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Differential effects of conjugated linoleic acid isomers on the biophysical and biochemical properties of model membranes

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Abstract

Conjugated linoleic acids (CLA) are known to exert several isomer-specific biological effects, but their mechanisms of action are unclear. In order to determine whether the physicochemical effects of CLA on membranes play a role in their isomer-specific effects, we synthesized phosphatidylcholines (PCs) with 16:0 at *sn*-1 position and one of four CLA isomers (*trans*10 *cis*12 (A), *trans*9 *trans*11 (B), *cis*9 *trans*11 (C), and *cis*9 *cis*11 (D)) at *sn*-2, and determined their biophysical properties in monolayers and bilayers. The surface areas of the PCs with the two natural CLA (A and C) were similar at all pressures, but they differed significantly in presence of cholesterol, with PC-A condensing more than PC-C. Liposomes of PC-A similarly showed increased binding of cholesterol compared to PC-C liposomes. PC-A liposomes were less permeable to carboxyfluorescein compared to PC-C liposomes. The PC with two *trans* double bonds (B) showed the highest affinity to cholesterol and lowest permeability. The two natural CLA PCs (A and C) stimulated lecithin-cholesterol acyltransferase activity by 2-fold, whereas the unnatural CLA PCs (B and D) were inhibitory. These results suggest that the differences in the biophysical properties of CLA isomers A and C may partly contribute to the known differences in their biological effects.

1. Introduction

Conjugated linoleic acids (CLA) are the naturally occurring isomers of linoleic acid, which are present in the dairy products and ruminant meats, and which have been reported to have several beneficial effects, including protection against cancer, obesity, heart disease and immune dysfunction [1–3]. Of the 16 known isomers of CLA, two (*cis* 9 *trans* 11 or c9 t11, and *trans* 10 *cis* 12 or t10 c12) are predominant in the natural products, as well as in the synthetic supplements sold commercially. These two major isomers differ significantly in their biological effects, although their structural differences are subtle. Thus the *c*9 *t*11 isomer has been shown to be responsible for most of the anti-carcinogenic effects, whereas the *t*10 *c*12 isomer appears to be responsible for most of the anti-obesity effects in mice [1,3]. Despite extensive investigations from several laboratories, neither the exact mechanisms involved in the multiple biological effects of CLA, nor the basis for the isomer-specific effects are clear. The majority of the proposed mechanisms involve differential effects of CLA isomers on gene transcription, specifically as ligands or inducers of transcription factors PPARs, SREBPs, and

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NFκB [2,4]. However, CLA are relatively weak ligands compared to the other (more abundant) unsaturated fatty acids [3], and the studies with PPARα knockout mice in fact showed that the effects of CLA on body fat distribution are independent of this transcription factor [5]. CLA, like other polyunsaturated fatty acids (PUFA), have been shown to be incorporated into membrane lipids, the extent of incorporation ranging from <1% to >50% of total phospholipid fatty acids in various cells [6–11]. The enrichment of membrane lipids with dietary polyunsaturated fatty acids (PUFA) is known to affect the distribution and function of several membrane raft-associated proteins [12–14], and therefore we were interested in investigating whether the effects of dietary CLA on membrane functions of the cells would account for at least part of their biological effects. Since several receptors, signaling proteins, and enzymes are associated with the membranes, the pleiotropic effects of CLA may be explained by their effects on membrane structure and function. As a first step in testing this hypothesis, we synthesized phosphatidylcholines (PCs) containing palmitic acid at the *sn*-1 position and one of four isomers of CLA, c9t11, t10c12, *cis* 9 *cis* 11 (c9c11), or *trans* 9 *trans* 11, (t9t11) at the *sn*-2 position, and tested their physicochemical effects on model membranes, including the monolayer properties, fluidity, permeability, oxidizability, and the ability to bind cholesterol. In addition, we tested the effect of the presence of CLA isomers at the *sn*-2 position, on the activity of LCAT, the enzyme that transfers the *sn*-2 acyl group from PC to cholesterol, and is responsible for the synthesis of most of the cholesteryl esters present in plasma [15]. The results show significant differences in the physicochemical effects of various CLA isomers on the model membrane properties, as well as on the enzyme activity.

2. Materials and Methods

2.1. Materials

All isomers of CLA (t10c12, t9t11, c9t11, and c9c11) were purchased from Matreya, Inc. Lyso PC (1-palmitoyl), 16:0–18:2 PC (with unconjugated LA), dipalmitoyl PC (16:0–16:0 PC), and egg sphingomyelin were obtained from Avanti Polar Lipids. Carboxyfluorescein, methyl B cyclodextrin, and unlabeled cholesterol were obtained from Sigma Chemical Co (St. Louis, MO). Labeled cholesterol (4-^{14}C) (53.0 mCi/mmol) was purchased from Perkin Elmer (Boston, MA) and dipalmitoyl-[1-^{14}C] PC (55.0 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc (St.Louis, MO). *Sn*-1-palmitoyl-2-diphenylhexatriene PC (DPH-PC) was obtained from Invitrogen (Carlsbad, CA). CLA-PCs, containing 16:0 at *sn*-1 and various isomers of CLA at *sn*-2 were synthesized by the procedure of Paltauf and Hermitter [16] using 1-palmitoyl lyso PC, and free CLA. The PCs were purified by silicic acid column chromatography, and the purity of the compounds assessed by TLC. Lipid phosphorus of the samples was determined by the modified Bartlett procedure [17]. More than 90% of CLA was present in the *sn*-2 position, as determined by snake venom PLA_2 treatment [18].

2.2. Monolayer measurements

Force-area isotherms of CLA-PCs and cholesterol were obtained using a computer- controlled Nima 302M Langmuir-type surface balance (Nima Technologies, Coventry, England) on a subphase of purified water (Nanopure Infinity filtration system, Dubuque, IA; resistivity α17.5 MΩ cm). The PTFE trough and barriers were cleaned before and after each run with chloroform and isopropanol. Glassware was cleaned in base (ethanol-2 M KOH, 1/1) and acid (1 M HNO_3) and rinsed thoroughly in purified water [19]. CLA-PC and cholesterol solutions were prepared by dissolving lipids in HPLC-grade chloroform at a concentration of ~1 mg ml^{-1}. CLA- PC-cholesterol solutions were prepared by mixing appropriate volumes of stock lipids and were spread on the aqueous surface using a Hamilton (Reno, NV) digital syringe. Monolayer compression at a rate of 10 cm min^{-1} was initiated after a 10 min delay to allow for solvent evaporation. All compressions were done in triplicate to ensure reproducibility.

Compression data were collected using proprietary software from Nima and analyzed using Origin software (Northampton, MA). Condensation of CLA-PCs by cholesterol was determined as the deviation from ideal mixing according to the equation

$$A_\pi = X_1(A_1)_\pi + (1 - X_1)(A_2)_\pi \quad \text{(Equation 1)}$$

where X_1 is the mole fraction of component 1 and $(A_1)_\pi$ and $(A_2)_\pi$ are the mean molecular areas of components 1 and 2, respectively, at surface pressure π. Deviations from the linearity expressed by Eq. 1 represent attractive (negative deviations) or repulsive (positive deviations) interactions between the components of the binary films [20]. The degree of condensation reported herein was calculated using equation 2:

$$\%\,\text{Condensation} = [(A_{ideal} - A_{exp})/A_{ideal}] \times 100_{[21]} \quad \text{(Equation 2)}$$

where A_{ideal} is the mean molecular area calculated assuming ideal additivity and A_{exp} is that observed experimentally.

2.3. Cholesterol binding studies

The affinity of the liposomes prepared with each CLA-PC for cholesterol was determined by the rate of transfer of labeled cholesterol from the methyl β cyclodextrin (MBCD)-labeled cholesterol complex to the liposomes by a modification of the procedure of Niu and Litman [22]. Liposomes of each CLA-PC were prepared in 10 mM PIPES buffer by extrusion through 0.1 μm polycarbonate filters, and incubated with 0.5 mM 4-^{14}C -cholesterol-10 mM MBCD complex for 2 h at 37 °C. The sample was diluted with an equal volume of 4M NaCl, and filtered through Microcon YM30 filter at 37 °C. The filter was washed 3 times with 2M NaCl and the radioactivity retained on the filter was determined in a liquid scintillation counter. The percent of labeled cholesterol bound to the liposomes was calculated and expressed as the % of the value obtained with control PC (16:0–18:2 PC, unconjugated LA). In the experiments where the effect of SM was studied, the liposomes contained 20 mol% of egg SM and 80 mol % of the test PC.

2.4. Permeability studies

The permeability property of the liposomes was measured by the rate of leakage of the trapped carboxyfluorescein, as described Roach et al [23]. Briefly, small unilamellar vesicles (SUV) of CLA-PCs were prepared by sonication in the presence of 60 mM carboxyfluorescein, and the unincorporated dye was removed by gel filtration on a Sepahdex G-50 column. The liposomes were spiked with a trace of dipalmitoyl[1-^{14}C]-PC in order to monitor the column fractions for liposomes. An aliquot of the caroboxyfluorescein-labeled liposomes was incubated with a 30- fold excess of buffer (10 mM PO_4, 90 mM KCl, pH 7.2) and the increase in fluorescence due to the leakage of the dye into the medium was monitored in a spectrofluorometer at 25 °C for 2 h (excitation 490 nm, emission 520 nm). At the end of this period, the total amount of carboxyfluorescein sequestered in the liposome (F_{max}) was determined by disrupting the liposomes with 0.1% Triton X100 (final concentration). The rate of carboxyfluorescein leakage in the linear range of the graph was calculated by the formula

$$\%\,\text{leakage/min} = ((F_2 - F_1)/(t_2 - t_1)/F_{max}) \times 100 \quad \text{(Equation 3)}$$

where F_2 is the fluorescence value at the end of the linear range (t_2), F_1 is the fluorescence at the beginning of the linear range (t_1), and F_{max} is the total fluorescence in the sample. This value for each CLA PC was then expressed as % of the value obtained with control liposomes (16:0–18:2 PC, containing unconjugated LA).

2.5. Differential scanning calorimetry (DSC)

Calorimetric measurements were performed using a Microcal VP-DSC calorimeter. Chloroform solutions of dipalmitoyl PC (2 mg) alone or with 10 mol% or 25 mol% CLA-PC were evaporated under nitrogen, dissolved in 300 μl of ethanol, re-evaporated under nitrogen, and traces of the solvent were removed under vacuum. The lipid was dispersed in 1 ml of degassed distilled water by vortexing, and the sample was incubated at 50 °C for 20 min before loading into the sample chamber of DSC. Distilled water was used in the reference cell. At least 4 heating scans were performed for each sample, with scan rate of 60 °C/h. Scan 4 was used for the calculation of transition temperature (T_m), calorimetric enthalpy (ΔH), van't Hoff enthalpy (ΔH_{vH}), and cooperativity units (CU), with the software (Microcal Origin) provided by the manufacturer (non-two state Cursor Init model).

2.6. Oxidizability of CLA-PC

CLA-PC liposomes (0.1 μmol PC), prepared by extrusion, were incubated with 1 mM AAPH at 37 °C for 2 h. Aliquots of the reaction mixture were extracted at 0 h,1 h, and 2 h after adding 17:0–17:0 PC as the internal standard. The total lipid extract was methylated with methanolic HCl, and the fatty acid methyl esters analyzed by GC as described previously [24], except for the modification of the temperature programming. Initial temperature was set at 150 °C for 1.0 min, raised to 210 °C at the rate of 3 °C/min, and then to 225 °C at the rate of 2 °C/min and maintained at this temperature for 15 min. The percent of CLA remaining intact was calculated by taking the 0 h value as 100%. Control PC (16:0–18:2 PC, unconjugated LA) was oxidized under identical conditions for comparison.

2.7. Fluidity measurements

The fluidity of the liposomes was determined by measuring fluorescence polarization of DPH-PC incorporated into the CLA-PC liposomes. The liposomes containing test PC and DPH-PC at a molar ratio of 200:1 were prepared by sonication in 10 mM Tris-0.15 M NaCl-1mM EDTA, pH 7.4. The polarization values were determined at various temperatures in a spectrofluorometer (model PC1, ISI, Champagne, IL) (excitation 362 nm, emission 430 nm). The temperature of the cuvette was maintained with a circulating water bath, and measured with a probe. The sample was equilibrated for 10 min at each temperature before measuring the polarization values. The anisotropy values were calculated with the formula

$$(I_v - I_h{}^{*}g)/(I_v + 2{}^{*}I_h{}^{*}g) \qquad \text{(Equation 4)}$$

where *Iv* is the intensity parallel to the excitation plane, *Ih* is the intensity perpendicular to the excitation plane, and *g* is the grating factor.

2.8. Lecithin-cholesterol acyltransferase (LCAT) assay

LCAT activity was assayed as described previously [25], using proteoliposome substrates containing the test PC: 4-^{14}C cholesterol: apoprotein A-I at a molar ratio of 250:12: 0.8, prepared by the cholate dialysis procedure [26]. The enzyme preparation was the partially purified human plasma LCAT (Phenyl Sepharose eluate, prepared as described by Chen and Albers [27]). The reaction mixture contained 20 μl of proteoliposomes corresponding to 50 nmol of PC and 2.4 nmol of labeled cholesterol, 20 μg of enzyme of enzyme preparation, 10

mM mercaptoethanol, and 0.5 mg of human serum albumin in a total volume of 200 μl. The reaction was carried out for 30 min at 37 °C, and stopped by the addition of 800 μl of ethanol. The lipids were extracted with hexane containing 30 μg each of unlabeled cholesterol and cholesteryl oleate (carriers) and the radioactivity in cholesterol and cholesteryl ester was determined after TLC separation, as described previously [25]. The enzyme activity was calculated as % of cholesterol esterified per h. The activity obtained with each CLA-PC substrate was then expressed as % of the activity obtained with unconjugated 16:0–18:2 PC substrate (control).

3. Results

3.1. Monolayer properties of CLA-PCs

The surface-area isotherms of the four CLA-PCs and the control (unconjugated) 16:0–18:2 PC were determined in the absence and presence of increasing mol fraction of cholesterol. As shown in Fig. 1a, the molecular areas occupied by the PCs containing the two naturally occurring CLA (c9 t11, and t10 c12) were significantly lower than that of the unconjugated 16:0–18:2 PC. The conjugation of the double bonds alone appears to decrease the limiting surface area by about 10% (compare unconjugated control vs c9 c11 isomer), although this effect is not apparent at low surface pressure. The presence of a *trans* double bond alone decreases the molecular surface area significantly (compare the curve of c9 c11 CLA-PC with that of c9 t11 isomer). The presence of two *trans* double bonds (t9 t11 isomer) decreased the surface area further. The decrease in surface area due to *trans* unsaturation in the *sn*-2 acyl group has previously been reported for PCs with unconjugated fatty acids [28].

Although the two PCs containing the natural CLA did not differ in their molecular surface areas, they differed significantly with respect to their interaction with cholesterol at 20 mN. (Fig 1b). Thus, at all cholesterol concentrations except 50 mol%, the t10 c12 CLA-PC was more condensed than the c9 t11 CLA-PC, suggesting a higher affinity of the former for cholesterol in monolayers. The maximum condensation occurred at 70 mol% cholesterol for the t10 c12 isomer, whereas it was at 50 mol% for the c9 t11 isomer. A cholesterol content of up to 50 mol% condensed the CLA-PC containing two *trans* double bonds (t9 t11) more than other isomers; above this cholesterol concentration, the double *cis* isomer was more condensed.

3.2. Differential scanning calorimetry

The effect of incorporating increasing amounts of CLA-PC on the phase transition temperature (T_m) of 16:0–16:0 PC (DPPC) is shown in Fig. 2. The main transition peak was broadened, and the Tm decreased in presence of all CLA-PCs and control PC. The two natural CLA-containing PCs differed in their effects, with the c9t11 CLA-PC showing a more ‘fluidizing’ effect than the t10c12 isomer. This is consistent with previous studies on free CLA, which showed that c9t11 CLA had lower Tm than that of t10c12 CLA [29]. The double *cis* CLA-PC showed the maximum decrease in T_m, while the double *trans* CLA-PC isomer showed the smallest change. The results show that the presence of a *cis* double bond at carbon 9 has the maximal ‘fluidizing’ effect. Furthermore, conjugation of double bonds increased the ‘fluidizing’ effect (compare the results for LA-PC vs c9c11 CLA-PC). The pre-transition peak (35.5 °C) exhibited by pure 16:0–16:0 PC disappeared in the presence of all CLA-PCs except the t9t11 CLA-PC, in which case it was diminished but not eliminated completely (not shown). In addition to the Tm, the cooperative unit (C.U.) value was calculated for each sample by the equation

$$\text{C.U.} = \text{van't Hoff enthalpy } (\Delta H_{vH}) / \text{calorimetric enthalpy } (\Delta H) \quad \text{(Equation 5)}$$

The C.U. value for 100% DPPC was 539. This value decreased significantly in the presence of 10 mol% and 25 mol% respectively, of LA-PC (33.8 and 23.9), t10c12 isomer (28.2 and 24.1), t9t11 isomer (54.8 and 48.6), c9t11 isomer (28.1 and 19.2), and c9c11 isomer (13.9 and 17.9). These results indicate that the c9t11 isomer interferes with phase transition cooperativity of disaturated PC more than the t10c12 isomer, at least at the higher concentration. As expected, the double-*trans* isomer has lower effect on C.U. of DPPC, than the double-*cis* isomer.

3.3. Cholesterol binding properties of CLA-PCs

The affinity of various CLA-PCs to cholesterol was studied by the ability of the PC liposomes to extract cholesterol from cholesterol-cyclodextrin complex, as described previously [30], but employing radioactive cholesterol. In addition to the binding studies employing PC alone, we studied the effect of the presence of 20 mol% sphingomyelin (SM), which is a normal constituent of the plasma membrane, and which is known to influence the affinity of the membrane to cholesterol [31]. As shown in Fig. 3, substitution of normal (unconjugated) 18:2 at *sn*-2 of PC with t10 c12 CLA increased the affinity to cholesterol by 20%, whereas substitution with c9 t11 CLA had no effect. Substitution with the double *cis* CLA (c9 c11) decreased the affinity to cholesterol, whereas the substitution with a double *trans* CLA (t9 t11) more than doubled the binding of cholesterol compared to the control PC. These results suggest that the presence of a *cis* double bond at carbon 9 is important in maintaining the normal cholesterol binding property of PC. The presence of a *trans* double bond at carbon 9 or 10 significantly increases the cholesterol binding property, and the presence of two *trans* double bonds increases the binding more dramatically, as has been reported for the unconjugated *trans* fatty acid PCs [30]. Inclusion of 20 mol% SM increased the binding capacity of all PCs, but the magnitude of the SM effect was not equal. Maximal impact of SM was observed with the PCs that had a *cis* double bond at carbon 9 (c9 c11 and c9 t11).

3.4. Binding of cholesterol to 16:0–18:1 PCs

The importance of the double bond at carbon 9 for cholesterol binding is more clearly demonstrated with 16:0–18:1 PCs, where there is only one double bond. As shown in Fig. 4, the PC containing a *cis* double bond at carbon 9 showed the lowest affinity to cholesterol, among the PCs containing a *cis* double at various positions of *sn*-2 18:1. Furthermore, the presence of a *trans* double bond increases the affinity to cholesterol at all positions, compared to the *cis* double bond, with the effect being most evident at carbon 9. Previous studies showed that the introduction of a *cis* double bond at carbon 9 of an 18 carbon fatty acid has the maximal effect on the transition temperature of the phospholipids containing the fatty acid [32]. Our studies show that the substitution of a *trans* double bond for *cis* double bond at this position also increases the cholesterol binding property to the maximum.

3.5. Membrane permeability

The permeability property of the membranes composed of various CLA-PCs was determined by the carboxyfluorescein leakage assay as described by Roach et al [23]. As shown in Fig. 5, the permeability was not affected by the presence of t10c12 CLA at *sn*-2, compared to the control PC containing c9c12 LA. However the presence of c9t11 CLA at *sn*-2 significantly increased the permeability, indicating a looser packing, whereas the presence of a double *trans* CLA (t9 t11) or double *cis* CLA (c9 c11) decreased the permeability indicating tighter packing of the bilayer.

3.6. Membrane fluidity

DPH-PC was incorporated into each CLA-PC liposome at the molar ratio of test PC: DPH-PC of 200:1, and the anisotropy values were determined at various temperatures in a spectrofluorometer. As shown in Fig. 6, the anisotropy values were similar for the two PCs

with natural CLA (c9 t11 PC, and t10 c12 PC) and the control PC (with unconjugated 18:2) at most temperatures except at 15 °C, where the c9 t11 isomer showed a slightly lower anisotropy. The double *trans* isomer (t9 t11) showed the highest anisotropy values at low temperature, but as expected, decreased with increasing temperature, to give a value comparable to the control PC at 37°C. However, the double *cis* isomer (c9 c11) behaved anomalously, exhibiting high anisotropy values at all temperatures except at 50 °C. The melting point (T_m) for the free fatty acid form of this CLA is also unusually high for a fatty acid with two *cis* double bonds (42.5 °C), compared with unconjugated 18:2 with two *cis* double bonds (−5 °C), showing that the conjugation of double bonds has profound effect on the physical structure of the fatty acid. (T_m values for other free fatty acids are: c9 t11: 20 °C, t10 c12: 22.5 °C; c9 c11:42.5 °C; t9 t11: 54 °C, data supplied by Matreya Inc).

3.7. Oxidizability of CLA-PCs

The oxidative susceptibility of CLA containing PCs was tested by treating the PC liposomes with 1 mM AAPH at 37 °C, and analyzing the fatty acid composition at 0, 1 and 2 h time points. As shown in Fig. 7, all CLA-PCs showed increased susceptibility to oxidation, compared to the unconjugated control PC. In contrast to the previous studies with PCs containing unconjugated linoleic acids which showed that the *trans* double bond renders the fatty acid more resistant to oxidation [33], the double-*trans* CLA-PC, (t9 t11 PC) was actually oxidized at the highest rate, whereas the double-*cis* CLA-PC (c9c11 PC) showed the highest resistance among the conjugated species.

3.8. Effect of CLA on LCAT activity

The effect of various CLA isomers at *sn*-2 position of PC on the LCAT reaction, which predominantly transfers the *sn*-2 acyl group to cholesterol, is shown in Fig. 8. Compared to the control PC substrate containing the unconjugated 18:2, the PCs containing the two natural CLA isomers (t10 c12 and c9 t11 isomers) were superior substrates for LCAT, showing on average about 2-fold higher activity. On the other hand, the presence of a double *trans* CLA (t9 t11) inhibited the enzyme activity by 85%, while the presence of a double *cis* isomer (c9 c11) inhibited the activity by about 20%. Previous studies from our laboratory showed that the presence of *trans* unsaturation in *sn*-2 fatty acids (unconjugated) was inhibitory to human LCAT [34]. Interestingly, the present studies show that the naturally occurring conjugated fatty acids promote LCAT activity, although they also contain a *trans* double bond.

4. Discussion

Trans unsaturated fatty acids (TUFA) produced by partial hydrogenation of vegetable oils are well known to be harmful to human health because of their adverse effects on the lipoprotein profile and on atherogenic risk. In addition, TUFA consumption has been associated with inflammatory disease, increased insulin resistance, impaired endothelial function, and increases in certain types of cancer [35,36]. A significant part of the adverse effects of TUFA is apparently due to their effects on membrane properties and consequent influence on receptor and signaling functions. In contrast to the deleterious effects of the manufactured TUFA, the naturally occurring TUFA in dairy products, namely CLA, have been shown to exhibit several beneficial effects in experimental animals including anti-obesity and anti-carcinogenic effects, and amelioration of cardiovascular risk. The major structural difference between the harmful and beneficial forms of TUFA in the diet is the presence of conjugated double bonds in the latter, although it is not known how this explains their differential biological effects. Like other unsaturated fatty acids in the diet, CLA are incorporated into cell membranes to varying extent, depending upon the experimental conditions. Thus, while some feeding experiments in humans and experimental animals showed that only about 1% of the phospholipid fatty acids were replaced by CLA [9,37], cell culture experiments showed that CLA could replace up to 50%

of phospholipid fatty acids in leukemic cells [6], up to 32% in human breast cancer cells [11], and up to 13% in macrophages [10]. Although some dietary *trans* unsaturated fatty acids have been shown to be incorporated partly into the *sn*-1 position of membrane phospholipids [38], the positional distribution of CLA in phospholipids is unknown. Our recent studies showed that the natural isomers of CLA were incorporated predominantly, but not exclusively into *sn*-2 position of PC and PE in Chinese Hamster Ovary cells (unpublished data). In this study we tested the effect of incorporation of the two major naturally occurring CLA isomers in the *sn*-2 position of PC, on the physicochemical properties of model membranes including surface area, fluidity, permeability, cholesterol binding property and oxidizability. To determine the relative importance of the double bond geometry and conjugation on the membrane properties, we included two unnatural CLA that contained either two *cis* or two *trans* double bonds, and compared the effects with the unconjugated double *cis* 18:2 isomer (linoleic acid). A summary of various parameters measured in this study is presented in Table 1.

The monolayer properties of the CLA PCs showed that the molecular surface areas of the two natural CLA-containing PCs were similar at various surface pressures, but lower than that of the unconjugated LA-PC. They also differed from each other significantly in presence of cholesterol, especially at very low or very high sterol/PC molar ratios. Thus the PC containing t10c12 CLA was condensed more than the PC that contains c9t11 CLA, in the presence of various concentrations of cholesterol, indicating that the former interacts with the sterol more avidly than the latter. These results were supported by the direct binding experiments with labeled sterol and PC bilayers, which showed that the t10c12 CLA-PC bound 20% more cholesterol compared to either c9t11 isomer or the unconjugated 18:2 isomer (Fig. 3). Previous studies showed that the presence of a *trans* double bond in the fatty acid increases its affinity to cholesterol, compared to the *cis* double bond at the same position [30]. Furthermore, feeding TUFA to experimental animals increases the cholesterol content of cell membranes [39]. However, since both c9t11 and t10c12 contain one *cis* and one *trans* double bond, the difference in their cholesterol affinity is most probably be due to the location of the double bonds in the acyl chain. In this context, it is interesting to note that Stillwell et al [40] showed that cholesterol binds weakly to the PCs containing a fatty acid with a *cis* double bond prior to the carbon 9 position, and binds more efficiently to PCs that contain no *cis* double bond at carbon 9. Since the t10c12 CLA does not have a *cis* double bond up to carbon 12, it may provide a deeper pocket to accommodate the sterol ring as depicted schematically in Fig. 9. Since the *trans* double bond does not induce a ‘kink’ in the chain as much as the *cis* double bond, it is similar to a single bond with respect to the overall shape and cholesterol interaction. The importance of the position of the double bond, as well as its configuration is also evident from the studies of the cholesterol binding properties of 16:0–18:1 PC species. The presence of a *cis* double bond at carbon 9 results in much weaker binding of cholesterol compared to a *trans* double bond at the same position. Previous studies showed that feeding t10c12 CLA increases insulin resistance in mice [41], although the authors proposed a mechanism that involved the effect of CLA on nuclear transcription factors. An alternative explanation is that an increase in membrane cholesterol may be responsible, because such an increase leads to a decrease in membrane fluidity and impaired function of insulin receptors [42].

Conflicting reports have appeared on the oxidizability of CLA and their potential antioxidant effects. The free radical scavenging property of CLA was reported by many laboratories [43, 44], with the t10c12 CLA showing stronger scavenging capacity than the c9t11 isomer [44]. This property may be responsible for the antioxidant effects reported in vivo [45] [46]. However, Basu et al [47] reported that CLA have pro-oxidant effects in vitro. Whereas some studies reported that CLA methyl esters of CLA are less oxidizable than methyl linoleate [48], others reported a greater oxidative susceptibility [49,50]. Although most of the in vitro studies have been performed with free CLA or methyl esters, the physiological form of CLA that is relevant to membrane function is the phospholipid that contains esterified CLA.

Therefore we focused on the oxidizability of PC species containing the different isomers of CLA at *sn*-2 and a saturated fatty acid at *sn*-1, a most likely configuration that occurs in vivo. Our results show that all the tested CLA-PCs are more susceptible to oxidative degradation compared to the PC with unconjugated 18:2. Furthermore, unlike the unconjugated LA, where the presence of *trans* double bonds decreases the oxidizability by up to six-fold [33], the presence of even two *trans* double bonds in CLA actually increased its oxidizability, suggesting that the conjugated structure in the fatty acid dramatically alters the physicochemical properties of the phospholipids.

Another important finding of the present study is that the presence of either c9t11 CLA or t10c12 CLA in the *sn*-2 position of PC activates the LCAT-mediated cholesterol esterification reaction by 2-fold, indicating a potentially beneficial effect on the reverse cholesterol transport pathway. In contrast to this, our previous studies showed that the presence of (unconjugated) TUFA at the *sn*-2 position of PC inhibits the LCAT reaction [34], indicating that the conjugated structure overcomes the negative effects of *trans* unsaturation with respect to some biological activities. Although the effect of CLA on LCAT has not been reported previously, the study of Burdge et al [51] showed that following the feeding of a CLA-enriched diet to humans, there was a disproportionate increase in the CLA content of plasma CE, compared to plasma PC. This indicates a preferential incorporation of CLA into CE by the plasma LCAT activity, since the LCAT reaction is the predominant source of all cholesteryl esters in human plasma [15] and since the CLA feeding is known to actually inhibit cholesterol esterification in liver by the acyl CoA: cholesterol acyltransferase (ACAT) reaction [52], the other source of plasma cholesteryl esters.

In summary, our results show that the two natural CLA, when incorporated into PC, exhibit significant differences in their physicochemical properties in model membranes. Compared to the c9t11 CLA-PC, the t10c12 CLA-PC was found to interact more strongly with cholesterol, and to decrease membrane permeability. Since the latter isomer is known cause insulin resistance in some animal models, this property may be relevant to its biological effect. Both the CLA-PCs, however, have similar surface areas and fluidity properties, and activate the LCAT reaction to a similar extent. The presence of two *cis* or two *trans* double bonds in the CLA, on the other hand, affected the physical properties of PC more strongly. Interestingly, the conjugation of double bonds alone appears to profoundly affect the physicochemical properties of the PC, as evident from the differences between c9c12 PC (unconjugated) and c9 c11 PC (conjugated), both with two *cis* double bonds. Thus, the c9c11 PC binds cholesterol less efficiently, shows decreased permeability, and increased oxidizability, compared to c9c12 PC. Furthermore, the effects of *trans* unsaturation appear to be neutralized to some extent by the presence of conjugation, as evident form the increased activation of LCAT reaction by the t10c12 PC and c9t11 PC, compared to the previously reported inhibition of LCAT by the unconjugated *trans* fatty acid PCs [34].

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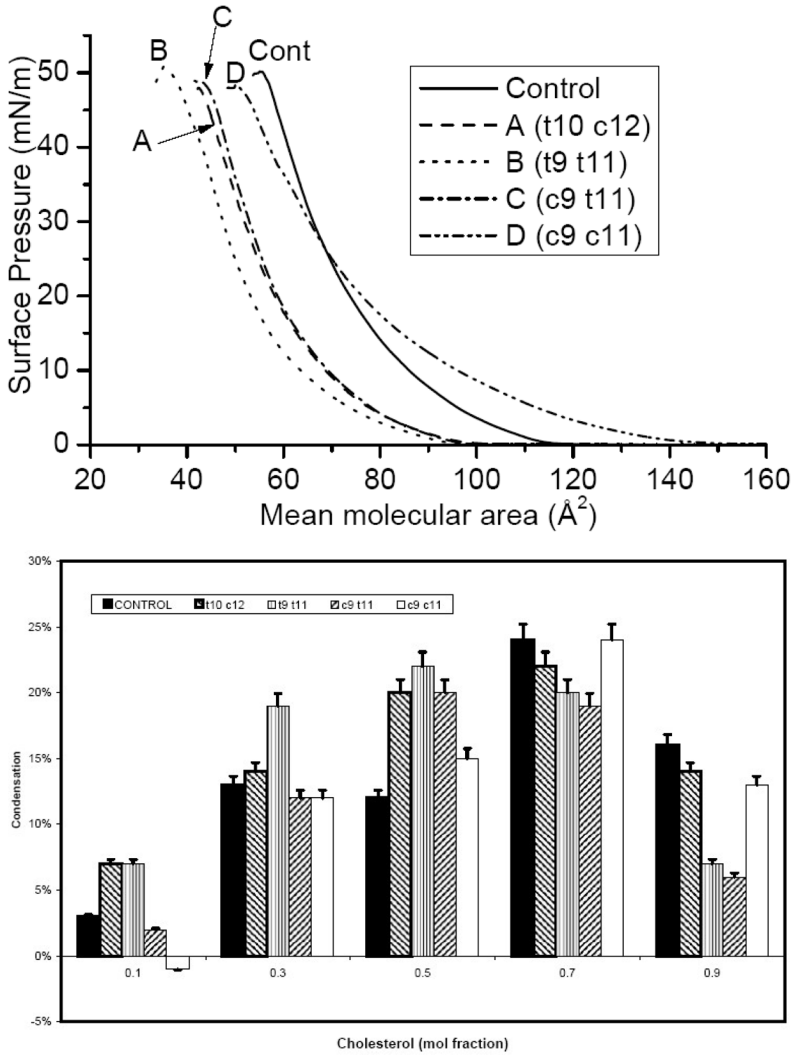

Fig. 1.

a) Surface area isotherms of CLA-PCs and control (unconjugated 18:2) PC. Compressions were performed at ambient temperature (~24 °C) on a surface of purified water at a rate of 10 cm^2/min. Isotherms are representative of at least 3 runs; error ranges were ± 3 $Å^2$ $molecule^{-1}$.

b) Condensation of CLA-PCs and control PC monolayers by cholesterol at 20 mN/m. Data for condensation were derived from the pressure-area curves similar to those shown in Fig. 1a, as described in Materials and Methods.

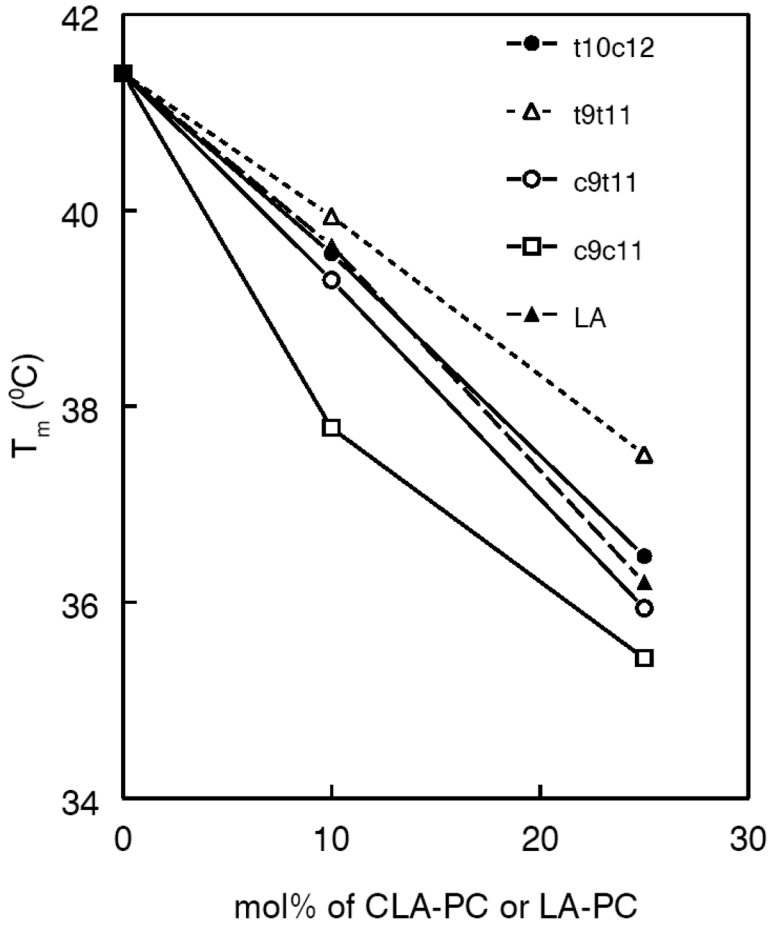

Fig. 2. Effect of CLA-PC isomers on the transition temperatures (T_m) of dipalmitoyl (16:0–16:0) PC

Multilamellar liposomes of 16:0–16:0 PC (2 mg/ml) alone or containing 10 mol% or 25 mol % of CLA-PC or LA-PC (control) were prepared in distilled water and at least 4 heating scans were performed in Microcal VP-DSC calorimeter. The transition temperatures (T_m) were determined from the fourth scan.

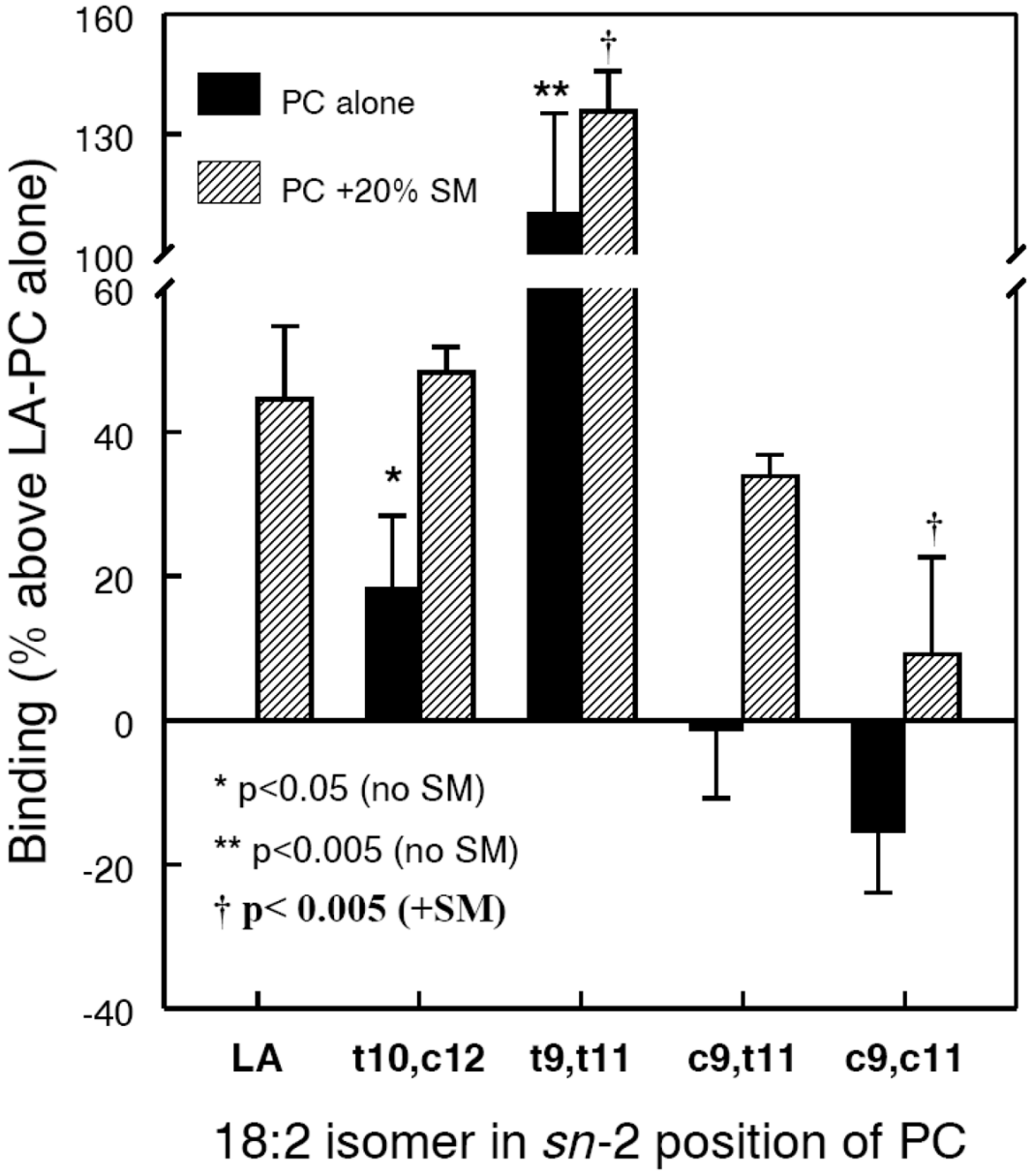

Fig. 3. Cholesterol-binding properties of CLA-PCs

The ability of the liposomes prepared from each of the CLA-PCs to extract labeled cholesterol from [^{14}C]-cholesterol-β-methyl cyclodextrin complex was determined as described in the text, in the absence and presence of 20 mol% egg SM. The amount of cholesterol extracted by control LA-PC (16:0–18:2, unconjugated) was taken as the baseline, and all other values were expressed as % above or below that value. The values are shown are mean ± S.D of 3–5 experiments. * p< 0.05, and ** p<0.005 compared to LA-PC alone.
† p<0.005, compared to LA-PC + 20 mol% SM.

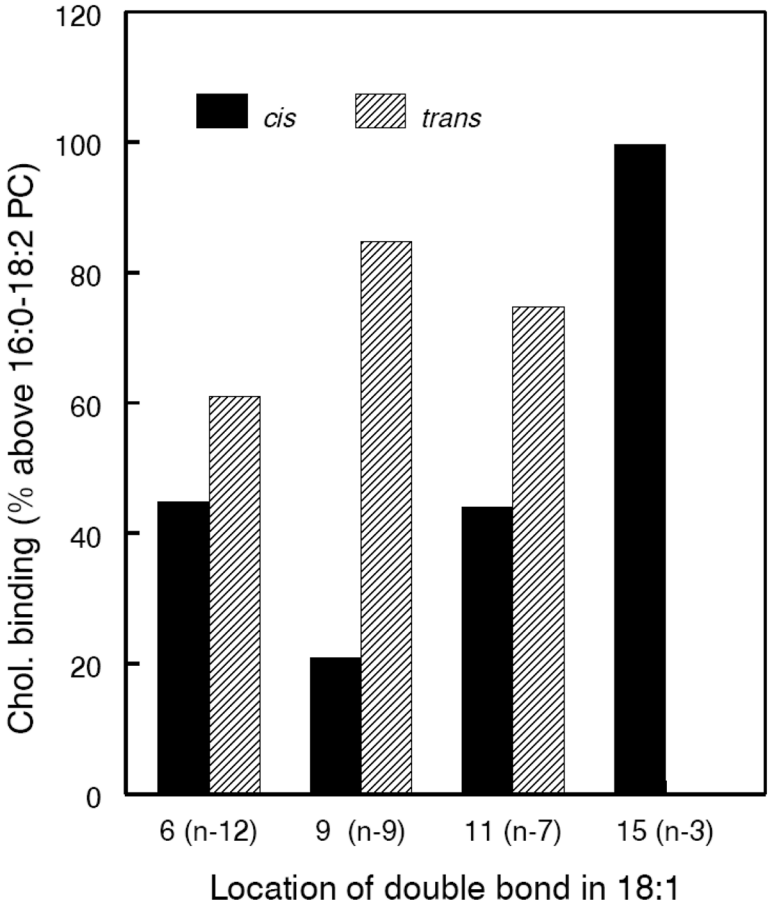

Fig. 4. Cholesterol-binding properties of isomers of 16:0–18:1 PC

PCs containing the different isomers of 18:1 at *sn*-2 position were chemically synthesized, and their affinity to cholesterol was determined as described in the text. The values are expressed as % of the value obtained with 16:0–18:2 (unconjugated) PC, and are average of two separate experiments. The n-3 *trans* isomer of 18:1 free fatty acid was not available, and therefore is not included in the study.

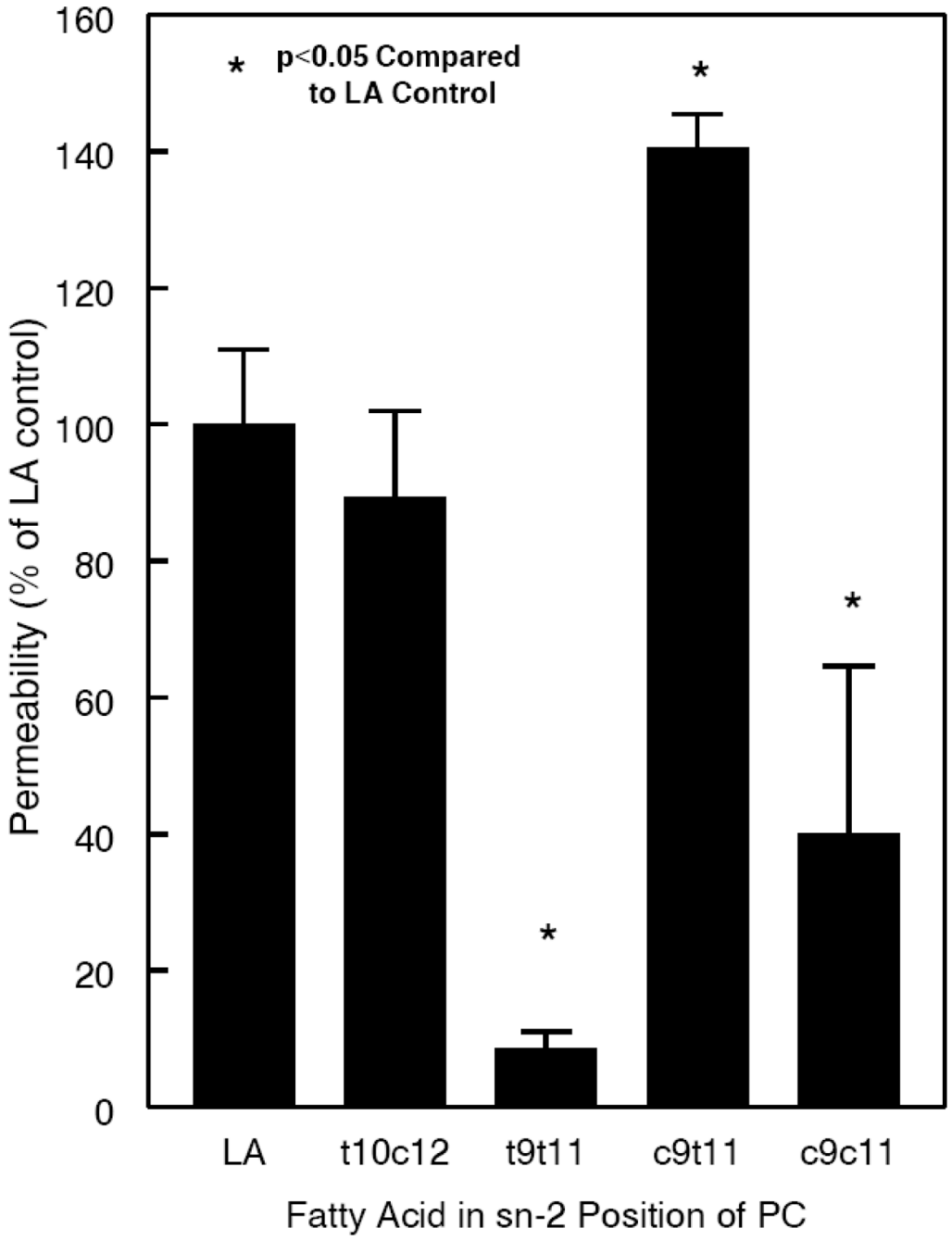

Fig. 5. Carboxyfluorescein permeability of CLA-PC liposomes

Small unilamellar vesicles of various CLA-PCs, containing the trapped carboxyflurescein were prepared by sonication, and the untrapped dye was removed by gel filtration chromatography. The leakage of the dye following its dilution with excess buffer was monitored in a flurometer as described in the text. The values shown are mean ± S.D of 4 separate experiments, and are expressed as % of the values obtained with unconjugated 16:0–18:2 PC.

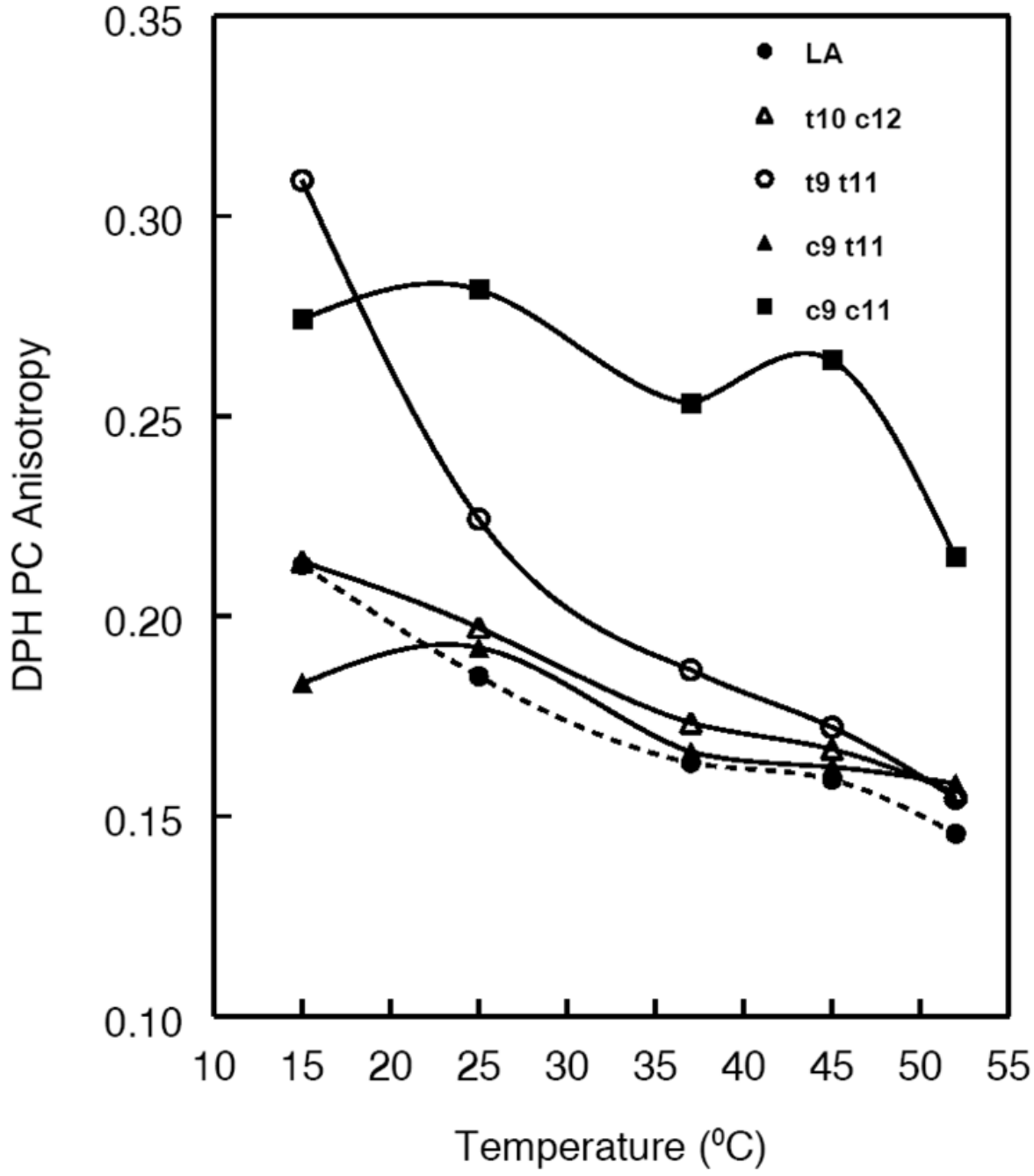

Fig. 6. Fluorescence anisotropy values of DPH-PC incorporated into various PC liposomes

DPH-PC was incorporated into the liposomes at probe: PC ratio of 1:200, and fluorescence anisotropy was determined at various temperatures.

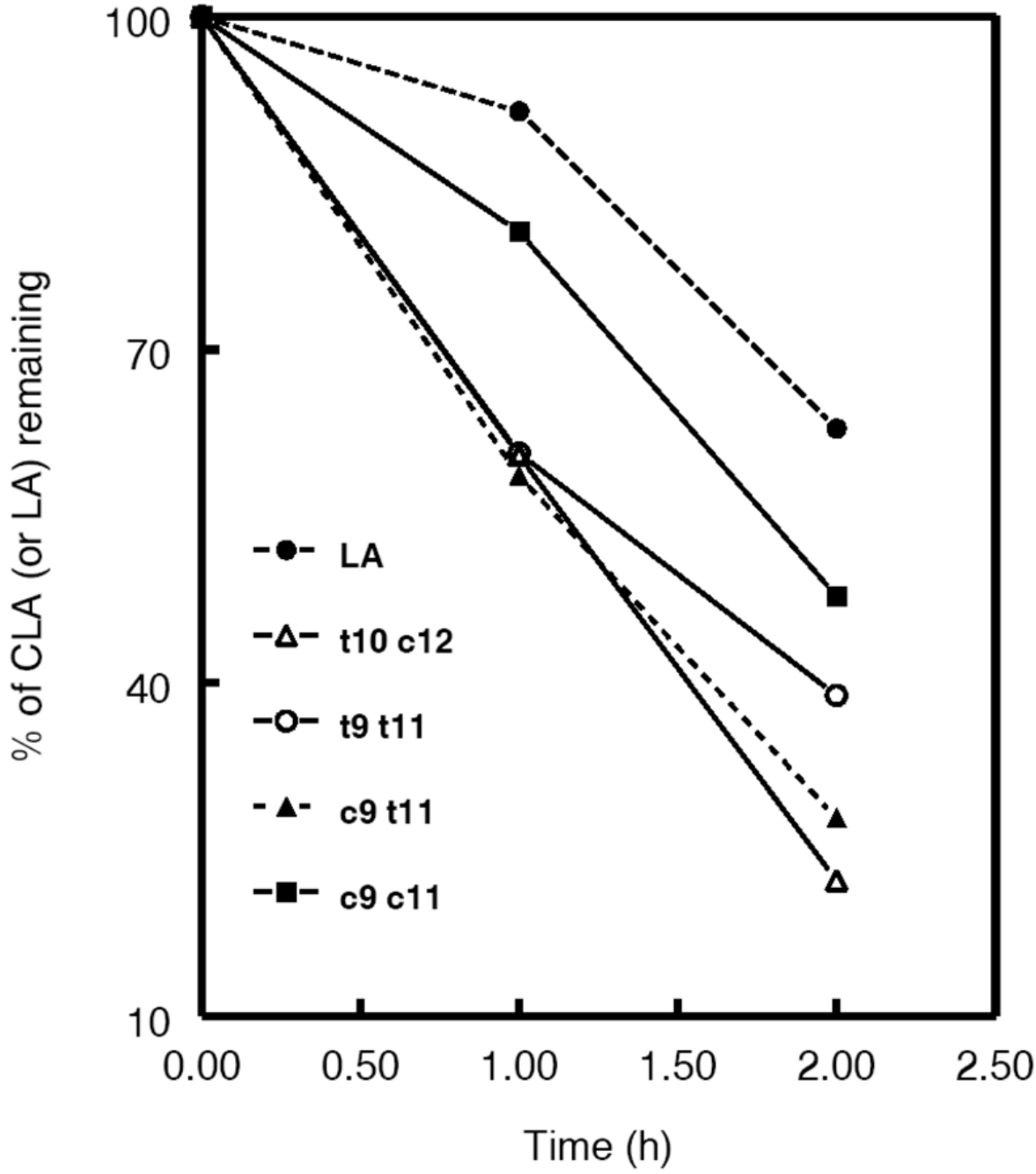

Fig. 7. Oxidation of various PC species in presence of 1 mM AAPH

PC liposomes (0.1 μmol PC) were incubated at 37 °C in presence of 1 mM AAPH. At the indicated period of time the total lipids were extracted and the fatty acid composition analyzed by GC, after adding 17:0–17:0 PC as internal standard. The % CLA remaining was calculated, relative to 17:0. The data shown are averages of 3 separate experiments.

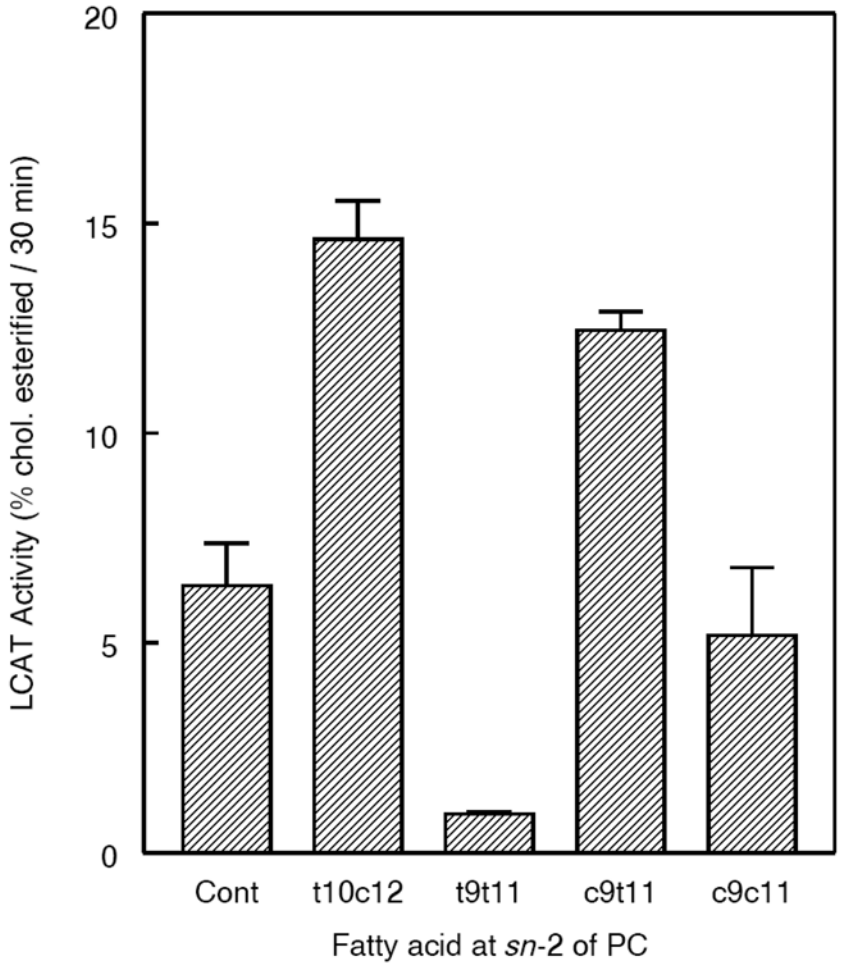

Fig. 8. Effect of CLA on LCAT activity

Proteoliposome substrates containing the respective PCs and labeled cholesterol were prepared as described in the text, and incubated with a semi-purified preparation of human LCAT for 30 min. The percent of labeled cholesterol esterified was determined after TLC separation of the lipid extract. The values shown are mean ± S.D of 3 separate assays.

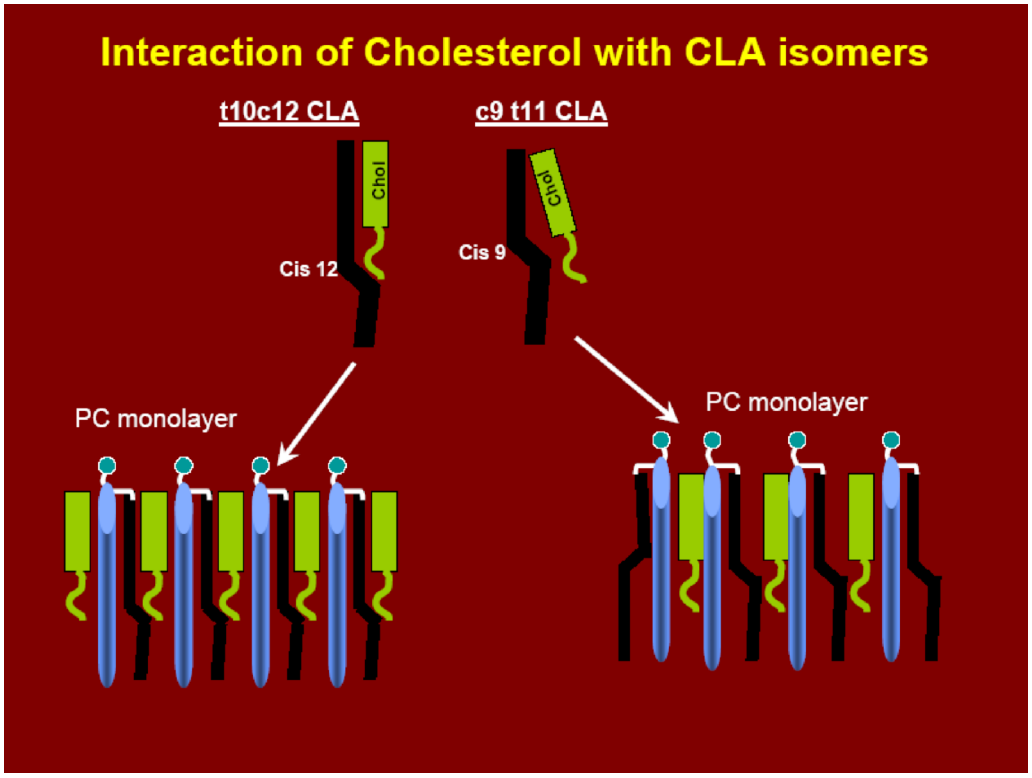

Fig. 9. A postulated mechanism for the increased binding of cholesterol by t10c12 CLA-PC

It is proposed that position of the first *cis* double bond (starting from the carboxy end) is critical in cholesterol binding. Because of the kink in the acyl chain generated by the presence of the *cis* double bond, the pocket formed by the *cis* 12 double bond in t10c12 CLA-PC is larger than the pocket formed by the *cis* 9 double bond in c9t11 CLA-PC, and therefore more easily accommodates the bulky and rigid sterol ring. Furthermore, in a membrane that contains a saturated fatty acid at *sn*-1 and t10c12 at *sn*-2, the cholesterol may interact with both sides of the PC molecule, whereas it interacts only with the saturated acyl group in the PC containing c9t11 CLA. Since the *trans* double bond does not introduce a kink in the acyl chain, it is considered to be equivalent to a single bond in this model.

Table 1

Summary of physicochemical properties of isomers of CLA-PC compared to LA-PC.

Property	Control (LA-PC)	t10 c12 CLA-PC	t9 t11 CLA-PC	c9 t11 CLA-PC	c9 c11 CLA-PC
Area at 20 mN/m (Å^2/mole)	73.8	58.2	53.2	58.8	76.2
Area condensation with 10% chol. at 20 mN/m	3%	7%	7%	2%	−1%
Chol. binding (% of Cont)	100	118.5	210.3	98.6	84.5
Tm of DPPC (+25% test PC)	36.2 °C	36.5 °C	37.5 °C	34.7 °C	35.4 °C
Cooperative unit[1] (DPPC +25% test PC)	23.9	24.1	48.6	19.2	17.9
DPH-PC anisotropy (37 °C) (% of Cont.)	100	106.1	114.3	101.7	154.9
Permeability (% of Cont.)	100	89.3	8.5	140.6	40.0
Oxidizability (% of Cont.)	100	336	312	339	184
LCAT activity (% of Cont.)	100	230	14	195	81

[1]Cooperative unit (C.U) was obtained by dividing the van't Hoff enthalpy (ΔH_{VH}) with the calorimetric enthalpy (ΔH). The C.U value for pure DPPC was 539.