The deacylation and reacylation process of phospholipids is the major pathway of turnover and repair in erythrocyte membranes. In this paper, we have investigated the role of carnitine palmitoyltransferase in erythrocyte membrane phospholipid fatty acid turnover. We have recently shown that phospholipid reacylation in intact human erythrocytes is confined to the deacylation of membrane phospholipids and their subsequent reacylation: the Land cycle (1-5). Phospholipid turnover can be studied both in intact erythrocyte and its isolated membrane, suggesting that the enzymes involved in the fatty acid turnover of membrane phospholipids are located in the membrane fraction. Thus, after a very fast nonmediated uptake of the fatty acid into the erythrocyte membrane from plasma albumin (6), a long chain acyl-CoA synthetase (EC 6.2.1.3) is required for the esterification of the fatty acid to CoA (1, 3, 7-9), since the true substrate for the reacylation step of lysophospholipid, catalyzed by acyl-CoA lyso phospholipid acyltransferase(s) (4, 10), is acyl-CoA. In addition, it has been shown that such enzymes use substrates from the inner hemileaf of the erythrocyte membrane (11) and that the reacylating enzyme is mainly expressed at the second position of the glycerol backbone of membrane phospholipids (12). In this sequence of events an endogenous phospholipase A2 activity provides the lysophospholipids (5). We have recently shown that phospholipid reacylation in erythrocyte membrane ghosts can also occur by using palmitoyl-CoA synthetase and the reacylating enzymes for palmitate and oleate, which support the importance of carnitine palmitoyltransferase in modulating the optimal acyl-CoA/free CoA ratio for the physiological expression of the membrane phospholipids fatty acid turnover.

The major metabolic pathway for membrane phospholipid fatty acid turnover in human erythrocytes is confined to the deacylation of membrane phospholipids and their subsequent reacylation: the Land cycle (1-5). Phospholipid turnover can be studied both in intact erythrocyte and its isolated membrane, suggesting that the enzymes involved in the fatty acid turnover of membrane phospholipids are located in the membrane fraction. Thus, after a very fast nonmediated uptake of the fatty acid into the erythrocyte membrane from plasma albumin (6), a long chain acyl-CoA synthetase (EC 6.2.1.3) is required for the esterification of the fatty acid to CoA (1, 3, 7-9), since the true substrate for the reacylation step of lysophospholipid, catalyzed by acyl-CoA lyso phospholipid acyltransferase(s) (4, 10), is acyl-CoA. In addition, it has been shown that such enzymes use substrates from the inner hemileaf of the erythrocyte membrane (11) and that the reacylating enzyme is mainly expressed at the second position of the glycerol backbone of membrane phospholipids (12). In this sequence of events an endogenous phospholipase A2 activity provides the lysophospholipids (5).
tine (ALC), which may be used whenever the activation step of the fatty acid becomes the limiting factor for the resylation process of erythrocyte membrane phospholipids (13, 15). In fact, because of the near equilibrium condition in which erythrocyte carnitine palmitoyltransferase catalyzes the reversible transfer of the fatty acid from carnitine to CoA, the net flux of carnitine palmitoyltransferase reaction is governed by the mass action ratio of the substrates. In addition, a closer look at some functional properties of erythrocyte carnitine palmitoyltransferase revealed, interestingly, that the affinity of this enzyme for those fatty acids commonly found in red cell phospholipids is higher than for carnitine palmitoyltransferases from organelles such as peroxosomes, microsomes, and mitochondria (16). This latter finding further supports a role for carnitine palmitoyltransferase in membrane phospholipid fatty acid turnover.

In this paper we explore the role of carnitine palmitoyltransferase in relation to membrane phospholipid fatty acid turnover.

EXPERIMENTAL PROCEDURES

Preparation of Washed Erythrocytes and Ghosts—Venous human blood was collected daily from healthy volunteers into heparinized syringes, and samples were washed three times with incubation buffer (NaCl, 120 mM; KCl, 5 mM; MgSO4, 1 mM; NaH2PO4, 1 mM; saccharose, 40 mM; glucose, 5 mM; Tris-HCl, 1 mM; 2-mercaptoethanol, 5 mM; Na2EDTA, 1 mM; 10 mM, at pH 7.4) and utilized immediately for the experiments.

Incubation Conditions—All incubations were carried out in a Rotaphab shacking bath at 37°C with air as the gas phase. For experiments with intact cells, the isolated erythrocytes were once again washed with incubation buffer (NaCl, 120 mM; KCl, 5 mM; MgSO4, 1 mM; Na2HPO4, 1 mM; saccharose, 40 mM; glucose, 5 mM; Tris-HCl, 10 mM, at pH 7.4) and resuspended in the same buffer at a final hematocrit of 5%. Saccharose was added to the incubation buffer in order to prevent colloid-osmotic lysis of the cells. The reaction was started by adding erythrocytes to the incubation buffer containing [1-14C]palmitic acid and/or [9,10-3H]oleic acid complexed to fatty acid-free bovine serum albumin (1.65 mg/ml). In some experiments, erythrocytes were loaded with [1-14C]palmitoyl-L-carnitine essentially as described by Classen et al. (18). 200 µl of 50% suspension in incubation buffer were incubated for 2 min at 22°C in a 50-ml polypropylene tube containing 2 nmol of dry lipid probe (8). Then the cells were transferred in a tube, washed three times with incubation buffer, and incubated as indicated above. Incubations were ended by adding 1 ml 3-deoxy-D-glucose washed with the same buffer, and then incubated as previously described. ATP levels in control erythrocyte suspensions, which contained glucose, did not vary throughout the experiments. The residual erythrocyte ATP content was determined using the method by Adams (19).

The experiments with membrane ghosts were performed at a final protein concentration of 1.5 mg/ml in calcium-free Krebs-Ringer buffer containing CaO, 5 mM ATP, 1 mM [1-14C]palmitic acid and/or [9,10-3H]oleic acid, 10 µM complexed to fatty acid-free BSA (1.65 mg/ml). The reaction was terminated by adding 5 volumes of cold incubation buffer and washing several times with the same buffer at 4°C.

Protein concentration was determined according to Bradford (20).

Lipid Extraction and Phospholipid Isolation—Lipids were immediately extracted from intact cells and ghosts by the method of Rose and Oklander (21) with slight modification. In order to prevent lipid oxidation, 0.1% butylated hydroxytoluene was added to the lipid extracts. Lipid extracts were dried under nitrogen, redissolved in 1:1 chloroform/methanol, and applied to thin-layer chromatography plates reactivated previously in a oven at 150°C for 10 min.

Two-dimensional thin-layer chromatography was used to separate the individual phospholipid classes (22, 23). Briefly, the chromatograms were developed using chloroform/methanol/28% ammonia (65:25:5) in the first dimension and then they were dried with a flow of warm air until the solvent had completely evaporated, particularly ammonia. The chromatograms were then developed using chloroform/acetone/methanol/acidic acid/water (6:6:2:2:1) (5, 6) in the second dimension and allowed to dry in air. Phosphatidyicholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) were visualized by brief exposure of the plates to iodine and identified using standards as a reference.

Measurement of Radioactive Fatty Acid Incorporation into Phospholipids—Individual phospholipid spots were scraped off the thin-layer chromatography plate and extracted from silica gel with 1:1 chloroform/methanol. Aliquots were used for determination of lipid phosphorus content (24), and radioactivity was determined by liquid scintillation counting. Radioactive lipids were also visualized by autoradiography of the thin-layer chromatography plate. This procedure allowed us to identify PLC and oleoyl-L-carnitine (OLC) in the thin-layer chromatogram, since the iodine stain did not visualize them (see also "Results and Discussion"). In order to identify PLC and OLC in the thin-layer chromatograms, 0.1 mg of genuine PLC was added to the lipid extracts immediately before the application to the thin-layer chromatography plates (OLC comigrate with PLC). In this way, PLC and OLC were easily identifiable with iodine stain, scraped off the thin-layer chromatography plate, extracted from silica gel with 1:1 chloroform/methanol, and radioactivity was determined by liquid scintillation counting. Counting efficiency was evaluated by an external standard. Calculations are based on the specific activity of [1-14C]palmitic acid, [9,10-3H]oleic acid, and [1-14C]palmitoyl-L-carnitine.

Enzymatic Assays—Long chain acyl-CoA synthetase was assayed by the incorporation of incorporated [1-14C]palmitoyl-CoA into palmitol-CoA (25). The incubation contained 100 mM Tris-HCl, pH 8.0, 150 mM KCl, 1.6 mM Triton X-100, 5 mM dithioerythritol, 15 mM MgCl2, and 10 mM ATP. Controls contained no ATP. The samples were incubated at 35°C for 30 min.

Acyl-CoA lysophospholipid acyltransferase was assayed essentially as described by Momchilova-Pankova and Markovska (26). The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.5% BSA, 5 mM MgCl2, 0.5 mM ATP, 1 mM dithioerythritol, 100 µM [1-14C]palmitoyl-CoA (0.5 Ci/mmol), and 150 µM lysophosphatidylcholine. Each sample was compared with a control without lysophosphatidylcholine. Samples and controls without radiolabeled palmitoyl-CoA were incubated at 30°C for 15 min and then radiolabeled palmitol-CoA was added. After 30 min the reaction was stopped by the addition of chloroform/methanol (v/v, 1:1), and the phospholipids were extracted as described above.

Carnitine palmitoyltransferase was assayed in the forward direction by the incorporation of [1-14C]l-carnitine into PLC as before (16, 27). The palmitoyl-CoA concentration was 50 µM (with 1.3 mg/ml BSA) and l-carnitine was 1 mM. Both forward and reverse reaction were measured spectrophotometrically at 232 nm in 20 mM potassium phosphate buffer, pH 7.4, in the absence of BSA, against a blank with no l-carnitine or CoA as appropriate.

Statistics—Values are the average of three experiments done in duplicate. The variation between experiments was not more than 5%.

RESULTS AND DISCUSSION

Identification of ALC in the Course of the Resylation Study—Erythrocyte membrane phospholipid fatty acid turn-
over can be examined by following the incorporation of the radiolabeled fatty acid into phospholipid fractions of a red cell suspension previously exposed to BSA-radioactive fatty acid complex. Fig. 1 shows the iodine stain and autoradiography pattern of a two-dimensional TLC chromatogram of membrane lipids extracted from an erythrocyte suspension incubated for 60 min at 37 °C with [1-14C]palmitic acid complexed to fatty acid-free BSA. The iodine stain (Fig. 1A) visualizes the major phospholipid classes, PC, PE, PS, and sphingomyelin, plus the major neutral lipids such as fatty acids and cholesterol. A comparison of the iodine stain pattern with the autoradiography pattern (Fig. 1B) of the same TLC plate revealed some intriguing differences. A new intense radioactive spot in the area between PC and PS, not visible in the iodine stain pattern, was present. The chromatographic behavior of the compound associated with the new spot was identical to authentic PLC. The alkaline hydrolysis of this new radioactive spot in the TLC plate gives rise to radioactive palmitate (data not shown). It should be mentioned that since erythrocytes do not possess any metabolic activity toward fatty acids, except for the ability to transfer the acyl moiety to different acceptor molecules, such as CoA, carnitine, and phospholipids, the most likely radioactive acyl-derivative found in our autoradiogram would be PLC. In addition, the pretreatment of the erythrocyte suspension with TDGA, a compound considered to be a specific inhibitor of carnitine palmitoyltransferase (28), completely suppressed the formation of the radioactive spot of PLC. Moreover, leukocytes may be disregarded as a potential source of either radioactive PLC or fatty acid by-products, since light microscopy examination of our erythrocyte suspension, routinely purified with the cellulose column method, showed that no white cells were present.

Because of the amphiphilic chemical nature of PLC, we decided to evaluate the recovery of PLC into the lipid extract we usually obtained with the method of Rose and Oaklander (21). Such extraction procedure allowed us to recover more than 95% of the [1-14C]palmitoyl-L-carnitine into the final lipid extracts, when a known exogenous amount of radioactive PLC was added to the erythrocyte suspension prior lipid extraction.

The absence of a radioactive spot in the area related to sphingomyelin agrees well with the concept that this phospholipid does not undergo active fatty acid turnover in erythrocytes (3-5).

**Fatty Acid Incorporation into Membrane Phospholipids and ALC by Intact Erythrocytes**—Fig. 2 shows a typical time course of [1-14C]palmitic acid incorporation into membrane PC, PE, and ALC by a normal erythrocyte suspension incubated with [1-14C]palmitic acid complexed to fatty acid-free BSA. The largest fraction of the radiolabeled fatty acid is recovered into ALC. Between membrane phospholipids, the reacylation process seems to be slightly more active on PC than PE. The incorporation of radioactive palmitate into all these acyl acceptor molecules increases linearly with the time of incubation for at least 3 h. In contrast, it has been reported that the incorporation of radiolabeled fatty acid (palmitic or oleic acid) into erythrocyte long chain acyl-CoA follows a different kinetic pattern (5). After a brief initial linear increase of radiolabeled fatty acid incorporation into acyl-CoA (30 min), at rates comparable with those of fatty acid incorporation into membrane PC, acyl-CoA formation did not
change significantly up to 3 h (5).

The amount of red cell PLC formation suggests that carnitine palmitoyltransferase is buffering the acyl-CoA produced by long chain acyl-CoA synthetase activity. In other words, the rate of utilization of acyl-CoA by the membrane phospholipid reacylation system is lower than its production by long chain acyl-CoA synthetase, which could lead to an alteration of the acyl-CoA/free CoA ratio. In addition, given the low amount of total CoA (~1 µM) (29) present in the red cells in comparison with total carnitine (~30 µM) (30), ALC may represent a convenient and useful way to store acyl units. In fact, this sort of intra-erythrocyte acyl reservoir, together with a proper carnitine palmitoyltransferase activity, may become an important source of acyl units when the availability of acyl-CoA generated directly by long chain acyl-CoA synthetase becomes too low for the reacylation needs. Carnitine palmitoyltransferase, a well known membrane-associated organelle enzyme, catalyzes the reversible conversion of long chain acyl-CoA to long chain ALC, using the L-isomer of carnitine (31). This reaction is fundamental for the shuttling of long chain fatty acids for β-oxidation into the mitochondrial matrix (14), but the enzyme is also found in peroxisomes and microsomes where its role has not been established. Erythrocyte carnitine palmitoyltransferase is not involved either in the fatty acid transport across the membrane, since the translocation of fatty acid across membrane erythrocyte is most likely a fast simple diffusive flip flop process (6), or in the oxidation of fatty acids, since mitochondria are absent.

When an erythrocyte suspension was incubated with equimolar concentrations of [9,10-3H]oleic acid and [1-14C]palmitic acid complexed to fatty acid-free BSA, differences in the incorporation rates of the radiolabeled fatty acids into membrane phospholipids and ALC were observed (Fig. 3). Thus, after 1 h of incubation PC and PE seemed to incorporate 22 and 38% more oleate than palmitate, respectively. On the contrary, the incorporation of palmitate into ALC was 42% more than for oleate. This competition study offers additional clues on the potential modulatory activity of carnitine palmitoyltransferase on erythrocyte membrane phospholipid fatty acid turnover. In fact, the lower palmitate incorporation into phospholipids fits well with the higher PLC formation, since oleate appears to be the preferred substrate for the reacylation process. As expected, OLC production was lower than that of PLC. Our data, thus, suggest that erythrocyte carnitine palmitoyltransferase is a potential modulator of the acyl trafficking between the fatty acid activ

![Fig. 3. [1-14C]Palmitic and [9,10-3H]oleic acid incorporation into human erythrocyte membrane phospholipids and ALC. Erythrocyte suspensions were incubated at 37 °C with an equimolar concentration of [1-14C]palmitic and [9,10-3H]oleic acid (10 µM each) complexed to fatty acid-free BSA (1.05 mg/ml). After 60 min of incubation at 37 °C, erythrocytes were washed, extracted, and chromatographed as described under "Experimental Procedures." Results are expressed as in Fig. 1.](image-url)

We have shown that erythrocyte membrane ghosts are able to utilize PLC as a source of fatty acid for the reacylation activity of membrane phospholipids in the absence of ATP (13). It was also shown that the mediator of the ATP-independent reacylating activity was carnitine palmitoyltransferase. These findings prompted us to test whether the incubation of an intact erythrocyte suspension with [1-14C]palmitoyl-L-carnitine was followed by the appearance of radio-labeled palmitic acid into membrane phospholipids. The result of this experiment is shown in Fig. 4, where radiolabeled fatty acid was indeed found in both PC and PE. The extent of palmitic acid incorporation into PC and PE was linearly correlated with the time of incubation, although PC contained

![Fig. 4. [1-14C]Palmitic acid incorporation into human erythrocyte membrane phospholipids. Erythrocytes were loaded with [1-14C]palmitoyl-L-carnitine, as described under "Experimental Procedures," and incubated at 37 °C. At fixed times, aliquots were removed and processed as described under "Experimental Procedures." Results are expressed as in Fig. 1.](image-url)

Effect of 2-deoxy-glucose on [1-14C]palmitic acid incorporation into ALC and ATP production in intact human red blood cells. Erythrocytes were pretreated with 1 mM 2-deoxyglucose, in the absence of glucose in the incubation buffer. At fixed times of the pretreatment, the cells were washed and reincubated for 60 min at 37 °C with [1-14C]palmitic acid complexed to fatty acid-free BSA. At the end of the incubation, an aliquot of the red cell suspension was used for ATP determination, and another aliquot was processed as reported under "Experimental Procedures" for the reacylation study. The abscissa shows the pretreatment times with the ATP-depletor, whereas the ordinate shows the percentage of ATP and radiolabeled fatty acid incorporation into ALC with respect to untreated red cells. In the inset is plotted the ATP concentration versus [1-14C]palmitoyl-L-carnitine formation (as expressed in Fig. 2). The line in the inset represents a linear fit of the ATP and [1-14C] palmitoyl-L-carnitine formation values, and the r is the correlation coefficient.
with 1 mM ATP-depleting agent for 3 h at 37 °C. Red cells were washed and reincubated with [1-14C]palmitic acid complexed to fatty acid-free BSA. Determinations of radiolabeled fatty acid incorporation into ALC were carried out as described in Fig. 2. Results are expressed as in Fig. 2. CPT, carnitine palmitoyltransferase; Cn, L-carnitine.

Erythrocytes were pretreated with 2-deoxy-glucose, and at fixed times aliquots were removed for the measurement of radiolabeled fatty acid incorporation rates into membrane PC, PE, and ALC. The PLC translocation process would then be the limiting step in terms of substrate availability for the fatty acid transfer into membrane phospholipids. Although, the difference in phospholipid reacylation rate still does not greatly vary either by using PLC or the BSA fatty acid complex, in comparison with the great difference in flip rate between PLC and free palmitate in erythrocyte membrane. However, one should also consider that another important limiting step of the reacylation process in intact erythrocytes is the activation of the free fatty acid, which may slow down the incorporation of the [1-14C]palmitic acid complex, in comparison with the great difference in flip rate between PLC and free palmitate in erythrocyte membrane. Furthermore, our preliminary measurements indicate that, for the substrate concentrations used, the reacylation step is the slowest, so that the effect of the faster initial access of the free fatty acid to the cell is not observed in the measured incorporation into phospholipids.

A specific and/or aspecific hydrolytic activity toward [1-14C]palmitoyl-l-carnitine, which could alternatively explain our findings through the release of labeled free fatty acid and its subsequent utilization via the combined activity of long chain acyl-CoA synthetase and the reacylating enzyme, may also be excluded: since (a) the commercial preparation of the labeled PLC in our buffer system is highly stable, (b) no potential erythrocyte hydrolyase activity toward PLC has ever been reported, (c) pretreatment of erythrocytes with TDGA completely suppressed radioactive palmitate incorporation into membrane PC and PE (data not shown).

Table I
Membrane phospholipid reacylation in TDGA-treated erythrocytes

| Time (h) | % of control Palmitate | % of control Oleate |
|---------|------------------------|---------------------|
| 2       | 98                     | 94                  |
|         | 55                     | 45                  |
| 4       | 96                     | 90                  |
|         | 62                     | 43                  |
| PC      | 98                     | 94                  |
| PE      | 96                     | 90                  |
| ALC     | 7                      | 9                   |
|         | 8                      | 8                   |

Fig. 6. Effect of 2-deoxy-glucose on [1-14C]palmitic acid incorporation kinetic into ALC. Erythrocytes were pretreated with 1 mM ATP-depleting agent for 3 h at 37 °C. Red cells were washed and reincubated with [1-14C]palmitic acid complexed to fatty acid-free BSA. Determinations of radiolabeled fatty acid incorporation into ALC were carried out as described in Fig. 2. Results are expressed as percentage of inhibition over the rate of untreated incorporation. The inset shows the effect of PDC pretreatment on the [9,10-3H]oleic acid incorporation into ALC in intact erythrocytes. The pretreatment time with PDC is reported on the abscissa.

Fig. 7. Membrane phospholipid incorporation study in ATP depleted erythrocytes by using [1-14C]palmitoyl-l-carnitine as a substrate. Erythrocytes were loaded with the radiolabeled fatty ALC as described under “Experimental Procedures.” Immediately after, [1-14C]palmitoyl-l-carnitine-loaded red cells were incubated with 2-deoxy-glucose, and at fixed times aliquots were removed for the measurement of radiolabeled fatty acid incorporation rates into membrane PC and PE and ATP determinations.

Fig. 8. Effect of PDC on [9,10-3H]oleic acid incorporation into ALC and membrane phospholipids. Erythrocytes were pretreated with 20 μM PDC for 6 and 12 h at 37 °C. Red cells were then washed twice with defatted BSA and reincubated with [9,10-3H]oleic acid complexed to fatty acid-free BSA for 60 min at 37 °C. Determinations of radiolabeled fatty acid incorporation into ALC, and membrane phospholipids were carried out as described in the legend of Fig. 2. Results are expressed as the percentage of inhibition over the rate of untreated incorporation. The inset shows the effect of PDC pretreatment on the [9,10-3H]oleic acid incorporation into ALC in intact erythrocytes. The pretreatment time with PDC is reported on the abscissa. At the end of the PDC treatment, radiolabeled fatty acid incorporation into ALC was carried out as reported above.
Carnitine Palmitoyltransferase Studies in Erythrocyte Membrane Ghosts—In a previous study, we have observed that some functional and molecular properties of the erythrocyte membrane carnitine palmitoyltransferase were very similar to those for the carnitine palmitoyltransferase activities present in microsomal, peroxisomal, and mitochondrial inner membranes, in particular the inhibition by malonyl-CoA and by 2-bromopalmitoyl-CoA in the presence of L-carnitine (16). In contrast, the solubilization by and stability of the carnitine palmitoyltransferase in detergents differed from the intracellular forms, and no cross-reaction was detected with the available antisera (peroxisomal and mitochondrial inner membrane forms) of carnitine palmitoyltransferase. In addition, the specificity for acyl-CoA substrates was quite different: the organelle forms of carnitine palmitoyltransferase have very low activity with acyl-CoA derivatives longer than palmitoyl-CoA, but erythrocyte carnitine palmitoyltransferase had good activity with the steareoyl-, linoleoyl-, arachidonyl-, and erucroyl-CoA derivatives (16).

In order to establish that erythrocyte carnitine palmitoyltransferase, like the other carnitine acyltransferases, is catalytically competent in both directions, we measured the forward and reverse reactions (Reactions 1 and 2, respectively) under the same conditions.

\[
Palmityl-CoA + L-carnitine \rightarrow \text{palmitoyl-L-carnitine} + \text{CoA (forward)} \quad (1)
\]

\[
Palmityl-L-carnitine + \text{CoA} \rightarrow \text{palmitoyl-CoA} + \text{L-carnitine (reverse)} \quad (2)
\]

Although the rates obtained cannot be compared directly with those for reacylation, the results demonstrate that the properties of the enzyme are compatible with a freely reversible flux, the direction of which is determined by the mass action ratio of the reactants. In the absence of BSA, the 229-nm spectrophotometric assay for the thioester bond yielded rates of 0.32 nmol/min/mg for the forward reaction with 50 \( \mu \text{M} \) palmitoyl-CoA and 1 mM L-carnitine and 0.65 nmol/min/mg for the reverse reaction with 50 \( \mu \text{M} \) PLC and 0.2 mM CoA. In one experiment, the equilibrium constant for the ghost carnitine palmitoyltransferase reaction was found to be 0.5, very similar to that determined for carnitine acyltransferase (32). Taken together these data support the proposed role of carnitine palmitoyltransferase in facilitating the turnover of the fatty acid moieties of the lipids by means of buffering the equilibrium between the acyl-CoA and free CoA pools.

In another series of experiments, we evaluated the contribution of contaminating reticulocytes to the total carnitine palmitoyltransferase activity in membrane preparations from reticulocyte-rich and poor fractions obtained by centrifuging the erythrocyte suspension over a discontinuous gradient of Dextran 40 (33). Four different fractions are obtained of which the first one, that contains the majority of younger erythrocytes and reticulocytes, and last one, that contains the majority of older erythrocytes and almost no reticulocytes, were used for carnitine palmitoyltransferase and cytochrome-c oxidase assays (16, 27, 34) in the membrane fraction (the latter enzyme is a true mitochondrial membrane associated protein). Cytochrome-c oxidase and carnitine palmitoyltransferase specific activities in fraction I were 2.21 and 0.68 nmol/min/mg of protein, respectively, and in fraction IV were 0.65 and 0.40 nmol/min/mg of protein, respectively. The fraction I/fraction IV ratios for cytochrome-c oxidase and carnitine palmitoyltransferase are 3.4 and 1.7. Thus, cytochrome-c oxidase activity is significantly more diluted (two times) than carnitine palmitoyltransferase activity in the reticulocyte-poor fraction, suggesting that an additional source of carnitine palmitoyltransferase has to be considered in this fraction, besides the potential mitochondrial one derived from the contaminant reticulocytes. Malonyl-CoA-sensitive carnitine palmitoyltransferases are found in three locations in liver cells, and these all appear to be different enzymes. On the outer face of mitochondria, carnitine palmitoyltransferase converts activated acyl groups to ALC for import into the mitochondrion. In peroxisomes (27) and in microsomes (35) the role of the enzyme is less clear, but is likely to be a mechanism to modulate the acyl-CoA/free CoA ratio without losing the energy expended in activating the acyl group. The large pool of intracellular carnitine is a suitable reservoir for these groups. In rat liver cells peroxisomes contribute about 12% of the total malonyl-CoA-sensitive carnitine palmitoyltransferase activity, microsomes 21% and mitochondria 67%. Since circulating reticulocytes are very poor in peroxisomes and microsomes, and we have demonstrated that mitochondrial marker enzymes are decreased at least 2-fold more than in carnitine palmitoyltransferase in our preparation, it is possible that this enzyme is indeed a component of the plasma membrane as are the acyl-CoA synthetase and the lysophospholipid acyltransferase.

Fatty Acid Incorporation into ALC by Intact ATP-depleted Erythrocytes—An important aspect of the potential carnitine palmitoyltransferase modulatory role on the intra-erythrocyte acyl trafficking would be to provide activated acyl units for the reacylation process under conditions where the cellular ATP generation has decreased. This would be mainly because long chain acyl-CoA synthetase, the enzyme involved in the activation step of fatty acids, requires ATP. Besides any potential physiopathological implications, the ATP-depletion studies may be considered as an additional experimental tool to improve the understanding of carnitine palmitoyltransferase function in membrane phospholipid fatty acid turnover.

Erythrocyte suspensions were pretreated with 2-deoxyglucose, a well known and specific ATP depletor. At different times of such pretreatment, aliquots were taken, washed, and reincubated for 60 min with [1-14C]palmitic acid complexed to fatty acid-free BSA. In this experimental approach, one may predict that, depending on the ATP content, which in turn would affect the acyl-CoA formation, the equilibrium of carnitine palmitoyltransferase would be shifted toward acyl-CoA, with the practical consequence of a decreased PLC formation. In other words, low acyl-CoA favors the transfer of acyl groups from ALC to free CoA rather than the opposite reaction. As expected, Fig. 5 clearly shows that the decrease of ATP generation is paralleled by a reduction of labeled palmitate incorporation into ALC. The ATP content and radioactive PLC production are well correlated (inset in Fig. 5). Even when ATP-depleted red cells were incubated with equimolar concentration of [9,10-3H]oleic acid and [1-14C]palmitic acid complexed to fatty acid-free BSA, a decreased incorporation of both radioactive fatty acids into ALC was observed (data not shown). Fig. 6 shows a time-dependent PLC production study, in which the radiolebered palmitate-BSA complex was added to the incubation mixture when the ATP erythrocyte content was below 30% of the control. The ATP-depleted cells showed a marked reduction of labeled PLC formation, which ceased completely after 60 min of incubation. In agreement with the mass action ratio of the substrates, acyl group transfer from acyl-CoA to carnitine predominates in the control resulting in ongoing formation of ALC (Fig. 6). In contrast, in the ATP-depleted cells, when

---

1 R. R. Ramsay, unpublished data.
the acyl-CoA pool decreases, acyl group transfer from ALC to CoA becomes more favorable.

In another series of experiments, we have evaluated the effect of ATP depletion on the erythrocyte membrane phospholipid fatty acid turnover. A reduction of ATP content down to 26% did not affect palmitate incorporation into PC and PE (data not shown). The observation that fatty acid incorporation into PC and PE is not altered even by a drastic reduction of ATP levels agrees well with the concept of a carnitine palmitoyltransferase activity sensitive to the mass action ratio of the reactants. When the "reverse" reaction (i.e., acyl-CoA formation) is favored, as in the ATP-depleted cells, the reversible carnitine palmitoyltransferase reaction is functioning to keep membrane phospholipid reacylation rates as physiological as possible. In other words, the undesirable incorporation of acyl groups into ALC would be a deterrent for the reacylation process, since carnitine palmitoyltransferase would compete with the reacylating enzyme(s) for acyl-CoA utilization. In keeping with such a competition for acyl groups, McLeod and Bressler (36) reported that high concentrations of carnitine inhibited the incorporation of free fatty acids into lipids in erythrocyte ghosts.

A more direct demonstration that the carnitine palmitoyltransferase reaction in the ATP-depleted red cells is shifted toward net acyl-CoA formation was obtained by following the incorporation of radioactive palmitate into PC and PE in a red cells suspension incubated with [1-14C]palmitoyl-L-carnitine in the presence of the ATP depletor. Given our rationale, an increase of palmitate esterification into membrane phospholipids in the ATP-depleted cells may be anticipated, since according to the mass action ratio of substrates, the decrease of acyl-CoA availability, as a result of the lowered ATP levels, would further shift the equilibrium of carnitine palmitoyltransferase toward acyl-CoA with respect to those cells with physiological ATP levels (see also reactions reported in Fig. 6). This was the case: erythrocyte membrane PC and PE accumulated more radioactive palmitate in inverse proportion to the ATP content (Fig. 7). It has been reported that the interleaflet transfer (flip rate) of PLC is unaffected by ATP depletion (18), so that the observed increase of palmitate incorporation into PC and PE is not due to an enhanced internal supply of PLC. Furthermore, all experiments used the same donors so that individual variation in the flip rate was eliminated.

Effect of Carnitine Palmitoyltransferase Inhibitors on Erythrocyte Membrane Phospholipid Reacylation—An appealing experimental approach to further corroborate that carnitine palmitoyltransferase may actively participate in the fatty acid turnover of membrane phospholipids, would be to inhibit carnitine palmitoyltransferase specifically. In our opinion the inhibition of carnitine palmitoyltransferase would lead to an alteration of the acyl-CoA/free CoA ratio, which in turn may affect the membrane phospholipid fatty acid turnover. This assumption is based on two important observations. 1) In a membrane ghost reacylation study, we have demonstrated that relatively low concentration of added free CoA (50 μM) produced a significant inhibition of fatty acid incorporation into erythrocyte membrane phospholipids (13). Similarly, Mok and McMurray (37) have found that rat liver glycerophosphate acyltransferase activity is severely depressed by high concentrations of free CoA. Taken together these data suggest that free CoA may act as an inhibitor of the lysophospholipid acyltransferases. 2) The relative acyl preference of human erythrocyte long chain acyl-CoA synthetase for the long chain fatty acids, palmitate and oleate, is not very dissimilar (8.58 and 11.78 nmol/min/mg of protein, respectively) (9). However, the difference in specificity for the transfer of the fatty acid from palmitoyl-CoA and oleoyl-CoA into human erythrocyte PC is large (2.0 and 5.3 nmol/min/mg of protein, respectively) (10). In addition, the range of the rates of incorporation reported for those long chain fatty acids commonly present in erythrocyte membrane phospholipids for long chain acyl-CoA synthetase is 8.58-12.21 and for the reacylating enzyme is 0.7-14.00 nmol/min/mg of protein (9, 10). Differences in rates and substrate specificities between long chain acyl-CoA synthetase and reacylating enzyme may potentially alter the acyl-CoA/free CoA ratio, if the buffering activity of carnitine palmitoyltransferase for these substrates is missing. For example, the higher rates of the reacylating enzyme for oleoyl-CoA with respect to palmitoyl-CoA would cause a relative increase of the endogenous free CoA when the erythrocytes are utilizing oleate as the substrate for the reacylation of membrane phospholipids (Scheme 1). Carnitine palmitoyltransferase would buffer the potential increase of the free CoA by shifting its equilibrium toward oleoyl-CoA. In other words, carnitine palmitoyltransferase may represent a sort of necessary molecular capacitor for a proper handling of activated fatty acids in membrane phospholipid reacylation.

Several carnitine palmitoyltransferase inhibitors, such as 2-bromo-palmitoyl-CoA, palmitoyl-d-carnitine, amino-carnitine, and TDGA, are available. However, due to the erythrocyte membrane impermeability to some of them (2-bromo-palmitoyl-CoA, amino-carnitine), we decided to explore the effect of TDGA and palmitoyl-d-carnitine on membrane phospholipid reacylation in intact erythrocytes.

TDGA is considered a specific irreversible inhibitor of long chain fatty acid oxidation at the carnitine palmitoyltransferase I site (28, 31). However, we did some preliminary experiments in order to evaluate whether TDGA interfered with the activation of the fatty acid and its subsequent utilization in

---

**Scheme 1. Proposed pathway for the involvement of carnitine palmitoyltransferase in the membrane phospholipid fatty acid turnover.**

**A**
- Without carnitine palmitoyltransferase inhibitor; A, after pretreatment with carnitine palmitoyltransferase inhibitors. Abbreviations used are: ACS, acyl-CoA synthetase; CPT, carnitine palmitoyltransferase; LAT, lysophospholipid acyl-CoA transferase; PLPs, phospholipids; POC, phospholipid; PL, phospholipid; Acyl-Ch, acyl-L-carnitine; Cn, carnitine; FFA, free fatty acid.

---

**B**
- With TDGA and palmitoyl-D-carnitine inhibitor.
the reacylation process. Concomitantly, we looked for the optimal concentration of TDGA to inhibit erythrocyte carnitine palmitoyltransferase. We found that incubation with 15 μM TDGA for 1 h at 37°C resulted in a complete loss of carnitine palmitoyltransferase activity measured in erythrocyte ghosts (data not shown). Under these experimental conditions, long chain acyl-CoA synthetase and acyl-CoA lysophospholipid acyltransferase activities were not affected. Table I shows the [1-14C]palmitic and [9,10-3H]oleic acid incorporation into membrane PC and PE and into ALC in TDGA-treated intact erythrocytes. Experimental conditions were the same as reported above, with the difference that after the TDGA treatment, erythrocytes were washed twice with isotonic buffer containing defatted BSA and then reincubated with the BSA-radiolabeled fatty acid complex containing either palmitate or oleate. [1-14C]Palmitic acid incorporation into membrane PC and PE in TDGA-treated erythrocytes is only slightly decreased. However, when TDGA-treated erythrocytes were incubated with [9,10-3H]oleic acid complexed to fatty acid-free BSA, membrane phospholipid reacylation showed a significant reduction of radiolabeled oleate incorporation into PC and PE in comparison to untreated erythrocytes. A possible explanation is that the higher rates of oleate utilization by the reacylating enzyme may result in an increased release of free CoA as a result of the activity of the reacylating enzyme. Carnitine palmitoyltransferase-inhibited red cells may tend to accumulate free CoA, which in turn may cause a feedback inhibition of acyl-CoA lysophospholipid acyltransferase (see also Scheme 1B). As expected, the incorporation of [1-14C]palmitic and [9,10-3H]oleic acid into ALC was almost completely abolished in TDGA-treated erythrocytes (Table I).

Palmitoyl-t-carnitine (PDC) is a well known reversible inhibitor of carnitine palmitoyltransferase which also inhibits erythrocyte carnitine palmitoyltransferase (38). Erythrocyte suspensions were pretreated with PDC, and at different times of such pretreatment, aliquots were taken, washed with defatted BSA, and reincubated for 60 min with [9,10-3H]oleic acid complexed to fatty acid-free BSA. Preliminary experiments have shown that in order to inhibit the formation of PLC in intact PDC-pretreated erythrocytes, long preincubation times with the inhibitor are required. In addition, to avoid any potential perturbing effect of PDC on the erythrocyte membrane, the incubations were carried out in the presence of defatted BSA. Under our experimental conditions no hemolysis was observed. Prior to the incubation of the erythrocyte suspension with the BSA-radiolabeled fatty acid complex, erythrocytes were washed at least two times with defatted BSA after the PDC pretreatment. This procedure will remove PDC present on the outer hemileaflet of the erythrocyte membrane (data not shown).

Fig. 8 shows that after 6 h of preincubation of an intact erythrocyte suspension with PDC, [9,10-3H]oleic acid incorporation into membrane PC and ALC is reduced with respect to untreated cells. An increase of the length of the PDC preincubation leads to a further decrease of [9,10-3H]oleic acid incorporation into PC and OLC. In addition, a slight decrease of fatty acid incorporation into PE was observed. The inhibitory pattern of these data are in good agreement with those obtained with TDGA. The inset of Fig. 8 shows the formation of radiolabeled OLC measured after exposure of the red blood cell suspension to PDC at various times. The fact that to observe a consistent reduction of radiolabeled PLC relatively long PDC incubation times are needed is in agreement with the very low translocation rate of long chain ALC through the erythrocyte membrane bilayer (18). It should be pointed out that in a control experiment, PDC up to 60 μM (twice as much as the intact cells experiments) did not affect the reacylation of membrane phospholipids measured in erythrocyte ghosts (see “Experimental Procedures” for experimental details), which suggests that PDC does not inhibit either long chain acyl-CoA synthetase or acyl-CoA-lysophospholipid acyltransferase activities.

Conclusions—Our results suggest that carnitine palmitoyltransferase may cooperate with the reacylation process of membrane phospholipids by modulating the size of the acyl-CoA/free CoA pool which lies between the activation step of the fatty acid and its transfer into lysophospholipids (Scheme 1). A consequence of our hypothesis is that long chain ALCs may represent an ATP-independent acyl reservoir for the reacylation process. The flux toward acyl-CoA would also prevent the potential harmful elevation of free CoA, under those conditions where the reacylating activity rate becomes higher than the acyl-CoA synthetase rate. In other words, the differences in rates between the enzyme devoted to the activation of the fatty acid and the one responsible for the reacylation of membrane phospholipids may be the cause of the alteration of the acyl-CoA/free CoA ratio, which in turn would affect the reacylation process, unless the cell can compensate for the alteration by means of the activity of carnitine palmitoyltransferase. The concept of a carnitine palmitoyltransferase participating in the acyl-trafficking control in the membrane phospholipid fatty acid turnover may be extended to more complex mammalian cells, where the remodelling activity of membrane phospholipids represents an important mechanism for membrane fluidity alteration (39) and formation of bioactive phospholipids (40). Finally, our findings propose for the first time that carnitine palmitoyltransferase, in addition to the well known role in the fatty acid oxidation, may be implicated in the lipid biosynthetic pathway.

Acknowledgments—We thank Dr. J. Kerner for the helpful discussion throughout the preparation of the manuscript.

REFERENCES
1. Mulder, E., and Van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 106–117
2. Mulder, E., and Van Deenen, L. L. M. (1965) Biochim. Biophys. Acta 106, 348–356
3. Shoet, S. B., Nathan, D. G., and Karnovsky, M. L. (1968) J. Clin. Invest. 47, 1096–1108
4. Lands, W. E. M., and Crawford, C. G. (1976) in The Enzymes of the Biological Membranes (Martonosi, A., ed) Vol. 2, pp. 3–85, Plenum Press Publishing, New York
5. Diee, C. A., Goodman, D. B. P., and Rasmussen, H. (1980) J. Lipid Res. 21, 292–300
6. Brotting, K., Haest, C. W. M., and Deuticke, B. (1988) Biochim. Biophys. Acta 986, 321–331
7. Robertson, A. P., and Lands, W. E. M. (1964) J. Lipid Res. 5, 88–93
8. Oliveira, M. M., and Vaughn, M. (1964) J. Lipid Res. 5, 156–162
9. Davidson, B. C., and Cantrill (1985) FEBS Lett. 193, 69–74
10. Waku, K., and Lands, W. E. M. (1968) J. Lipid Res. 9, 12–18
11. Renooji, W., Van Golde, L. M. G., Zwaal, R. F. A., Roelofsen, B., and Van Deenen, L. L. M. (1974) Biochim. Biophys. Acta 363, 287–292
12. Oliveira, M. E., and Vaughn, M. (1964) J. Lipid Res. 5, 156–162
13. Arduini, A., Mancinelli, G., and Ramsay, R. R. (1990) Biochem. Biophys. Res. Commun. 173, 212–217
14. Bremer, J. (1983) Physiol. Rev. 63, 1429–1480
15. Arduini, A. (1992) Am. Heart J. 123, 1729–1727
16. Ramsay, R. R., Mancinelli, G., and Arduini, A. (1991) Biochem. J. 275, 685–688
Membrane Phospholipid Reacylation and Carnitine Palmitoyltransferase

17. Beutler, E., West, C., and Blume, K. G. (1976) J. Lab. Clin. Med. 88, 328-333
18. Classen, J., Deuticke, B., and Haest, C. W. M. (1989) J. Membr. Biol. 111, 169-178
19. Adams, H. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 539-543, Academic Press, New York
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
21. Rose, H. G., and Oklander, M. (1965) J. Lipid Res. 6, 428-431
22. Rouser, G., Kritohovsky, G., Yamamoto, A., Simon, G., Calli, C., and Bauman, A. J. (1969) Methods Enzymol. 14, 272-317
23. Rouser, G., Fleisher, S., and Yamamoto, A. (1970) Lipids 5, 494-496
24. Bottcher, C. J. F., Van Gent, C. M., and Pries, C. (1961) Anal. Chim. Acta 24, 203-208
25. Tanaka, T., Hosaka, K., and Numa, S. (1981) Methods Enzymol. 71, 334-341
26. Momchilova-Pankova, A. B., and Markovska, T. T. (1990) Biochimie 72, 863-866
27. Derrick, J. P., and Ramsay, R. R. (1989) Biochem. J. 262, 801-806
28. Tutwiler, G. F., Ho, W., and Mohrbacher, R. J. (1981) Methods Enzymol. 72, 533-551
29. Pennel, R. B. (1964) in The Red Blood Cell (Bishop, C., and Surgener, D. M., eds) pp. 29-60, Academic Press, New York
30. Cooper, M. B., Forte, C. A., and Jones, D. A. (1988) Biochim. Biophys. Acta 959, 100-105
31. Bieber, L. L. (1988) Annu. Rev. Biochem. 57, 261-283
32. Fritz, I. B., Schiltz, S. K., and Sreve, P. A. (1963) J. Biol. Chem. 238, 2509-2517
33. Kunimoto, M., Kunimitsu, K., and Takashi, M. (1988) Lipids 19, 443-448
34. Yonetani, T. (1967) Methods Enzymol. 10, 332-335
35. Lilly, K., Bugaisky, G. E., Umeda, P. K., and Bieber, L. L. (1990) Arch. Biochem. Biophys. 280, 167-174
36. McLeod, M. E., and Bressler, R. (1967) Biochim. Biophys. Acta 144, 391-396
37. Mok, A. Y. P., and McMurray, W. C. (1990) Biochem. Cell. Biol. 68, 1380-1392
38. Wittels, B., and Hochstein, P. (1967) J. Biol. Chem. 242, 126-130
39. Thompson, G. A. Jr., and Martin, C. E. (1984) in Physiology of Membrane Fluidity (Shinitzky, M., ed) Vol. 1, pp. 99-129, CRC Press, Boca Raton, FL
40. MacDonald, J. I. S., and Sprecher, H. (1991) Biochim. Biophys. Acta 1084, 105-121