Effect of Inhalation Anesthetics on Spin-Labeled Cholesterol Containing DPPC Vesicles

N. Gulfo, R. Bartucci, and L. Sportelli

Dipartimento di Fisica, Laboratorio di Biofisica Molecolare, Università della Calabria, 87036 Arcavacata di Rende, Italy

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We have investigated by means of electron spin resonance (ESR) spectroscopy the influence of three inhalation anesthetics, *i.e.* halothane, chloroform and diethyl ether, on the interfacial and hydrophobic region as well of 38 mol% cholesterol containing DPPC unilamellar vesicles.

The study has been carried out in the temperature range 25–45 °C. The variation of the order parameter, *S, vs* temperature of the lipid phase indicates that with this content of cholesterol the characteristic gel → liquid crystalline main phase transition of DPPC, normally occurring at $T_t \sim 41$ °C, disappears.

When halothane and chloroform are added to the vesicles suspension up to [DPPC]/[anesthetic] molar ratio of 1:1 the main phase transition, as detected with the stearic acid spin label *I*(12,3), reappears again and it results down shifted at $T_t \sim 35$ and 39 °C, respectively.

In presence of diethyl ether, instead, the main phase transition is not observable also at the highest concentration of anesthetic used.

Moreover, halothane and chloroform affect similarly the hydrophobic core of cholesterol + DPPC vesicles which, in turn, results to be different from the action exerted by diethyl ether in the same region.

The ESR findings are discussed in terms of competitive effects shown by cholesterol and inhalation anesthetics. Moreover, the interfacial region of CHOL + DPPC vesicles results to be the target of anesthetics.

Introduction

Cholesterol (CHOL) is an important component of many biological membranes. It is known from many reports that it plays very important roles concerning either the structural or functional properties of natural membranes [1, 2]. Moreover, its fraction may reach as much as 50% of the total lipid content.

From this point of view all informations obtained from studies performed on artificial model membranes should be considered only partial if this steroid is not considered as intrinsic component of the lipid bilayer.

In this respect the interaction between volatile anesthetics and phospholipid bilayer has been investigated including 38 mol% CHOL in the sonicated DPPC unilamellar vesicles. This CHOL fraction has been chosen since it represents the middle CHOL content of a lot of natural membranes [3].

The influence of anesthetics on cell membranes it is well established [4–6]. Moreover, in literature data are lacking about the influence on order, hence on the thermotropic behaviour of lipid bilayer, produced by proton donor, *i.e.* halothane and chloroform, and proton acceptor, *i.e.* diethyl ether, anesthetics. Such a classification of general inhalation narcotics was recently proposed by Sandorfy and coworkers [7, 8] on the base of IR investigations.

To get more insight we have performed the present electron spin resonance study (ESR). The interpretation of the ESR spectra coming from stearic acids spin labeled phospholipid vesicles indicates that, on the contrary of the Meyer–Overton [9, 10] theory of anesthesia, inhalation anesthetics influence essentially the interfacial region of CHOL + DPPC vesicles. Moreover, proton donor narcotics are effectiver perturbing molecules of lipid order than the proton acceptor one. In fact, the perturbing anesthetic capacity results in the order halothane > chloroform ⩾ diethyl ether which also agree with anesthetic potency.

Materials and Methods

Synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Sigma (W. Germany) and used without further purification. The spin labels *I*(12,3) [2-(3-carboxypropyl)-4,4-dimeth-

Reprint requests to L. Sportelli.

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yl-2-tridecyl-3-oxazolidinyloxyl] and *I*(1,14) [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl] were Aldrich products stored below 0 °C as 3.02×10^{-2} M ethanol solution. 10 mM phosphate buffer solution (PBS) at pH 7.4 with 0.15 M NaCl was prepared with salts reagent grade from C. Erba.

30 mg CHOL and 91.7 mg DPPC were dissolved in $CHCl_3$ and the solvent evaporated on a rotary evaporator under reduced pressure and with a stream of dry nitrogen. After PBS addition the mixture was vigorously vortexed and sonicated for 30 min at 50 °C with a MSE instrument (100 W, 20 kHz, 9 μm amplitude).

Defined amount of spin labels in ethanol were placed in test tubes and the solvent evaporated. After addition of the translucent vesicles suspension to the tubes ([label]/[DPPC] = 1/100) samples were incubated for 1 h at 45 °C under shaking at 60 rpm. Amounts of liquid halothane ($CF_3CBrClH$), chloroform ($CHCl_3$) and diethyl ether ($(CH_3-CH_2)_2O$) at the dry ice temperature were added to 1 ml of vesicles suspension to obtain DPPC/anesthetic molar ratios of 4:1, 2:1 and 1:1. Sample tubes were quickly sealed minimizing the dead volume above the liquid, strongly vortexed and shaken overnight at RT to equilibrate.

ESR measurements were performed with a Bruker ER-200D-SRC X-band spectrometer using 100 kHz field modulation and equipped with an ER 4111VT variable temperature control unit (accuracy ± 0.3 °C). Samples in sealed capillary tubes were put in standard ESR quartz tube located at the center of the resonant TE_{102} cavity of the spectrometer. The standard ESR quartz tube was filled with silicon oil to avoid temperature gradient across the sample. Measurements were performed at thermic equilibrium and the experimental set-up for all measurements was: 10 mW microwave power; 1.6 gauss modulation amplitude; 0.2 s time constant; 500 s scan time.

Results

The ESR spectra of the stearic acid spin label *I*(12,3) located into the interfacial region of 38 mol% CHOL containing DPPC vesicles results to be powder like spectra on the whole range of temperature investigated (as example see insert of Fig. 1).

The $T'_{\parallel}$ and $T'_{\perp}$ components of the motionally averaged axial symmetric nitrogen hyperfine tensor, $\tilde{T}$, are evaluated from these spectra and used to calculate the order parameter, *S*, with the expression given by Marsh [11]:

$$S = 0.5407 \times \frac{T'_{\parallel} - T'_{\perp}}{\frac{1}{3}(T'_{\parallel} + 2\,T'_{\perp})} .$$

The *S*-values *vs* temperature for vesicle suspensions with and without CHOL are reported in Fig. 1.

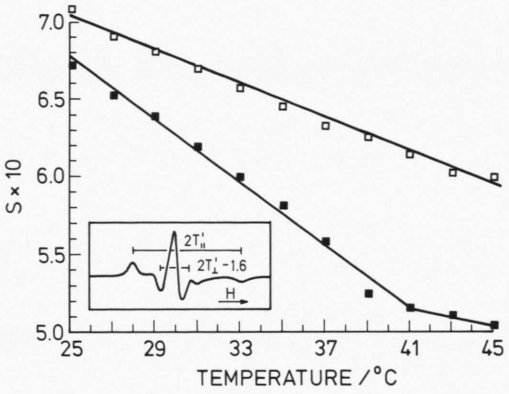

Fig. 1. Order parameter, *S*, *vs* temperature of spin labeled DPPC unilamellar vesicles (■——■) without and (□——□) with 38 mol% CHOL. Insert: ESR spectrum of *I*(12,3) spin label into CHOL + DPPC vesicles; the inner and outermost resonances are shown.

From the comparison of the two sets of values it is straightforward to observe that 38 mol% CHOL increase all values of *S* and induce the disappearance of the single discontinuity occurring at $T_t \sim 41$ °C characteristic of the gel → liquid crystalline main phase transition of pure DPPC [12]. In presence of halothane and chloroform the values of the order parameter, *S*, of the CHOL + DPPC lipid bilayer decrease and at the [DPPC]/[anesthetic] molar ratio of 1:1 the single discontinuity of the gel → liquid crystalline phase transition reappears (Fig. 2A and B). Moreover, the transition temperature results down shifted at ≃ 35 and ≃ 39 °C for halothane and chloroform, respectively, as compared with pure DPPC vesicles (Fig. 1 and Fig. 2A and B).

Diethyl ether less influences the molecular order of the interfacial region of the CHOL + DPPC system than other anesthetics do. In addition, with this anesthetic the discontinuity does not reappear also at the highest molar ratio used (Fig. 2C).

The hydrophobic region of CHOL + DPPC vesicles tested by the spin label *I*(1,14) results perturbed

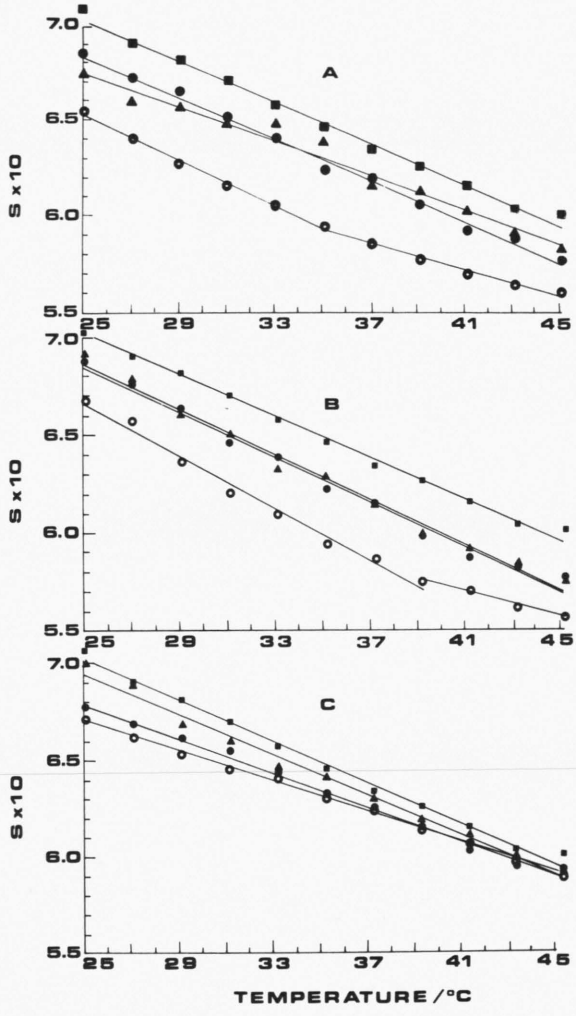

Fig. 2. Order parameter, *S*, *vs* temperature of the interfacial region of CHOL + DPPC + anesthetic ternary systems with A) halothane, B) chloroform and C) diethyl ether. (■——■) pure CHOL + DPPC vesicles; DPPC/anesthetic molar ratios of (▲——▲) 4:1, (●——●) 2:1 and (○——○) 1:1.

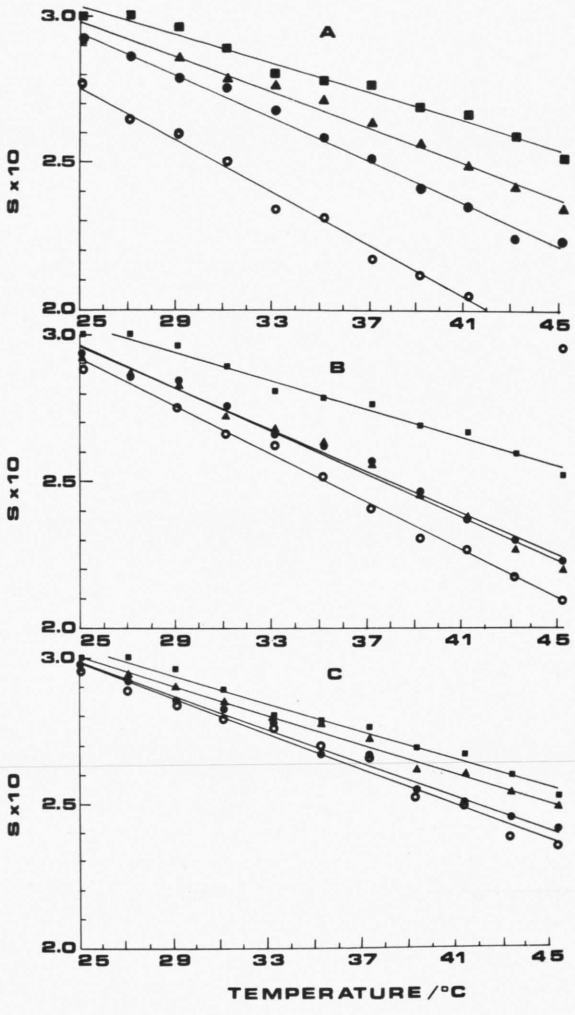

Fig. 3. As in Fig. 2 but with the spin label *I*(1,14).

from the three anesthetics about in the same manner though with different extent. At each temperature the order parameter decreases with increasing anesthetic concentration. Moreover, the linear best fits of *S*-values *vs* temperature show slopes which depend on anesthetic properties and their concentration (Fig. 3A, B and C).

Discussion

The CHOL + DPPC binary system is quite well characterized either in the anhydrous or full hydrated state. It has been, in fact, widely studied with different techniques including X-ray diffraction [13], differential scanning calorimetry [14], NMR [15], ESR [1, 16, 17], infrared and Raman spectroscopy [18–20].

From these investigations it comes out that CHOL incorporated in fully hydrated DPPC vesicles, which is our case, anchors with its −OH group at the level of the ester carbonyl groups of DPPC [19–21].

The CHOL binding eliminates the conformational inequivalence existing in the anhydrous state be-

tween the two fatty acids carbonyl groups by specifically perturbing the *sn*-2 acyl chain of DPPC [19], *i.e.* it gets all-*trans* configuration.

Moreover, the sterol can either increase or decrease the phospholipid bilayer packing density when its concentration is raised up to 50 mol% [15]. CHOL + DPPC vesicle dispersions with high mol% of sterol in presence of full hydration show vibrational $\rangle C = O$ Raman features like that of gel systems [20].

Such a literature data agree fairly good with our ESR findings obtained working with 38 mol% CHOL containing DPPC unilamellar vesicles. In fact, this sterol contents increases the phospholipid bilayer packing density, hence the degree of anisotropic motion of the interfacial region of CHOL + DPPC vesicles which results, now, more gel like on the whole range of temperature investigated. This results also evident from the comparison of order parameter values in Fig. 1. In fact, it is always $S_{CHOL+DPPC} > S_{DPPC}$. The rotational isomerisms of hydrocarbon chains observed in pure DPPC vesicles are reduced, too. Moreover, the ESR spectra of *I*(1,14) located into the hydrophobic core of the binary system look as one component spectra contrarily to what observed with pure DPPC vesicles [22]. This observation confirms what already known, *i.e.* that the structural inequivalence between the *sn*-1 and *sn*-2 acyl chains of DPPC is eliminated by CHOL [19].

In presence of halothane and chloroform an increase of the molecular dynamics of the polar head region as well as of the hydrophobic one of CHOL + DPPC vesicles takes place (Fig. 2 and 3 A and B). In fact, the calculated *S*-values are smaller than that found for the anesthetics free binary system. However, the slopes of the linear fits of *S vs* temperature in the cases of DPPC/anesthetic molar ratio of 4:1 and 2:1 are very similar to that obtained without anesthetics.

From these data can be argued that the molecular mechanism of forming cooperative unit between 25–45 °C, *i.e.* around the phase transition, remains unchanged in presence of these concentrations of both narcotics. The gel → liquid crystalline phase transition reappears at about 35 and 39 °C, respectively, in presence of one molecule halothane and chloroform per phospholipid molecule. This suggests that chlorofluorocarbons interact with the hydrophilic segment of DPPC. In fact, the dynamics of the *I*(12,3) nitroxide group located into the hydrogen belt region [23], *i.e.* near the glycerol backbone and/or ester carbonyl groups, reveals an increase in fluidity of this region which is clearly due to the presence of the anesthetics. Moreover, the transition temperature does not reach the values of 27 and 33 °C, respectively, observed in CHOL free DPPC vesicles in presence of same content of two narcotics [22]. Therefore, it can be deduced that sterol and anesthetics exert antagonistic action on the molecular dynamics of the interfacial region of DPPC.

In fact, the two anesthetics partially restore the molecular motion of phospholipid polar heads previously reduced by 38 mol% CHOL. Very likely the target of such a proton donor anesthetic molecules is the hydrogen belt region where they might perturb either the interpolar heads hydrogen bonds equilibrium or the electric interactions. It should be pointed out that Sandorfy and co-workers [24] have already observed the former effect. Diethyl ether behaves quite differently.

The data in Fig. 2C for this anesthetic suggest a binding site into the interfacial region of the CHOL + DPPC system different from that of the proton donor ones. In fact, although at 25 °C it influences the molecular order as other two, *i.e.* fluidizes the hydrophobic region, when the temperature is increased the anesthetic does not further increase the fluidity of the region but it seems to cooperate to pack the hydrogen belt region investigated by the radical moiety (Fig. 2C). This means that the binding site of $(CH_3CH_2)_2O$ in the lipid bilayer remains slightly outside than that of other narcotics.

The $-(CH_2)_2-$ segment of choline seems to be the most probable site of interaction. The binding should be also favored by hydrophobic interactions between this segment of DPPC and CH_3-CH_2-groups of diethyl ether. It is quite clear, therefore, that the hydrogen belt region results less perturbed and that at 45 °C the degree of anisotropic motion of this region is the same as found for anesthetic free CHOL + DPPC system.

Starting from this point of view the observed changes of the order parameter, *S*, hence of the molecular dynamics of the deep portion of the acyl chains (Fig. 3), should be a consequence of the interfacial binding of anesthetic molecules. In fact, the binding of halothane and chloroform in the hydrogen belt region increases the surface area per lipid polar head [4]. An increase in the acyl chains rotational

isomerisms of DPPC takes place already at 25 °C which depends, as expected, on anesthetic concentrations (Fig. 3A and B), *i.e.* higher for the highest DPPC/anesthetic molar ratio used. Moreover, temperature affects the rotational isomerisms formation, in turn, molecular cooperativity, as show the different slopes of the linear best fits in Fig. 3. Of course hydrogen bond breaking between ester carbonyl group of DPPC and −OH group of CHOL occurs as shown by Sandorfy and co-workers [24] with IR measurements on model systems.

The lower effects observed in presence of $(CH_3-CH_2)_2O$ as a function of anesthetic concentration as well as of temperature can be easily explained if the above hypothesis on the different binding site of this narcotic respect to halothane and chloroform is again considered. In fact, its binding near the $-CH_2-CH_2$-choline segment at 25 °C does not influence in any extent the hydrophobic core of vesicles also at the highest lipid/diethyl ether molar ratio.

As temperature is raised up the weakly bound narcotics molecules probably diffuse towards the hydrogen belt region. Consequently, polar heads separation increases, although in a very small extent, and the two DPPC acyl chains rotate somewhat more freely than without anesthetics as show *S*-values in Fig. 3C.

From the above results a picture of the interaction between volatile anesthetics and CHOL + DPPC vesicles can be drawn out. Proton donor anesthetics, halothane and chloroform, bind near the phosphate group of DPPC and perturb hydrogen bonds equilibrium. The influence on the interfacial lipid molecular order is exerted in the order halothane > chloroform which reflects their anesthetic potency. In the same order the two narcotics induce disorder in the spin labeled hydrophobic core of the CHOL + DPPC system.

The proton acceptor narcotic, diethyl ether, binds near the hydrophobic $-CH_2-CH_2-$ segment of choline with oxygen atom oriented probably towards water of bulk solution. Due to this spatial arrangement of diethyl ether either the interfacial region or the hydrophobic core of vesicles result influenced in a less extent even if temperature is raised up to 45 °C.

At present NMR studies are in progress to investigate the diffusional behaviour of these anesthetics between the different regions of the lipid bilayer.

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