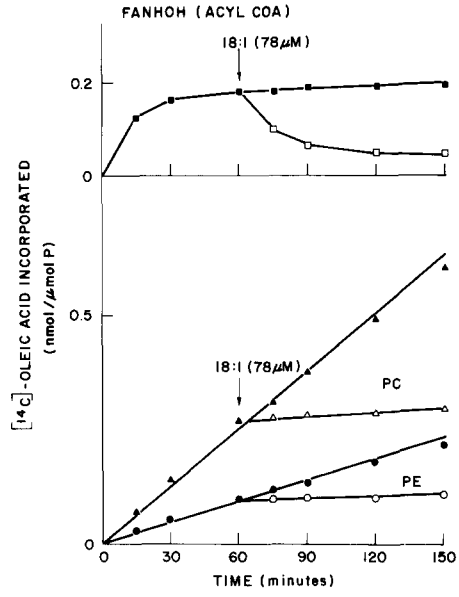

Fig. 2. Effect of addition of unlabeled oleic acid on incorporation of [1-^{14}C]oleic acid by human erythrocytes. Erythrocytes were incubated at 37°C with [1-^{14}C]oleic acid-BSA (oleic acid 5 μM BSA 1.25 mg/ml) for 60 min. Unlabeled oleic acid was then added to produce a final concentration of 76 μM (BSA 2.5 mg/ml). Aliquots of the incubation mixture were washed, extracted, and chromatographed as described in Materials and Methods. FANHOH: control (■), excess oleic acid (□). PC: control (▲), excess oleic acid (△). PE: control (●), excess oleic acid (○). Results are expressed as in Fig. 1.

TABLE 1. Effect of neutral methanolic hydroxylamine on extraction of [1-^{14}C]oleoyl CoA

Initial Extraction	Aqueous Phase	Recovered cpm	Chloroform Phase	Recovered cpm
	cpm	%	*cpm*	%
Methanol	92200	97.2	2700	2.8
Methanolic hydroxylamine	8000	9.7	74800	90.3

[1-^{14}C]Oleoyl CoA in 0.2 volume buffer (NaCl 35 mM, KCl 5 mM, $MgSO_4$ 1 mM, Tris 10 mM pH 7.4) was added to 1 volume of methanol, or methanol containing neutral hydroxylamine (0.5 M). After 30 min, 1 volume of chloroform was added. After an additional 30 min, 0.6 volume of 50 mM KCl was added to effect phase separation. Aliquots of the upper and lower phases were counted.

the absence of neutral hydroxylamine. Of [1-^{14}C]palmitoyl CoA added to erythrocytes immediately prior to treatment with neutral methanolic hydroxylamine, 85% is recovered in the chloroform phase. However, less than 20% of [1-^{14}C]palmitoyl CoA added to erythrocytes immediately prior to extraction in the absence of hydroxylamine is recovered in the extract (**Table 2**). The remainder appears to bind to, or be entrapped by, cellular debris. Since the CoA portion of the molecule is hydrophilic, underivatized acyl CoA would be poorly soluble in organic solvents and would precipitate with the cellular proteins. Thus, extraction of erythrocytes with chloroform and methanol alone would not permit accurate measurement of acyl thioester.

When erythrocytes are incubated with [1-^{14}C]oleic acid and then washed and treated with neutral methanolic hydroxylamine prior to lipid extraction, [1-^{14}C]-oleic acid is recovered as *N*-acyl hydroxamic acid (FANHOH). Initially, [1-^{14}C]oleic acid appears in the FANHOH fraction more rapidly than in phospholipid. Subsequently, little change in concentration occurs during further incubation for up to 2.5 hr (Figs. 1, 2). A corresponding fraction is not present in cells extracted in the absence of hydroxylamine. Extraction of erythrocytes with neutral methanolic hydroxylamine does not alter the amount of exogenous fatty acid detected in phospholipid as *O*-acyl ester or the recovery of phospholipid. Incorporation of [1-^{14}C]oleic acid into PC was 0.236 ± 0.016 nmol/μmol P/hr in the absence and 0.239 ± 0.008 nmol/μmol P/hr in the presence of hydroxylamine. [1-^{14}C]Oleic acid incorporation into PE was 0.093 ± 0.002 nmol/μmol P/hr in the absence and 0.094 ± 0.009 nmol/μmol P/hr in the presence of hydroxylamine. Phospholipid recovery was 3.64 ± 0.13 μmol P/ml erythrocytes in the absence and 3.63 ± 0.08 μmol P/ml erythrocytes in the presence of hydroxylamine. Thus, the ^{14}C-labeled FANHOH is formed by derivatization of [^{14}C]acyl groups which react with hydroxylamine only at neutral pH as do acyl thioesters (15, 27, 28) and is not formed by reaction of hydroxylamine with *O*-acyl esters formed by ^{14}C-labeled fatty acid esterified to phospholipid.

Effect of inhibitors of fatty acid activation in incorporation of fatty acid into FANHOH and phospholipid

If the FANHOH represents an acyl thioester formed by the action of acyl:thiol ligase, reducing the amount of ^{14}C-labeled fatty acid available for activation should reduce the amount of ^{14}C-labeled FANHOH formed. Furthermore, if formation of such an acyl thioester intermediate is required prior to acylation of lysophospholipid, reducing the formation of ^{14}C-labeled acyl thioesters should also reduce the rate of incorporation of ^{14}C-labeled fatty acid to acyl thioester and subsequent incorporation into phospholipid would be reduced by: *1*) reducing the specific activity of FFA;

TABLE 2. Effect of neutral methanolic hydroxylamine on extraction of [^{14}C]palmitoyl CoA from erythrocytes

	Aqueous Phase		Chloroform Phase	
	cpm	*% added cpm*	*cpm*	*% added cpm*
Methanol	4300	10	3450	8
Methanolic hydroxylamine	3500	8	38000	85

Equal amounts of [^{14}C]palmitoyl CoA (44,500 cpm) in buffer as in Table 1 were added to a lysate prepared by mixing equal volumes of water and erythrocytes. Five volumes of neutral methanolic hydroxylamine (0.5 M) or methanol were added immediately, and extraction and analysis carried out as in Table 1.

TABLE 3. Activation of [1-^{14}C]oleic acid and incorporation of [1-^{14}C]oleic acid into phospholipid by erythrocyte membranes

	[1-^{14}C]Oleic Acid Incorporation		
	FANHOH	PC	PE
	nmol/μmol lipid phosphorus		
Complete	0.96	1.50	0.20
−ATP	0.24	0.01	0.01
−CoA	0.39	0.60	0.04

Erythrocyte membranes prepared by hypotonic hemolysis were incubated with [1-^{14}C]oleic acid-BSA (oleic acid 10 μM, BSA 1.65 mg/ml), ATP 1.5 mM, CoA 5 μM, dithiothreitol 15 μM, $MgSO_4$ 1.5 mM, Tris 10 mM pH 7.4. After 30 min, the incubation mixture was treated with neutral methanolic hydroxylamine and lipids extracted and chromatographed as described in Materials and Methods.

or *2*) by adding fatty acids, differing in chain length or degree of unsaturation, that utilize available cofactors and can also be incorporated into phospholipid (6, 10, 11, 13); or *3*) by adding 2-bromopalmitate, which has been shown to inhibit fatty acid activation and transport in liver microsomes (29, 30). To investigate these possibilities, erythrocytes were preincubated with [1-^{14}C]oleic acid for 60 min. This preincubation permits the [1-^{14}C]oleic acid in the FANHOH and FFA fractions to reach a steady state and the incorporation of fatty acid into phospholipid to attain a constant linear rate (Figs. 1, 2). Cells were then treated with: *1*) a large excess of unlabeled oleic acid; *2*) an excess of palmitic acid or linoleic acid; or *3*) 2-bromopalmitate. In each case, the amount of [1-^{14}C]oleic acid present in FANHOH declines rapidly and the rate of appearance of [1-^{14}C]oleic acid in phospholipid is reduced. The effect of addition of a large excess of oleic acid is shown in Fig. 2. Similar results were observed following addition of either 2-bromopalmitate or excess palmitic or linoleic acid.

Incorporation of fatty acid into phospholipid and FANHOH by erythrocyte membranes

The acylation of endogenous LPC and LPE with exogenous fatty acids also occurs in isolated erythrocyte membranes (6, 10, 11, 12). The acylation of endogenous LPC and LPE by exogenous fatty acids is dependent on the presence of ATP and stimulated by addition of CoA (**Table 3**). When erythrocyte membranes are incubated with [1-^{14}C]oleic acid and treated with neutral methanolic hydroxylamine, [1-^{14}C]oleic acid is detected as FANHOH. The incorporation of [1-^{14}C]oleic acid into FANHOH is stimulated by the addition of ATP and CoA (Table 2). Addition of an excess of unlabeled oleoyl CoA reduces the incorporation of [1-^{14}C]oleate into PC and PE and increases the amount of [1-^{14}C]oleate present as FANHOH. This inhibition is overcome by raising the concentration of [1-^{14}C]oleic acid (**Table 4**).

Release of free fatty acid from phospholipids of intact erythrocytes and erythrocyte membranes

Release of fatty acid previously incorporated into phospholipid can be detected in erythrocytes and erythrocyte membranes. To increase the specific activity of endogenous phospholipid, erythrocytes are incubated with [1-^{14}C]oleic acid–BSA. The cells are then washed with fatty acid-free BSA to remove excess [1-^{14}C]oleic acid and reincubated in buffer without oleic acid–BSA. The appearance of [1-^{14}C]oleic acid as FFA is measured. After an initial rapid increase, the amount of free [1-^{14}C]oleic acid increases at a constant rate for at least 2 hr. [1-^{14}C]Oleoyl hydroxamate decreases rapidly initially and then undergoes no further change with time (**Fig. 3**). As the increase in the amount of [1-^{14}C]oleic acid present as FFA is greater than the decrease in the amount present as [1-^{14}C]oleoyl thioester, the appearance of free [1-^{14}C]-oleic acid cannot be accounted for solely by hydrolysis of [1-^{14}C]oleoyl thioester and must be derived in part from [1-^{14}C]oleic acid previously incorporated into phospholipid. The amount of [1-^{14}C]oleic acid released as FFA represents less than 2% of that incorporated during preincubation. For this reason, no significant change in the amount of [1-^{14}C]oleic acid esterified to either PC or PE is detected, and it is not possible to identify the source of the [1-^{14}C]oleic acid released. Because it is possible that the fatty acid may be released from multiple pools of phospholipid of different specific activities, the release is expressed as radioactivity released per μmol P. If the release of [1-^{14}C]oleic acid detected in intact cells under these circumstances represents endogenous phospholipase activity, it represents only a minimal estimate of activity since reincorporation of fatty acid released is possible.

Release of fatty acid also occurs upon incubation of isolated membranes labeled by previous overnight incubation with [9,10-^{3}H]oleic acid. Membranes of cells labeled with [9,10-^{3}H]oleic acid are prepared by hypotonic hemolysis and incubated in the presence of ATP and CoA to prevent reincorporation of [9,10-^{3}H]oleic acid released. Free [9,10-^{3}H]oleic acid is released at a constant rate of 1.6×10^{-5} cpm/μmol P/hr. Since [9,10-^{3}H]oleic acid is present in only PC and PE initially, free [9,10-^{3}H]oleic acid must be derived from that previously incorporated into PC and PE and could arise by the action of an endogenous phospholipase. Since the [9,10-^{3}H]oleic acid released represents less than 1% of [9,10-^{3}H]oleic acid present in phospholipid initially, no significant decrease in the amount of [9,10-^{3}H]oleic acid esterified to PC or PE is detected,

TABLE 4. Effect of oleoyl CoA on incorporation of [1-^{14}C]oleic acid by erythrocyte membranes

	[1-^{14}C]Oleic Acid Incorporation		
	FANHOH	PC	PE
	nmol/μmol lipid phosphorus		
[1-^{14}C]Oleic acid (1 μM)	0.21	1.10	0.13
[1-^{14}C]Oleic acid (1 μM) + oleoyl CoA (10 μM)	0.51	0.55	0.06
[1-^{14}C]Oleic acid (10 μM)	0.96	1.53	0.20
[1-^{14}C]Oleic acid (10 μM) + oleoyl CoA (10 μM)	1.02	1.42	0.17

Erythrocyte membranes prepared by hypotonic hemolysis were incubated with [1-^{14}C]oleic acid-BSA (oleic acid 1 μM, BSA 0.17 mg/ml) in the presence of ATP 1.5 mM, CoA 5 μM, dithiothreitol 15 μM, $MgSO_4$ 1.5 mM, Tris 10 mM pH 7.4. After 30 min at 37°C, the incubation mixture was treated with neutral methanolic hydroxylamine, and lipids extracted and chromatographed as described in Materials and Methods.

and it is not possible to identify the phospholipid from which fatty acid is released.

DISCUSSION

Recent studies in a variety of biological systems, including the mammalian erythrocyte, have shown that changes in membrane phospholipid fatty acid turnover and composition influence membrane properties and membrane protein function (31–35). The pathway for fatty acid incorporation into erythrocyte membrane phospholipids has been only partially characterized previously (2, 6, 8, 10, 11, 13, 14). Hence, before the relationship between membrane phospholipid fatty acid composition and turnover and membrane function can be examined in the erythrocyte, further definition of this pathway is necessary.

The incorporation of fatty acids into erythrocytes by acylation of endogenous lysophospholipids has been assumed to proceed through an initial activation step, in which fatty acids taken up by the cell are esterified to Coenzyme A in an ATP-dependent reaction by acyl CoA ligase (2, 10, 11, 13, 15). Transesterification of the acyl group from acyl CoA thioester to lysophospholipid by one or more acyl CoA-lysophospholipid acyltransferases would then follow. This assumption is based on the observations that: *1*) acyl CoA thioesters can serve as acyl donors for acylation of lysophospholipid by erythrocyte membranes (11, 12) and *2*) acylation of lysophospholipid with exogenous fatty acid by erythrocyte membranes is dependent on the presence of ATP and is stimulated by addition of CoA (2, 10, 11). The formation of acyl thioesters from exogenous fatty acid by erythrocyte membranes has been observed (15), but demonstration that such acyl thioesters are intermediates in the acylation of endogenous lysophospholipid by human erythrocytes has not been reported.

In order to determine if incorporation of fatty acids into phospholipid proceeds through a pathway involving an acyl thioester intermediate, it was necessary to develop a procedure for selective detection of acyl thioesters. Since acyl thioesters are not soluble in the solvents employed for lipid extraction (Table 1), derivatization is required. Hydroxylamine is a suitable derivatizing agent because it reacts selectively with acyl thioesters at neutral pH and with *O*-acyl esters only at more alkaline pH (15, 27, 28). Since acyl thioesters

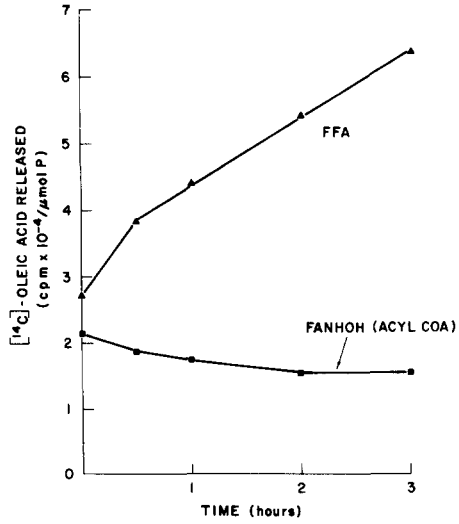

Fig. 3. Release of [1-^{14}C]oleic acid from human erythrocytes. Erythrocytes were incubated for 17 hr at 37°C with [1-^{14}C]oleic acid-BSA (oleic acid 10 M, BSA 1.65 mg/ml) in standard buffer pH 7.4, containing glucose 10 mM, penicillin 69 μg/ml, and streptomycin sulfate 64 μg/ml. After this incubation, cells were washed three times with buffer containing BSA (3%). Cells were then resuspended to 10% (v/v) in buffer containing glucose 10 mM at t = 0. Aliquots of this incubation mixture were treated with neutral methanolic hydroxylamine and extracted and chromatographed as described in Materials and Methods. Free fatty acid (FFA) (▲), *N*-acyl hydroxamic acid derived from acyl thioester (FANHOH) (■). Results are expressed as cpm × 10^{-4}/μmol total lipid phosphorus.

can undergo transesterification to *O*-acyl esters or hydrolysis to fatty acid, it is necessary to carry out derivatization under conditions which prevent further reaction. Further reaction is prevented by carrying out the derivatization in methanol (20). Since methanol is the initial solvent employed for lipid extraction, derivatization and extraction are carried out simultaneously, prior to separation of neutral and phospholipids from hydroxamic acids by thin-layer chromatography. The two-stage one-dimensional system employed in this study permits rapid separation of neutral ligands and hydroxamic acids from phospholipid. It should be pointed out that this system does not completely resolve all phospholipid classes (24). Lysophospholipids, which are minor or trace components of erythrocyte phospholipids (17), are not completely separated from phospholipids. Two-dimensional systems which are capable of resolving all lipid components have been described (36). The procedures described in this paper permit separate and simultaneous measurement of activation of fatty acids to acyl thioesters and acylation of endogenous lysophospholipid by the acyl thioester.

When human erythrocytes are incubated with exogenous ^{14}C-labeled fatty acids, uptake of labeled fatty acid by the cell as free fatty acid and acylation of endogenous LPC and LPE occur (Figs. 1, 2) (8, 13). When these erythrocytes are treated with neutral methanolic hydroxylamine prior to lipid extraction, an additional fraction is detected as ^{14}C-labeled *N*-acyl hydroxamic acid (FANHOH) (Figs. 1, 2). Since the incorporation of fatty acid into phospholipid is not altered by hydroxylamine extraction, FANHOH is not derived from reaction with fatty acid esterified to phospholipid as *O*-acyl esters, but represents acyl linkages having the same reactivity with hydroxylamine as acyl thioesters.

Acyl thioesters derived from exogenous fatty acids have not been detected previously in intact erythrocytes, although formation of FANHOH by erythrocyte membranes during incubation in hydroxylamine buffer has been described (15). The observation that acyl CoA added to erythrocytes is not recovered by extraction with chloroform and methanol but is recovered by extraction with neutral methanolic hydroxylamine (Table 2) indicates that acyl thioesters are not extracted by chloroform and methanol unless they are rendered lipid-soluble by derivatization. This property of acyl thioesters accounts for the failure of previous studies attempting to define the pathway for incorporation of exogenous fatty acids into erythrocyte phospholipids to identify these compounds in erythrocytes.

Formation of FANHOH occurs more rapidly initially than either fatty acid binding or incorporation into phospholipid (Fig. 1). This suggests that the FANHOH fraction represents a precursor for both of the other two pools. The tightly bound FFA not extracted by fatty acid-free BSA may be derived from acyl thioester hydrolyzed by endogenous esterases. The experimental results presented in Fig. 2 demonstrate that a reduction in the specific activity or concentration of FANHOH is associated with a decrease in incorporation of fatty acid into phospholipid. These results support the concept that FANHOH represents an acyl thioester intermediate in the acylation of endogenous lysophospholipid by exogenous fatty acids. However, since the added fatty acids function as competitive inhibitors, they would inhibit any pathway for utilization of exogenous fatty acids. Consequently, these results do not rule out the possibility that incorporation of fatty acid into phospholipid could occur by a pathway not involving acyl thioesters. However, the existence of such a pathway involving direct esterification or esterification via another type of intermediate has not been demonstrated in any mammalian system (37).

Acylation of LPC and LPE by erythrocyte membranes is dependent on the presence of ATP and stimulated by addition of CoA (Table 3). Formation of FANHOH is also enhanced by addition of ATP and CoA. These results indicate that formation of FANHOH and acylation of endogenous lysophospholipid proceed through a common pathway requiring ATP. The ability of oleoyl CoA to depress incorporation of [1-^{14}C]oleic acid into phospholipid indicates that [1-^{14}C]oleic acid incorporation occurs via the same pathway utilized by oleoyl CoA. These results also support the conclusion that acyl thioesters are intermediates in the incorporation of fatty acids into phospholipids, but they do not show that thioesterification is the only pathway for ATP-dependent acylation. Such proof would require complete elimination of endogenous thiols which might act as acyl acceptors so that the contribution of non-thiol dependent pathways could be assessed. The ability of erythrocyte membranes to form FANHOH and incorporate fatty acid into phospholipid in the absence of added CoA (Table 3) may reflect retention of endogenous CoA or participation of other endogenous thiols in acyl activation and transesterification. The formation of FANHOH in the absence of added ATP when fatty acid incorporation into phospholipid is prevented may reflect the formation of acyl esters that are not involved in fatty acid incorporation into phospholipid. The observation that [^{14}C]FANHOH does not disappear completely following addition of excess fatty acid (Fig. 2) or during long term reincubation (Fig. 3) also suggests that not all acyl esters

derivatized as FANHOH are involved in transesterification of fatty acid into phospholipid.

Release of the fatty acid previously incorporated into phospholipid via the acyl-thioester intermediate was also observed in this study. It is possible that this release of FFA reflects the presence of endogenous phospholipase which can act upon endogenous phospholipids. Release of fatty acids incorporated into PC by acylation has been reported previously, but the release of fatty acid was dependent on the presence of a heat-labile serum factor, and was thought to involve transfer of fatty acid from PC to PE prior to release into the medium (8, 38). Paysant et al. (39) have detected endogenous phospholipase activity in human erythrocytes previously. Detection of this activity required prior treatment of the membranes with trypsin or detergent. Endogenous phospholipase A_2 activity has also been detected in sheep erythrocytes treated with Triton X-100 (40). However, the physiologic significance of these observations is unclear.

The release of FFA from erythrocytes and erythrocyte membranes observed in this study occurs in the absence of serum. The FFA which appears is that which had previously been incorporated into phospholipid. Transfer of fatty acid from PC to PE prior to release into the medium, in the manner described by Shohet for cells in serum (38), was not observed in this study with cells suspended in buffer. The release of FFA observed could represent the action of an endogenous phospholipase. However, it is not possible to exclude the possibility that the release of FFA reflects nonspecific, nonenzymatic hydrolysis of acyl thioester or phospholipid or release of tightly bound FFA or that it reflects release from multiple sources. Because the acyl groups are the only moieties of PC and PE which undergo turnover in the erythrocyte (42), it is not possible to label other portions of endogenous PC and PE so that formation of LPE and LPC can be detected. Measurement of these compounds would provide more conclusive evidence supporting the presence of an endogenous phospholipase in erythrocytes. It would also permit determination of the source of the FFA released.

Previous studies concerned with the relationship between phospholipid fatty acids and membrane function have examined the effects of major changes in phospholipid fatty acid composition on membrane physical properties and function (31–35). While these studies have provided significant insight into the relationship between membrane lipids and membrane function, it is unlikely that such major shifts in membrane phospholipid fatty acid composition occur in vivo. It is more likely that small changes in fatty acid turnover in specific pools of phospholipid are involved in the regulation of the microenvironment of membrane proteins. Cellular phospholipases thought to be important in the regulation of membrane lipid composition have recently been demonstrated in mammalian cells in culture (41). We have recently shown that a selective stimulation of fatty acid incorporation accompanies decreases in erythrocyte volume induced by treatment with A23187, nigericin, or hypertonic buffer (42).[3] Thus, it is apparent that small changes in fatty acid turnover accompany changes in membrane function and morphology, and that the acylation-deacylation pathway for regulation of phospholipid fatty acid composition in situ may be an important component in the endogenous regulation of membrane function. The erythrocyte provides a simple system for examining the relationship of these changes in membrane function to membrane phospholipid fatty acid turnover. Utilizing the information obtained in this study that extends the characterization of the acylation-deacylation pathway, it should be possible to further elucidate this relationship.

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