Biochimica et Biophysica Acta, 1146 (1993) 229–235

BBAMEM 75892

Effects of L-carnitine and its acetate and propionate esters on the molecular dynamics of human erythrocyte membrane

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(Received 27 July 1992)
(Revised manuscript received 23 November 1992)

Key words: L-Carnitine; Acetyl-L-carnitine; Propionyl-L-carnitine; Molecular packing; Membrane fluidity; Erythrocyte

EPR and fluorescence probes were used in this study to define the effects of L-carnitine and its short-chain esters, acetyl-L-carnitine and propionyl-L-carnitine, on the natural fluidity gradient and molecular packing of phospholipid headgroups of erythrocyte membrane in intact cells. Purified erythrocyte suspensions, labeled with different stearic acid derivatives containing a stable doxyl radical ring at the C-5, C-7, C-12 and C-16, were incubated with 0.5–5 mM L-carnitine and its esters for 60 min at 37°C and washed twice with an isosmotic buffer. A decrease in the order parameter, calculated from the EPR spectra of the 5-doxylstearic acid derivative, was observed at all the concentrations of propionyl-L-carnitine and the extent of the decrease was dose and temperature dependent. An increase of the chain length between the doxyl ring and the carboxylic group of the spin label, resulted in a much lower efficacy of propionyl-L-carnitine in decreasing the order parameter. Acetyl-L-carnitine also showed a significant effect of decreasing the molecular order but only at the lower temperatures of red cells labeled with 5-doxyl and treated with the highest concentration of the drug. L-Carnitine did not modify the molecular dynamics at all the temperatures and concentrations used in this study. L-Carnitine and its short-chain derivatives did not alter significantly membrane fluidity of deeper regions of the erythrocyte membrane, measured by means of the excimer/monomer fluorescence intesity ratio of pyrene incorporated into the membrane of intact erythrocytes. However, these compounds were all capable of loosening the molecular packing of the polar head of erythrocyte membrane phospholipids evaluated by the membrane binding fluorescence properties of merocyanine-540. The binding of the fluorescent probe decreased in the order propionyl-L-carnitine > acetyl-L-carnitine > L-carnitine. Our findings suggest that this category of compounds affect the molecular dynamics of a membrane bilayer region close to the glycerol backbone of phospholipids, which might be relevant for the expression of membrane functions.

Introduction

Human erythrocytes represent a useful model for studying the effect of amphiphilic compounds on the physico-chemical status of biological membranes. In this context, it has been shown that palmitoyl-L-carnitine significantly affected the molecular dynamics of purified suspension of human erythrocytes, suggesting that the documented accumulation of this metabolic intermediate in the course of myocardial ischemia may lead to profound alteration of the membrane functions of myocytes [1]. The addition of 2 mM L-carnitine (LC) was able to normalize the membrane molecular dynamics of those erythrocyte exposed to palmitoyl-L-carnitine [2]. Haeyaert et al. [3] have also found that long-chain acylcarnitines decreased the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), an index of the lipid bilayer molecular dynamics, in pure and mixed phospholipid vesicles, and that this effect was dependent on the length of the acyl chain esterified to carnitine.

A recent study from our laboratory has shown that L-carnitine and acetyl-L-carnitine (ALC) were capable of modulating erythrocyte membrane stability, most likely via a specific interaction with cytoskeletal membrane proteins, when resealed ghost containing 20–300

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; ALC, acetyl-L-carnitine; LC, L-carnitine; PLC, palmitoyl-L-carnitine; DSA, doxylstearic acid.

μM of these compounds were subjected to ektacytometric analysis [4]. Interestingly, Fritz et al. have found that LC inhibited fibrinogen induced-clustering of erythrocytes, suggesting a mechanism of action in which LC would affect the surface charge distribution of the red cell membrane [5]. In addition to these in vitro observations on the potential modulatory effects of LC and its short-chain esters on membrane functions, in vivo studies provided evidence that ALC administration influenced the lipid and protein composition of biological membranes [6–10]. From these latter studies, however, it is not clear whether ALC is acting directly or indirectly on the membrane lipid and/or protein turnover. On the other hand, by affecting the physical status of the lipid bilayer one may influence a variety of cellular physiological activities that are confined to the cell membrane.

In this study, we decided to examine in some detail the effect of LC and its short-chain esters on parameters concerning the physical status of the erythrocyte membrane in intact human red cells. Propionyl-L-carnitine (PLC), ALC and LC seem to decrease the molecular packing of the polar head of membrane phospholipids, with PLC being the most effective molecule. However, only PLC and ALC increased the molecular dynamics of erythrocyte membrane components in a region close to the membrane surface, but no effect was recorded in deeper regions of the erythrocyte membrane bilayer.

Materials and Methods

Erythrocyte preparation

Freshly taken venous human blood from healthy volunteers, anticoagulated with heparin, was processed to remove leukocytes and platelets [11] and washed three times with 0.9% NaCl. Isolated erythrocytes were then washed with incubation buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 10 mM Tris-HCl, at pH 7.4) and resuspended in the same buffer at a final hematocrit of 50%.

Spin labeling and EPR studies

Stearic acid derivatives labeled by stable doxyl radical ring at the C-5 (5-DSA), C-7 (7-DSA), C-12 (12-DSA) and C-16 (16-DSA) position (counted from the carboxylic group of the stearic acid derivatives) were used. Spin labels were added to 500 μl erythrocyte suspension at a concentration of 10 μg and incubated for 60 min at 4°C in a shaking water bath. Spin labeled erythrocytes were washed twice with the incubation buffer and reincubated for 60 min at 37°C with either LC, ALC or PLC (final hematocrit 50%). Incubations were ended by washing cells twice with cold incubation buffer and the pellet was aspirated into a flat quartz cuvette thermostated at various temperatures. EPR spectra were obtained using a spin-resonance spectrometer (Instrument built at the Institute of Electrochemical Engineering, St. Petersburg, Russia) operating at a center field strength of 3280 G with an 8 min scan-time to scan 100 G, a 0.25 s time constant, a modulation amplitude of 2.0 G and 20 mW microwave power. Order parameter (S_{EPR}) values were calculated according to Gaffney [12].

Merocyanine-540 and pyrene fluorescence studies

Changes occurring on the molecular packing of membrane surface in intact cells were measured by following the binding of the fluorescent probe merocyanine-540 to the outer hemileaflet of the red cell membrane [13]. Erythrocytes treated with LC, ALC and PLC, as described above, were labeled with merocyanine-540 as recently reported [13,14]. The amount of dye bound to the erythrocytes membrane was evaluated by observing fluorescence emission taken with a Perkin Elmer LS-5B Luminescence Spectrophotometer, equipped with a thermostated cell compartment. The excitation wavelength was 560 nm while the emission wavelength was 594 nm (ex. and em. slit width 5 nm). In order to minimize photobleaching and photolysis phenomena, all procedures were carried out in the dark and the time for fluorescence measurement was less than 30 s. In addition, to prove the membrane surface location of the dye, quenching experiments were carried out by adding, in the cell compartment containing the labeled erythrocytes, the water soluble compound bis[3-(trimethylamino)propylbenzothiazole-(2)]pentamethinecyanine bromide, diS-$C_3N(Cl)_3$-(5) (a kind gift from Dr. A. Waggoner).

Fluidity studies were performed by measuring the excimer/monomer (E/M) fluorescence ratio of pyrene incorporated in intact erythrocytes [15,16]. Red cells were incubated with 0.1 μM pyrene in the incubation buffer for 60 min at 37°C (final hematocrit of 4%). Pyrene labeled erythrocytes were washed twice with incubation buffer and the cells were treated with LC, ALC and PLC as described above. The maximum excitation wavelength was found at 332 nm and the maximum monomer and excimer emission wavelengths were 375 and 460 nm, respectively (ex. and em. slit width 5 nm). It should be noted that hemoglobin did not affect the E/M fluorescence ratio of the erythrocyte membrane in intact red cells.

All the fluorescence measurements were corrected for the contribution of intrinsic fluorescence and light scattering.

Statistics

Values are the average of four experiments performed in duplicate $\pm$S.D. A Student's t-test was used to determine the statistical differences between the

experimental groups, assuming $P = 0.05$ as the limit of significance.

Results and Discussion

In the past decade, several studies have addressed the potential harmful effects of amphiphilic molecules, such as long-chain acylcarnitines, lysophospholipids and acyl-CoAs, on the physiological expression of biological membranes [17,18]. Long-chain acylcarnitines, in particular, have been shown to alter profoundly the molecular dynamics of membrane phospholipids, measured either with fluorescent or EPR probes [1,3]. However, it is known that depending on the concentration of either long-chain acylcarnitine or lysophospholipids, they also improve the mechanical stability of erythrocyte membrane [19,20]. Studies from our laboratory have demonstrated that LC and ALC induced changes in the membrane stability, but not deformability, in resealed ghosts [4]. The extent of the increase of membrane stability was inversely correlated with the concentration of LC and ALC, such that at the lowest concentration used, which was close to that physiologically present in intact erythrocytes, the highest value of membrane stability was observed. Since this parameter is considered to be important in the understanding of how red cells can undergo passive deformation in blood capillaries [21], our findings would suggest that LC and ALC may improve the stability of the erythocyte membranes when subjected to high shear stress. Moreover, it was shown that PLC also had a similar effect and the extent of the increase in membrane stability was slightly higher than ALC (unpublished data). These findings, however, do not help us to understand whether these molecules are also capable of affecting the physico-chemical status of the erythrocyte membrane, although at the doses used for the membrane stability measurements, we could not detect any changes of the lipid order parameter in resealed ghosts [4]. In order to obtain a more detailed picture on the potential modulation of the membrane physicochemical status by such compounds, we have carried out studies on the effect of LC, ALC and PLC on natural fluidity gradient and molecular packing of phospholipid headgroups in intact erythrocytes.

The failure to find any effect of both LC and ALC on the order parameter in resealed ghosts, must somehow be related to the low concentration of these compounds used and/or the non-appropriate experimental approach utilized [4]. In fact, by using fluorescence steady-state anisotropy of DPH to measure the order parameter, as we did, one is mainly looking at the molecular order of the membrane core, where the highly apolar fluorescent probe is embedded [22,23]. To overcome this regional limitation, it is possible to use spin-labeled stearic acid derivatives in which the spin probe, a doxyl radical ring, is attached to different carbon number of the fatty acid. In this way, one may scan the methylene group dynamics of the membrane from the region close to the surface of the lipid bilayer to those situaded more deeply [24]. Thus, the anisotropic motion of fatty acid spin probes incorporated into biological membranes can be characterized by the measure of an empirical order parameter, S_{EPR} [12]. This value can be calculated from the spectral line splittings reflecting the amplitute of the motional anisotropy and will be dependent on the particular intramembrane location sampled by the probe [12,24]. It should be noted that, because of the anisotropy of the erythrocyte membrane, the calculated order parameters are not true ones [25]. However, the apparent order parameter, which describes essentially the spin-label freedom of motion, can be used to gain informations on the molecular dynamic of the red cell membrane.

Fig. 1 shows that when 5-DSA labeled erythrocytes were treated with PLC, a significant decrease of the S_{EPR} was observed at all the temperatures tested. A clear dose-dependent effect on the S_{EPR} was also evident, although at lower temperatures a better decreasing ordering effect was seen. As a matter of fact, at the lowest concentrations of PLC (0.5 and 1 mM), an increase of the temperature caused the S_{EPR} to return to the control values. In addition, a similar

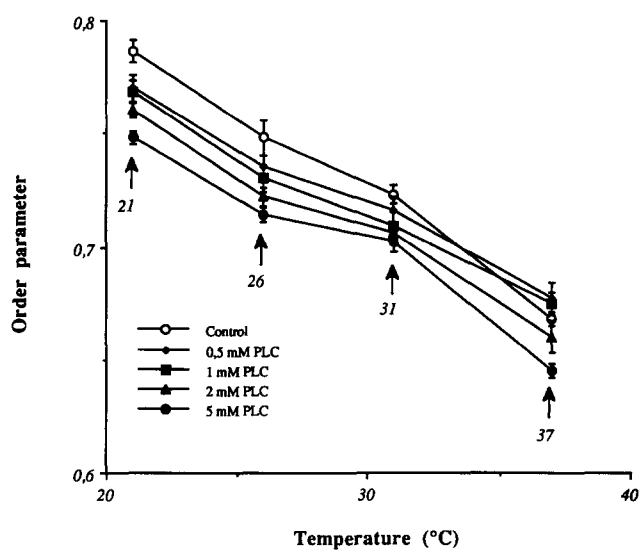

Fig. 1. Effect of PLC on the membrane order parameter of intact human erythrocytes. Human erythrocytes, labeled with 5-DSA, were incubated with different concentrations of PLC as reported in Materials and Methods. The cells were washed twice with an isosmotic buffer and S_{EPR} was calculated from the EPR spectra recorded at the temperatures indicated by arrows in the graph. Significant changes of PLC-treated versus control values at different temperatures: 21°C, $P < 0.01$ with 0.5, 1 and 2 mM, and $P < 0.001$ with 5 mM; 26°C, $P < 0.05$ with 0.5 and 1 mM, and $P < 0.01$ with 1 and 2 mM; 31°C $P < 0.05$ with 1 and 2 mM, and $P < 0.01$ with 5 mM; 37°C, $P < 0.01$ with 5 mM.

TABLE I

Effect of acetyl-L-carnitine on the membrane order parameter of intact human erythrocytes

Human erythrocytes, labeled with 5-DSA, were incubated with ALC as reported in Materials and Methods. The cells were washed once with an isosmotic buffer and S_{EPR} was calculated from the EPR spectra recorded at the temperatures indicated. Significant changes with respect to control values: * $P < 0.05$; ** $P < 0.001$.

Temperature (°C)	Control	Acetyl-L-carnitine (mM)	
		2.5	5
21	0.773 ± 0.008	0.768 ± 0.005	0.749 ± 0.007 **
26	0.737 ± 0.005	0.730 ± 0.006	0.723 ± 0.005 *
31	0.709 ± 0.009	0.712 ± 0.005	0.698 ± 0.008
37	0.672 ± 0.006	0.678 ± 0.008	0.676 ± 0.005

dose-dependent effect of the S_{EPR} calculated with the methyl-ester derivative of the 5-DSA was obtained (data not shown). This latter finding would rule out the possibility that PLC is causing changes on the motional freedom of the spin-label through an ion-pair association of its quaternary ammonium group with the carboxylate anion of the 5-DSA.

It is known that due to the heterogeneous composition of biological membranes, gel-phase transitions or phase separations are events difficult to detect [26]. However, the better efficacy of PLC in increasing the molecular dynamics at the lower temperatures may suggest that this compound, like other chaotropic molecules [27], is decreasing the temperature of the gel-to-liquid crystalline transition. It should be taken into account that higher temperatures, as a result of an increase in kinetic energy, may also affect the binding capacity of PLC to the surface of the membrane, thus decreasing the effective amount of this LC ester bound to the membrane. On the other hand, PLC seems generally to increase the molecular dynamics in a region of the membrane bilayer close to the polar heads of membrane phospholipids. ALC showed a significant decreasing ordering effect at lower temperatures, although this effect was present only at the highest concentration (Table I). LC did not modify the S_{EPR} at all the temperatures and concentrations used in this study. In addition, by using the D-stereoisomers of PLC, ALC and LC, we have obtained results similar to those reported above for the L-stereoisomers (data not shown).

Taken together, these data suggest that the increase of the molecular dynamics induced by PLC may be related to the better efficacy of the more hydrophobic propionate moiety to intercalate between the polar heads of membrane phospholipids, perturbing in this way the alkane-chain environment close to the polar headgroups. The slight effect of ALC on the molecular order of membrane red cell components may be ascribed to the shorter chain ester moiety. According to this assumption, LC did not show any alteration of the S_{EPR} at all the concentrations used. In addition, our observations are reminescent of earlier findings in which the enhanced disordering effect elicited by acylcarnitines on pure and mixed phopsholipid vesicles, measured by means of steady-state fluorescence polarization of DPH, was linearly correlated with the increased chain length (C-12 to C-16) of the carnitine esters [3]. Fig. 2, however, shows that PLC possesses only a minor pertubing effect on the molecular order of the membrane red cell component, when the doxyl ring was located deeper in the membrane core. A significant decrease of the S_{EPR} value was recorded with 7-DSA, but only at the highest concentration (5 mM) of PLC treatment. With 12-DSA and 16-DSA, we were not able to detect any changes in the S_{EPR} values. These data are in good agreement with the idea that the deeper the hydrocarbon belt region of the red cell membrane examined for molecular order lies, the lower is the influence of the propionate moiety on such a biophysical parameter.

In another series of experiments, we wanted to explore the potential effect of LC and its derivatives on the E/M fluorescence intesity ratio of pyrene incorporated into the membrane of intact erythrocytes. Given the concept that the pyrene E/M ratio represent a measure of the fluidity in a membrane bilayer region between the hydrophobic core and the polar

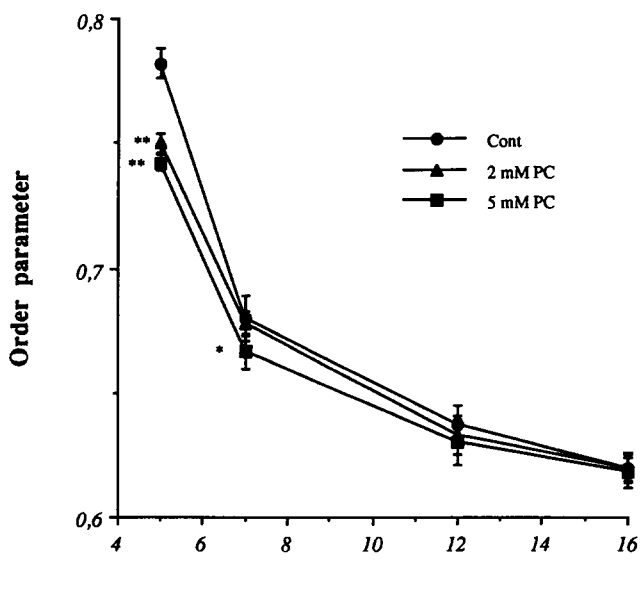

Fig. 2. Order parameter profile of PLC-treated erythrocytes. Human erythrocytes labeled with either 5-DSA, 7-DSA, 12-DSA or 16-DSA, were incubated with PLC as reported in Materials and Methods. The cells were washed twice with an isosmotic buffer and S_{EPR} was calculated from the EPR spectra recorded at 21°C of the various spin labels. Significant changes of PLC-treated versus control values: * $P < 0.05$, ** $P < 0.01$.

surface of a lipid bilayer [16], such measurements would further aid us in the understanding the depth-dependent effects reported above. Thus, the E/M ratios in erythrocytes treated with LC, ALC or PLC at 37°C, even at concentrations up to 5 mM, were not significantly different from those ratios measured in untreated red cells (data not shown). This lack of effect on the membrane fluidity in deeper regions would confirm the above EPR findings. It should be noted that the EPR measurements of S_{EPR} with the 7-, 12- and 16-DSA were carried out at 21°C (Fig. 2) and that the S_{EPR} value obtained with the 7-DSA from 5 mM PLC treated-erythrocyte at 37°C was comparable to untreated red cells.

Arienti et al. found that ALC and LC were able to increase membrane fluidity in rat brain microsomes and liposomes made with rat brain lipid microsomes. In this study, membrane fluidity was computed by measuring the steady-state fluorescence anisotropy of DPH. These data are not in agreement with our findings, particularly if one considers that the changes in fluidity reported by these authors were obtained with a probe located in the membrane core of the lipid bilayer [22,23]. However, the concentration of LC and ALC utilized in our study are lower than in the rat brain microsomes study (2.5–25 mM), but more importantly, in our study the effective concentration of these compounds interacting with the membrane were much lower, since after the incubation, erythrocytes were washed and then resuspended in a buffer free of LC and ALC. On the other hand, LC and ALC, at very high doses, may still affect the molecular dynamics of the membrane core, most likely through changes occurring in the glycerol backbone region of the membrane bilayer, which indirectly might alter the fluidity of the deeper hydrocarbon belt of the membrane.

Previous studies have demonstrated that the fluorescent probe merocyanine-540 provides satisfactory information on the molecular packing of the polar head of erythrocyte membrane phospholipids [13,14,28]. The binding of merocyanine-540 to the surface of a lipid bilayer changes its fluorescence spectral characteristic: a red shift of the emission peak is accompained with an increase of fluorescence intensity [13]. This is mainly caused by the interaction of the fluorescent probe with the less hydrophilic enviroment of the outer emileaflet of the erythrocyte membrane [13,28]. This prompted us to extend our observations on the effects of LC, ALC and PLC on the degree of molecular packing of the glycerol backbone region of erythrocyte membrane. Table II shows that all the compounds tested significantly increased the red shifted fluorescence emission of merocyanine-540 with respect to control erythrocytes. In addition, an increase of PLC concentration (from 1 to 5 mM) caused a parallel increase of the red shifted fluorescence emission (Fig. 3). The addition of diS-$C_3N(Cl)_3$-(5) in the cuvette before the fluorescence measurements, completely suppressed the merocyanine-540 fluorescence emission in both control and LC, ALC, and PLC treated erythrocytes. This latter observation confirmed that the location of the probe is truly the outer hemileaflet of the erythrocyte membrane [14,28].

TAble II

Effects of L-carnitine and its short-chain esters on merocyanine-540 binding on human erythrocytes

Human erythrocytes were incubated at 37°C for 60 min with 5 mM of LC, ALC or PLC. Cellular suspensions were washed with an isosmotic buffer and labeled with merocyanine-540. Fluorescence measurements are given as arbitrary units. Significant changes with respect to control values: * $P < 0.05$; ** $P < 0.001$.

	Fluorescence intensity
Control	17.3 ± 0.5
L-Carnitine	22.8 ± 0.7 *
Acetyl-L-carnitine	31.3 ± 0.5 **
Propionyl-L-carnitine	37.6 ± 0.5 **

As merocyanine-540 binds preferentially to membranes which have a loosened lipid packing near the headgroup of phospholipids [13], our data would suggest that in addition to a decrease of the order paramater detected in a hydrocarbon belt region close to the membrane surface, PLC and ALC are also decreasing the molecular packing of those phospholipid head-

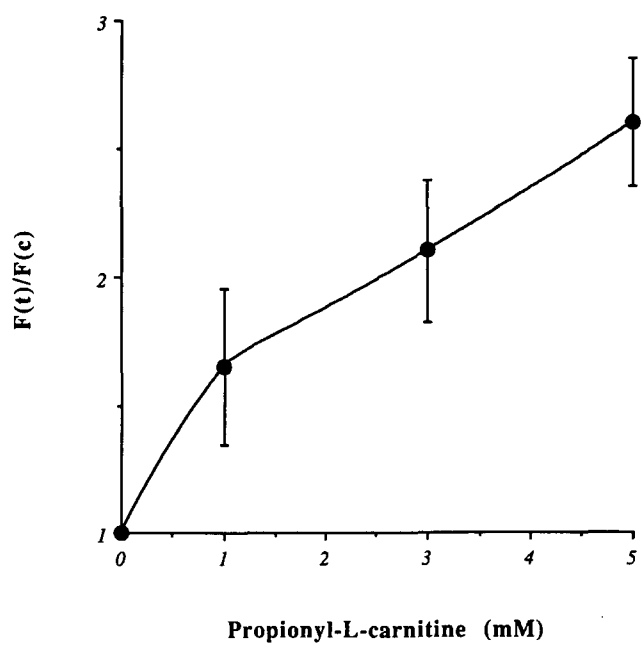

Fig. 3. Dose-dependent effect of PLC on the merocyanine-540 binding on human erythrocytes. Red cells were incubated at 37°C for 60 min with different concentrations of PLC. Cellular suspensions were washed with an isosmotic buffer and labeled with merocyanine-540 as reported in Materials and Methods.The fluorescence ratios of PLC-treated/control erythrocytes are reported on the ordinate.

groups located in the outer hemileaflet. Interestingly, even LC, which did not influence the molecular order of the red cell membrane, significantly loosened the packing of phospholipid headgroups, although to a lesser extent than PLC and ALC (merocyanine-540 binding decreased in the order PLC > ALC > LC). On the other hand, the short acyl chain present in ALC and PLC, which appears to be required for the decrease of the S_{EPR}, would further affect the molecular packing of erythrocyte membrane phospholipids. Fritz et al. have shown that LC inhibited the clustering of human red cells induced by either fibrinogen or clusterin [5]. This phenomena was evident even when washing red cells preincubated with LC, indeed a non-saturable binding of radiolabeled LC to red cell membrane was demonstrated. Although the nature of the plasma membrane binding domain of LC is not known, our observations may well fit with this intriguing effect of LC on the dispersion of red cells. In addition, since we have demonstrated that these compounds modulate the membrane stability, probably by interacting with specific cytoskeleton protein domains [4], one may reasonably assume that outer membrane surface domains of intrinsic membrane proteins are involved in the binding with LC and its short-chain esters, which in turn would cause the alterations of the molecular packing. In addition, the treatment of intact erythrocyte with a thiol group alkylating reagent resulted in a large decrease of LC binding to the membrane surface [5]. Our findings, however, would not rule out the possibility that LC and its short-chain esters may also affect the ionization state of phospholipid domains distributed in the outer hemileaflet of membrane red cell, a process also known to alter the molecular packing [29]. In this context, it has been proposed, on the basis of crystal X-ray diffraction, neutron diffraction and NMR studies, that the overall conformation of the PC headgroup, a phospholipid which is mainly distributed on the outer hemileaflet of the membrane erythrocyte, is folded to form a layer of polar heads oriented parallel to the plane of the membrane [30]. Thus, the quaternary ammonium group protons of PC interact intermolecularly with a neighboring PC phosphate group at an average angle of 90°. This kind of configuration may be easily tilted by carnitine and its short chain derivatives, since the positive quaternary ammonium and negative carboxylic group present at the two ends of its skeleton carbon may weaken the intermolecular interaction between two adjacent PC, most likely favoring a decrease of the head group molecular packing.

Recently, several research groups have provided evidence that LC and its short-chain esters, ALC and PLC, posses benificial therapeutic effects against the so-called ischemia-reperfusion injury of the heart in both human and animal studies [31–35]. In addition, acute treatment with ALC caused an alteration in the age-induced modification of the inner mitochondrial membrane protein composition in the rat cerebellum [6]. The same type of treatment was also capable of restoring the cardiolipin content and the phosphate carrier function in heart mitochondria from old rats [9]. The pharmacological mechanism of action is not well understood, although the two most credited hypotheses are the energy-linked and antioxidant activity. In this respect, our results may offer an additional element of discussion in the interpretation of the ameliorative effects of LC and its short chain esters in such pathological conditions. In conclusion, besides the potential pharmacological and physiological implications of our findings, this class of compounds are effective in modulating the physico-chemical behaviour of the biological membrane when they are present either inside or outside the cell.

Acknowledgments

This research was supported in part by Consiglio Nazionale delle Ricerche (Progetto Strategico per il Mezzogiorno) and Sigma Tau S.p.A., Pomezia, Italy.

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