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Palmitoyl-L-Carnitine, A Metabolic Intermediate Of The Fatty Acid Incorporation Pathway In Erythrocyte Membrane Phospholipids

Arduino Arduini[1]*, Giovanna Mancinelli[1] and Rona R. Ramsay[2,3]

[1]Istituto di Scienze Biochimiche, Universita' degli Studi "G. D'Annunzio", 66100 Chieti, Italy

[2]Department of Biochemistry and Biophysics, University of California, San Francisco, and
[3]Molecular Biology Division, Veterans Administration Medical Center, San Francisco, CA 94121

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In this paper we report that palmitoyl-L-carnitine can be a metabolic intermediate of the fatty acid incorporation pathway into erythrocyte membrane phosphatidylcholine, and phosphatidylethanolamine. Phospholipid acylation was evaluated by measuring the incorporation of radioactive [1-^{14}C]-palmitoyl-L-carnitine in membrane erythrocyte ghost phospholipids in the presence or absence of CoA. CoA highly stimulated the incorporation of [1-^{14}C]-palmitic acid into both the phospholipids examined, although the incorporation was also evident in the absence of added CoA. Incorporation of [1-^{14}C]-palmitic acid into phosphatidylcholine was greater than into phosphatidylethanolamine. 2-Bromo-palmitoyl-CoA, an irreversible inhibitor of the erythrocyte carnitine palmitoyltransferase, inhibited the acylation process.

The role of carnitine and its acyl-esters in erythroeytes is still obscure, despite the fact that erythrocytes contain relatively high amount of these compounds [1]. It has also been demonstrated that the erythrocyte membrane contains carnitine palmitoyltransferase which is involved in the translocation of the activated long chain fatty acyl groups across intracellular biological membranes [2,3]. This enzyme, however, does not seem to be involved in the transfer of fatty acids through the erythrocyte membrane, a process which it has been widely investigated particularly with respect to the phospholipid fatty acid turnover [4-7]. Since erythrocytes do not contain any specialized organelles, the turnover of its phospholipid fatty acids is mainly confined to deacylation of membrane phospholipids and subsequent acylation of the lyso-derivatives. The incorporation of fatty acids into erythrocyte membrane phospholipids occurs through two major steps. 1) Esterification of the fatty acid to coenzyme A in an ATP-dependent reaction catalyzed by acyl-CoA ligase, and 2) transesterification of the acyl group from the acyl-CoA to a lysophospholipid by a specific acyltransferase [5,7-9].

In this study, we report that the incorporation of fatty acids into erythrocyte membrane phosphatidylcholine (PC), and phosphatidylethanolamine (PE) may also take place in the absence of ATP. Our study suggests that palmitoyl-L-carnitine and other long chain acylcarnitine derivitives may represent an additional pool of intracellular fatty acids available for the reacylation of phospholipids and that carnitine palmitoyltransferase mediates this process.

* To whom correspondence should be addressed.

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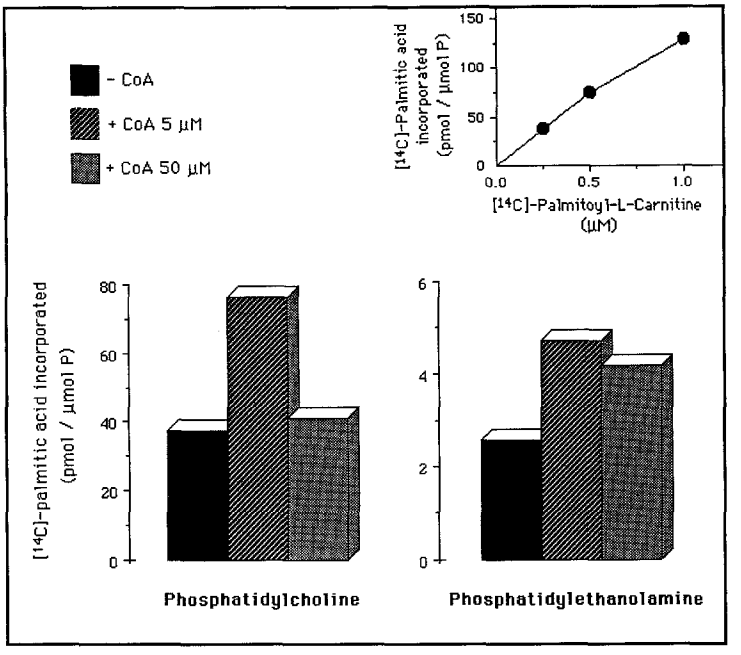

Figure 1. Palmitoyl-L-carnitine dependent acylation of erythrocyte membrane PC and PE. Erythrocyte membrane ghosts were incubated with [1-^{14}C]-palmitoyl-L-carnitine in the absence or in the presence of CoA. Incorporation of [1-^{14}C]-palmitic acid into erythrocyte membrane PC and PE was measured as described in the Materials and Methods section. In the inset is reported the incorporation of [1-^{14}C]-palmitic acid into erythrocyte membrane PC at different concentrations of [1-^{14}C]-palmitoyl-L-carnitine in the presence of 5 uM CoA. The values are the average of three experiments. The variation between one experimental value to another was not more than 5 %. Results are expressed as pmol of [1-^{14}C]-palmitic acid incorporated/umol lipid phosphorus.

reacylation of phospholipids, although a possible direct transesterifiction of the acyl group from palmitoyl-carnitine to the lysophospholipid cannot be excluded. The presence of endogenous cofactor is supported by the finding that the ATP dependent incorporation of fatty acids into PC and PE in erythrocyte ghosts in the absence of CoA was nearly 50 % of that measured in the presence of added CoA [7; see also Fig. 2].

The enhancement of the incorporation of [1-^{14}C]-palmitic acid from [1-^{14}C]-palmitoyl-L-carnitine to PC and PE by CoA (Fig. 1) strongly suggests that a carnitine palmitoyltransferase has to be involved in the reacylation process. In other words, if we consider that the classic route for the incorporation of fatty acids into membrane phospholipids assumes palmitoyl-CoA as the physiological substrate for the acyl CoA-lysophospholipid acyltransferase, the acylation observed under our experimental conditions would likely proceed through the transfer of the acyl group of palmitoyl-carnitine to CoA (to form palmitoyl-CoA) catalyzed by the carnitine palmitoyltransferase, an enzyme present in the erythrocyte membrane [2,16]. It has been demonstrated that both erythrocyte and mitochondrial outer membrane carnitine palmitoyltransferase are inhibited by thiol compounds like glutathione and dithioerythrytol [16,17]. This data seems to be consonant with the involvement of carnitine palmitoyltransferase in the acylation of phospholipids since thiol agents, like glutathione and dithiothreitol significantly decreased the incorporation of the label into PC and PE when added to an incubation mixture containing [1-^{14}C]-palmitoyl-L-carnitine, CoA, and erythrocyte membrane ghosts

Materials and Methods

Preparation of Erythrocyte Membranes. Venous human blood from healthy volunteers was daily collected in heparin, and leukocytes and platelets were removed by passage through a column of α-cellulose and microcrystalline cellulose [10]. Erythrocytes were then washed three times with 0.9 % NaCl and ghost membranes were obtained by hypotonic lysis using 20 volumes of 5 mM phosphate buffer, 1mM EDTA, pH 7.4 at 4° C. The hemolysate was centrifuged at 4° C and 25,000 x g. The resulting ghosts were resuspended in ice-cold hypotonic buffer and this process was continued until the red cell membranes were free of hemoglobin. Protein concentration was determined according to Bradford [11].

Incubation, Lipid Extraction, and Phospholipid Isolation of Erythrocyte Membrane. Incubations were carried out in a Rotabath shaking water bath at 37 °C with air as the gas phase. The reaction was started by adding erythrocyte membranes, at a final concentration of 1.5 mg/ml, to the incubation buffer (calcium free Krebs-Ringer phosphate buffer at pH 7.4) containing either [1-^{14}C]-palmitic acid (58 Ci/mol) complexed to fatty acid free albumin (1.65 mg/ml) or [1-^{14}C]-palmitoyl-L-carnitine (58 Ci/mol). Other additions were ATP, CoA, L-carnitine, 2-bromo-palmitoyl-CoA, glutathione and dithiothreitol (see text). After 45 min, the reaction was terminated by adding 5 vol. of cold incubation buffer and washing several times with the same buffer at 4 °C. Lipids were immediately extracted by the method of Rose and Oklander with slight modifications [12]. 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 a thin-layer of silica gel (Whatman LK6). Thin layer plates were developed in chloroform-methanol-28% ammonia (65:25:1, v/v), dried, and then developed in chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1, v/v) to separate individual phospholipid classes [13,14]. PC and PS were visualized by brief exposure of the plates to iodine, and identified using standards as a reference.

Measurement of [1-^{14}C]-palmitic acid incorporation into PC and PE. Individual PC and PE spots were scraped out from the TLC plate and extracted with 1:1 chloroform/methanol. Aliquots were used for determination of lipid phosphorus [15] 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-^{14}C]-palmitic acid.

Results and Discussion

In the last three decades, many studies on the phospholipid metabolism of the erythrocyte membrane have been published. A major focus of these accounts has been the turnover of fatty acids in the membrane phospholipids where a deacylation/acylation cycle seems to be the predominant process [4-9]. Although the incorporation of fatty acid in the lysophospholipid moiety has been well characterized, little or no information is available on one type of fatty acid derivative, namely the acyl-carnitine, as potential metabolic intermediates of the acylation process. Figure 1 presents clear evidence that acyl-carnitine can act as a source of fatty acid moieties for the acylation of membrane phospholipids. The addition of [1-^{14}C]-palmitoyl-L-carnitine to an erythrocyte membrane ghost suspension in the presence of coenzyme A results in the incorporation of [1-^{14}C]-palmitic acid into PC, and PE. The incorporation is greater for PC than PE, and does not require ATP. The maximum rate of phospholipid acylation is obtained when the concentration of CoA is 5 μM, but a further increase of the CoA concentration to 50 μM resulted in a decrease of [1-^{14}C]-palmitic acid incorporation (Fig. 1). An increase in the concentration of [1-^{14}C]-palmitoyl-L-carnitine is paralleled by a linear increase of [1-^{14}C]-palmitic acid incorporation into PC (inset of Fig. 1).

As can be seen in fig. 1, it is possible to observe active incorporation of the fatty acid into PC and PE, even in the absence of coenzyme A. Since it is difficult to eliminate endogenous thiols without causing major changes in the membrane phospholipid and protein organization, it is likely that endogenous CoA remains in the preparation. This CoA and/or other thiols could participate in the

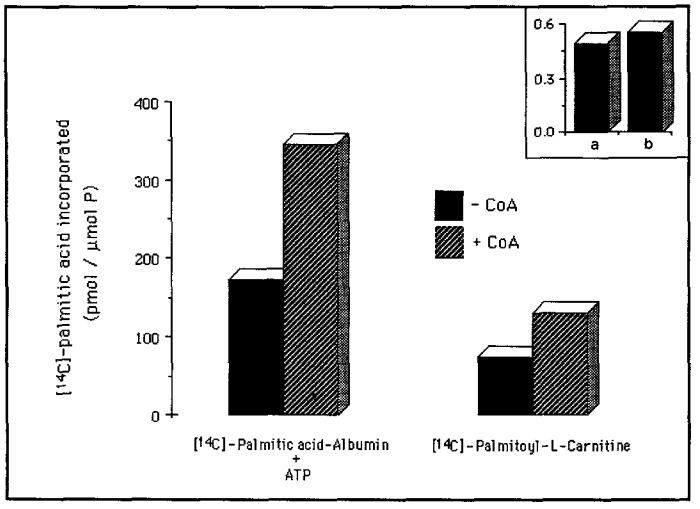

Figure 2. Comparison between the ATP and palmitoyl-L-carnitine dependent acylation system. The ATP dependent acylation of erythrocyte membrane PC (left bars) has been studied by measuring the incorporation of [1-^{14}C]-palmitic acid in an incubation mixture containing erythrocyte membranes, [1-^{14}C]-palmitic acid complexed to fatty acid free albumin and 1.5 mM ATP, in the absence or in the presence of 50 uM CoA. The palmitoyl-L-carnitine dependent acylation of erythrocyte membrane PC (right bars) has been studied by measuring the incorporation of [1-^{14}C]-palmitic acid in an incubation mixture containing erythrocyte membranes, [1-^{14}C]-palmitoyl-L-carnitine in the absence or in the presence of 5 uM CoA. In the inset is reported the acylation ratio between the samples incubated in the absence and presence of CoA in the ATP (a) and palmitoyl-L-carnitine (b) dependent acylation system. The values are the average of three experiments. The variation between one experimental value to another was not more than 5 %. Results are expressed as pmol of [1-^{14}C]-palmitic acid incorporated/umol lipid phosphorus.

(Table 1). The inhibition of PC and PE acylation observed at higher CoA concentrations (see above), may be due to similar effects from the free thiol group in CoA. To further substantiate the role of carnitine palmitoyltransferase, we have preincubated erythrocyte membrane ghosts with 1 mM L-

TABLE 1. Inhibition of palmitoyl-L-carnitine dependent acylation system in erythrocyte membranes*

Additions		Relative incorporation (%)
	µM	
None		100
Glutathione	$5 \cdot 10^3$	82
Dithiothreitol	$1 \cdot 10^3$	40
‡2-Bromo-palmitoyl-CoA	15	
+ L-carnitine	$1 \cdot 10^3$	4

* Incubation conditions and measurement of the incorporation of [1-^{14}C]-palmitic acid into erythrocyte membrane PC were carried out as described in the Materials and Methods section.

‡Erythrocyte membranes were preincubated with 2-bromo-palmitoyl-CoA and carnitine at 30 °C for 30 min in calcium free Krebs-Ringer phosphate buffer at pH 7.4. The reaction was terminated by adding 5 vol. of cold incubation buffer and the membranes were washed three times with the same buffer at 4 C°. Incubation conditions and measurement of the incorporation of [1-^{14}C]-palmitic acid into PC in this erythrocyte membrane preparation were carried out as described in the Materials and Methods section.

carnitine and 15 uM 2-bromo-palmitoyl-CoA, an irreversible inhibitor of the erythrocyte carnitine palmitoyltransferase [2,18,19]. 2-Bromo-palmitoyl-CoA treated ghosts, incubated with [1-^{14}C]-palmitoyl-L-carnitine and CoA, fail to incorporate [1-^{14}C]-palmitic acid into PC and PE (Table 1). This latter result demonstrates that erythrocyte carnitine palmitoyltransferase drives the acylation process in the absence of ATP further, it suggests that it is unlikely that direct incorporation of [1-^{14}C]-palmitic acid from [1-^{14}C]-palmitoyl-L-carnitine to phospholipids occurs.

Fig. 2 shows a comparison between the two acylation systems: one which uses as substrate [1-^{14}C]-palmitoyl-L-carnitine, and the other which requires ATP, since the substrate is the free [1-^{14}C]-palmitic acid complexed to albumin. The incorporation of [1-^{14}C]-palmitic acid into PC is 2.7 times higher in the acylation system requiring ATP than in the acylation system active with the acyl-derivative. As discussed previously, exogenous CoA is not an essential cofactor for both the reacylation systems. The acylation ratio between the samples incubated in the absence and presence of CoA is nearly the same in the two acylation systems so far examined (inset of Fig. 2), supporting the assumption that endogenous CoA is available and required for a step common to the two pathways. The greater incorporation observed in the acylation system requiring ATP may be explained by the low level of carnitine palmitoyltransferase in the erythrocyte [16]. In addition, the concentration of [1-^{14}C]-palmitoyl-L-carnitine was kept low (10 times less than [1-^{14}C]-palmitic acid complexed to albumin for the ATP-dependent acylation system) due to the well known toxic effects of the long chain acyl-derivatives on the integrity of erythrocyte lipid bilayer [20].

In conclusion, this is the first evidence that palmitoyl-L-carnitine as well as other long chain acyl-carnitines may be an intermediate in the reacylation process of phospholipids. The near equilibrium, reversible reaction catalyzed by carnitine palmitoyltransferase can buffer the pool of activated fatty acids, similar to the established role of carnitine acetyltransferase [21], and will guarantee optimal acyl-CoA concentrations to acylate lysophospholipids in all those conditions where the ATP generation by erythrocytes is decreased, i.e. glucose deprivation, oxidative stress.

Acknowledgments

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