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Dantrolene inhibits halothane-induced membrane reorganization

A study using ^{31}P-NMR and differential scanning calorimetry

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Abstract. The action of the relaxing agent dantrolene on dipalmitoylphosphatidylcholine (DPPC) model membranes in the presence and absence of the general anesthetic halothane has been investigated by DSC and ^{31}P-NMR. Dantrolene has a weak effect on both the thermodynamic and NMR parameters of the pure model membrane. When halothane is present in the system, the relaxing agent acts to counterbalance the strong anesthetic-induced membrane pertubation. This is reflected in DSC experiments by a change of the enthalpy variation (ΔH) and of the main gel-to-fluid phase transition temperature (T_c) towards the values of the pure lipid system. The amount of halothane-induced small tumbling vesicles, as detected by ^{31}P-NMR by the superposition of an isotropic line on a lamellar-type powder spectrum, is considerably reduced upon dantrolene addition. This means that the relaxing agent "cures" the membrane de-structuring action promoted by halothane. Membranes first treated with dantrolene are also protected from the halothane perturbation. So, the relaxing agent is both "curative" and "preventative" against halothane. The optimum effect is obtained for 1 dantrolene molecule per ca 34 halothane molecules. The mechanisms of action were discussed in relation to membrane fluidity.

Key words: Halothane – Dantrolene – DPPC – 31P-NMR – DSC – Model membranes

Introduction

Malignant hyperthermia is a pharmacogenetic disorder that affects skeletal muscles of susceptible humans and pigs (Cheah and Cheah 1984; Ellis and Heffron 1985; Gronert et al. 1988). It is triggered either by stress or pharmacological agents such as halothane, the volatile anaesthetic. However, its action can be reversed by dantrolene, a relaxing agent (Ellis and Heffron 1985; Harrison 1988).

According to many studies, halothane alters the gel-to-fluid phase transition temperature of DPPC membranes (Jain et al. 1975; Vanderkooi et al. 1977; Koehler et al. 1978; Mountcastle et al. 1978 and Craig et al. 1987), and may induce a lateral phase separation (Ueda et al. 1974) and a modification of the membrane fluidity (Trudell et al. 1973; Boggs et al. 1976; Rosenberg et al. 1975). Recently, Yoshida et al. (1988) reported studies on halothane solubilized in sodium dodecyl sulfate micelles, and proposed a dose-related biphasic mechanism for the interaction. It is quite clear by DSC, ^{31}P-NMR, and freeze-fracture electron microscopy that halothane interacts with DMPC and DPPC membranes and induces drastic changes in the structure and dynamics of the lipid bilayers (Gaillard et al. 1991). Thus the effects of halothane on model membranes take place in two different steps: at low halothane content the bilayer is disturbed but its macrostructure remains unaffected whereas in the case of higher drug concentrations, a new organization of lipids, identified as small vesicles, is stabilized for temperatures greater than T_c, the transition temperature of pure lipids.

Dantrolene is known to inhibit the effect of halothane on biological membranes (Denborough 1980; Ohnishi 1987; Harrison 1988); however, the mechanism of this action is not yet well understood. The aim of this work is to monitor the effects of dantrolene on the DPPC system with or without halothane, by DSC and ^{31}P solid state NMR. A comparison will be drawn between our present work on the ternary system dantrolene-halothane-DPPC membranes and previous work on the structure and dynamics of the halothane-DPPC system (Gaillard et al. 1991).

Materials and methods

Halothane and DPPC were purchased from ICI Pharma (Enghien, France) and Sigma (St. Louis, USA) respective-

Abbreviations: DPPC, Dipalmitoylphosphatidylcholine; DMPC, Dimyristoylphosphatidylcholine; DSC, Differential Scanning Calorimetry; NMR, Nuclear Magnetic Resonance; EDTA, Ethylenediaminetetraacetic acid; DMSO, Dimethyl sulfoxide; R_i, Halothane-to-lipid molar ratio; R_d, Dantrolene-to-lipid molar ratio; ΔH, Enthalpy variation; T_c, Main gel ($L_{\beta'}$)-to-fluid($L_{\alpha'}$) phase transition temperature

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ly, and were used without further purification. Halothane-DPPC systems were prepared as previously described (Gaillard et al. 1991).

Dantrolene (1-[(5-[p-nitrophenyl]furfurylidene)amino] hydantoin, sodium salt) was obtained from Lipha (Lyon, France) as a liposoluble compound. The dantrolene-DPPC mixtures were prepared as follows: 50 mg of pure DPPC and the amount of dantrolene necessary for the desired drug-to-lipid molar ratio (R_d) were dissolved in ca. 3 ml of methanol. The organic solvent was eliminated under vacuum, the resulting mixture suspended in 1 ml of buffer (20 mM *Tris*, 1 mM EDTA, pH = 7.5) and homogenized using several freeze-thaw cycles on a vortex mixer. In order to study the effect of dantrolene on preformed halothane-DPPC systems, the drug (20 mg) including NaOH and mannitol (Norwich Eaton Pharmaceuticals, Norwich) was solubilized in 7 ml of distilled water and then added to the preformed system.

Differential scanning calorimetry and ^{31}P-NMR experiments were carried out as previously described (Gaillard et al. 1991). DSC studies were performed on a DSC Setaram differential calorimeter. The sample volume was 100–120 µl and the sample was scanned twice using a heating rate of 3 °C/min, from −5 up to 70 °C. Thermograms were digitalized with a Hewlett Packard 85 calculator which allowed the determination of the temperature, T_c, relating to the lamellar gel-to-fluid phase transition of phospholipid dispersions, to which 50% of the total energy had been supplied. The enthalpy variation, ΔH, was obtained from the area of the endothermic peak.

^{31}P-NMR spectra were obtained at 162 MHz on a Bruker AM 400 spectrometer operating as described previously (Gaillard et al. 1991). Typically, spectra were obtained with the Hahn-echo sequence (Rance and Byrd 1983). Data processing was carried out on Bruker-Aspect 3000 and VAX/VMS 8600 computers.

Results

Differential scanning calorimetry

Thermograms of DPPC dispersions in the absence and the presence of halothane and/or dantrolene were recorded. The temperature of transition (T_c) and the enthalpy variation (ΔH) were calculated as described (Gaillard et al. 1991) and are reported in Table 1. Dantrolene slightly modifies the thermodynamic parameters (ΔH, T_c) of the main transition and the pretransition temperature (T_c'), whereas the values of $\Delta H'$ corresponding to the pretransition decrease as the amount of dantrolene (R_d) in DPPC increases. These results indicate that the effect of dantrolene is weak. The action of halothane on DPPC dispersions (Table 1, $R_d = 0$, $R_i \neq 0$) has previously been described (Gaillard et al. 1991). It is reported to depend upon the halothane-to-lipid molar ratio, R_i. For $R_d = 0$, when R_i increases T_c always decreases whereas a minimum of ΔH is observed for $R_i = 0.8$. ΔH and T_c values were obtained from the ternary system dantrolene-halothane-DPPC and are reported in Table 1. For any R_i, the presence of dantrolene makes both ΔH and T_c change towards the values that correspond to the pure DPPC system. When R_i is equal to 0.8, dantrolene addition results in an increase in ΔH towards 35 kJ, and an increase in T_c towards 41.8 °C. When R_i is equal to 2 or 4.4, dantrolene addition results in a T_c increase and a ΔH decrease (from 42 kJ/mol towards ΔH of the pure lipid).

Table 1. Thermodynamic parameters for DPPC dispersions in the presence and absence of halothane and/or dantrolene

R_i	R_d	T_c (°C)	ΔH (kJ mol^{-1})	T_c' (°C)	$\Delta H'$ (kJ mol^{-1})
0	0	41.8±0.2	35.0±1.5	35±0.5	5.7±0.6
	0.13	41.3±0.1	33.0±0.7	33±0.7	3.3±0.4
	0.26	41.5±0.5	37.0±0.9	34±0.6	2.8±0.4
0.8	0	36.7±0.4	29.9±1.2		
	0.13	38.4±0.2	31.5±0.9		
	0.26	38.5±0.2	32.7±0.9		
2	0	26.7±1.0	42.7±1.2		
	0.13	33.4±0.7	33.5±1.6		
	0.26	35.9±0.4	31.4±1.4		
4.4	0	21.9±0.1	42.3±3.3		
	0.13	22.1±0.2	39.5±0.9		
	0.26	25.0±0.2	32.1±0.9		

R_i = Halothane-to-lipid molar ratio; R_d = dantrolene-to-lipid molar ratio. T_c' and $\Delta H'$ correspond to the temperature and the enthalpy variation of the pretransition whereas T_c and ΔH are the equivalent thermodynamic parameters characterizing the main transition

^{31}P-NMR spectroscopy

The effect of dantrolene on DPPC was investigated first. Figure 1 shows typical ^{31}P-NMR powder patterns of DPPC multilayers in the absence ($R_d = 0$) and the presence ($R_d = 0.13$) of dantrolene. Slight differences are observed. Values of the second spectral moment (M_2) have been calculated from these spectra and are shown in Fig. 2 A. M_2 values reflect the changes occurring in the structure and dynamics of the phosphate head groups. In the fluid phase ($T > T_c$), there is a slight increase in M_2 values in the presence of dantrolene. In the gel phase, M_2 values decrease slightly as dantrolene is added. Since the observed powder pattern is characteristic of a lamellar structure, the resulting changes can be attributed to a difference between the motions of the phospholipid head groups in the gel and fluid phases. An isotropic line, estimated to represent less than 1% of the total spectral area occurs in all spectra and is attributed to micelles of lysolipids present in the sample.

Figure 3 shows thermal variations of spectra corresponding to halothane-DPPC ($R_i = 4.4$) systems either with or without dantrolene. In the absence of dantrolene, an isotropic line prevails in the fluid phase ($T > 25$ °C). This effect has already been described (Gaillard et al. 1991). The isotropic line is due to small vesicles undergoing very fast isotropic reorientation. For $R_d = 0.13$ and 0.26, the resulting spectra obtained at $T = 50$ °C are composed of a lamellar powder pattern and an isotropic line.

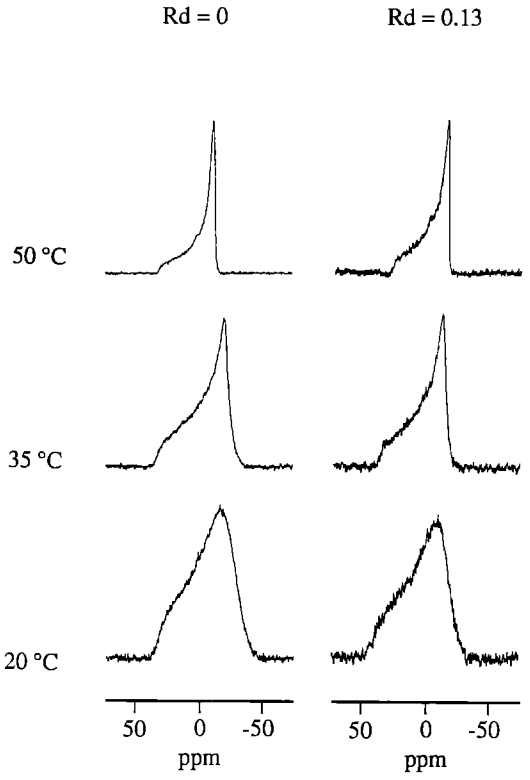

Fig. 1. Solid state ^{31}P-NMR spectra of DPPC dispersions with dantrolene $R_d=0.13$ and without dantrolene $R_d=0$, as a function of temperature. Experimental parameters: spectral window: 50 kHz; 90° pulse width: 13 μs; delay between 90° and 180° pulses: 40 μs; recycling delay: 4–5 s; 1600 scans; gated high-power proton decoupling

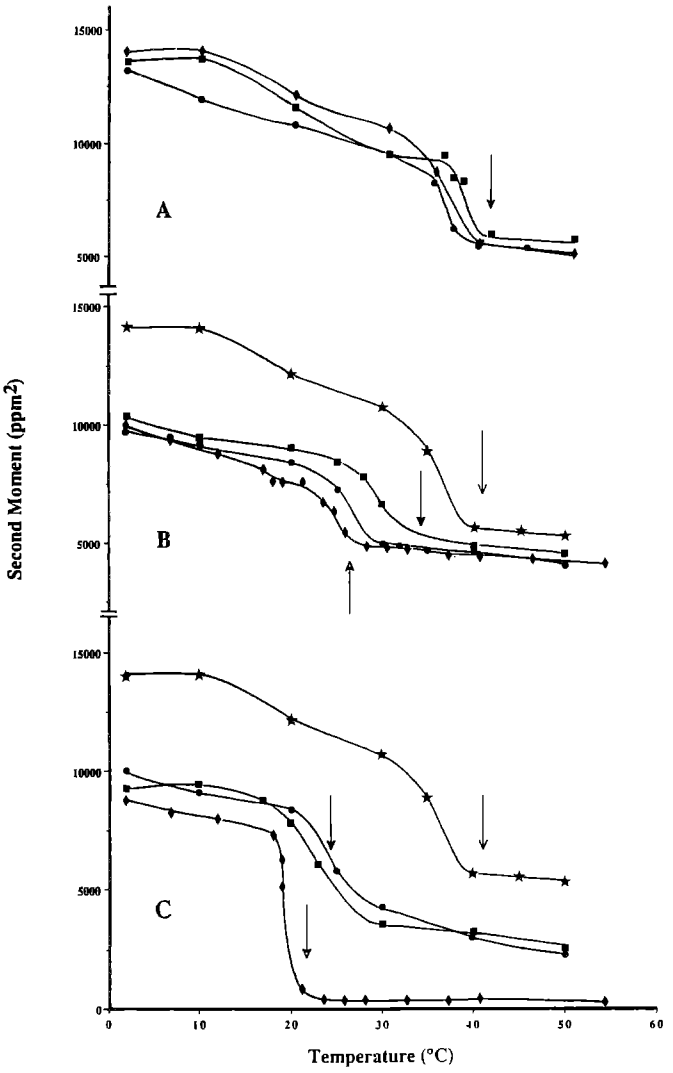

Fig. 2 A–C. Temperature dependence of the second moment (M_2) calculated from ^{31}P-NMR spectra of DPPC dispersions in the presence of various amounts of dantrolene. **A** $R_i=0$, **B** $R_i=2.0$, **C** $R_i=4.4$. in all curves (♦): $R_d=0$; (●): $R_d=0.13$; (■): $R_d=0.26$. M_2 thermal variation for pure DPPC (★) is also given for **B** and **C**. Arrows give T_c as obtained from DSC (see text)

This isotropic line becomes less important as dantrolene is added. The fall in temperature towards 25 °C reduces the proportion of the isotopic line. At 10 °C, in the gel phase, at any dantrolene and halothane concentration, the system is characterized by a lamellar gel-like powder pattern. These results clearly point to the fact that the dantrolene effect is stronger when the halothane-DPPC system is in the fluid phase.

Figure 4 illustrates the effect of dantrolene on the ^{31}P-NMR spectral shape for systems corresponding to various halothane-DPPC molar ratios, in the fluid phase at 41 °C. At $R_i=0$ and 0.8, the systems show a powder pattern reflecting a fluid lamellar phase both in the presence and absence of dantrolene. When more halothane is added ($R_i=2$), an isotropic line occurs on the powder pattern. Adding dantrolene to the system leads to a decrease in this sharp line. For $R_i=4.4$, the spectrum shows a prevailing isotropic line and a drastic decrease in intensity of this line is induced upon dantrolene addition. Thus, the dantrolene effect appears to be stronger for higher halothane contents.

The proportions of the lamellar powder pattern and the isotropic line can be determined by spectral simulations (Gaillard et al. 1991) and are expressed with respect to the total spectrum area. Figure 5 illustrates the percentage of the lamellar phase as a function of temperature for $R_i=2$ (B) and $R_i=4.4$ (A) in the presence of various amounts of dantrolene (R_d). In the presence of halothane only, one detects 100% of the lamellar gel phase. When the temperature increases towards T_c the lamellar phase content decreases owing to the appearance of the isotropic line (Gaillard et al. 1991). Adding dantrolene reduces the amount of this line, and therefore restores the powder pattern characteristic of the lamellar phase. Such an effect is stronger when dantrolene is present in a membrane treated with an excess of halothane (Fig. 5A). In this case, at 50 °C the percentage of the lamellar powder pattern goes from almost 0 to 50. In the fluid phase, the percentage of lamellar powder pattern is almost temperature independent, for the system halothane-DPPC (Fig. 5). In contrast, for the ternary systems (dantrolene-halothane-DPPC) an increase in temperature results in an almost linear decrease in the powder pattern sub-spectrum. This is obvious for $R_i=4.4$ (Fig. 5A).

Figure 6 shows the percentage of the isotropic line as a function of the dantrolene-DPPC ratio in the fluid phase at 41 °C. For R_d in the range 0–0.13, the decrease

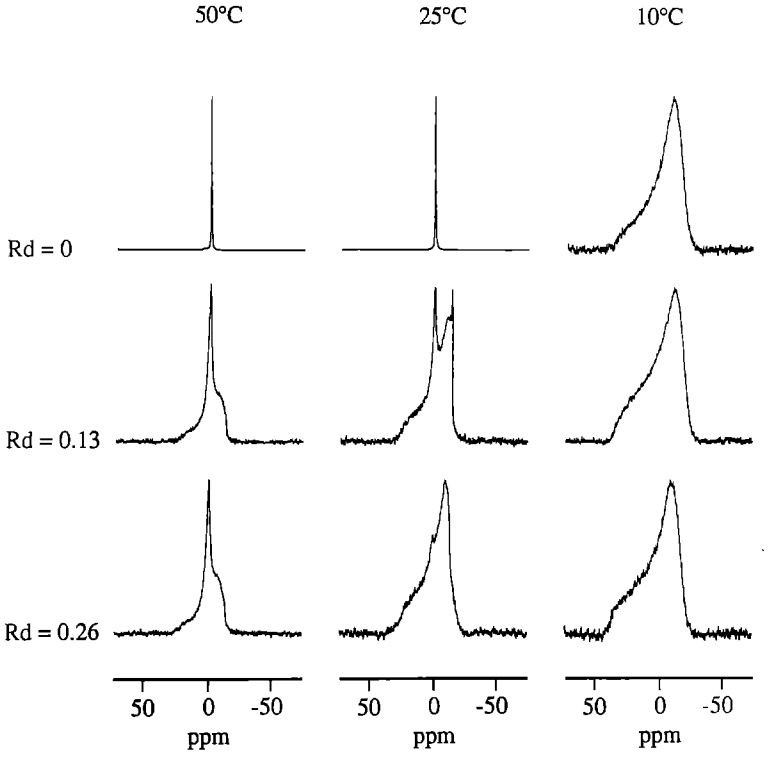

Fig. 3. Thermal variation of solid state ^{31}P-NMR spectra of dantrolene/halothane/DPPC systems. $R_i = 4.4$ and R_d as indicated on spectra. Experimental parameters as in Fig. 1

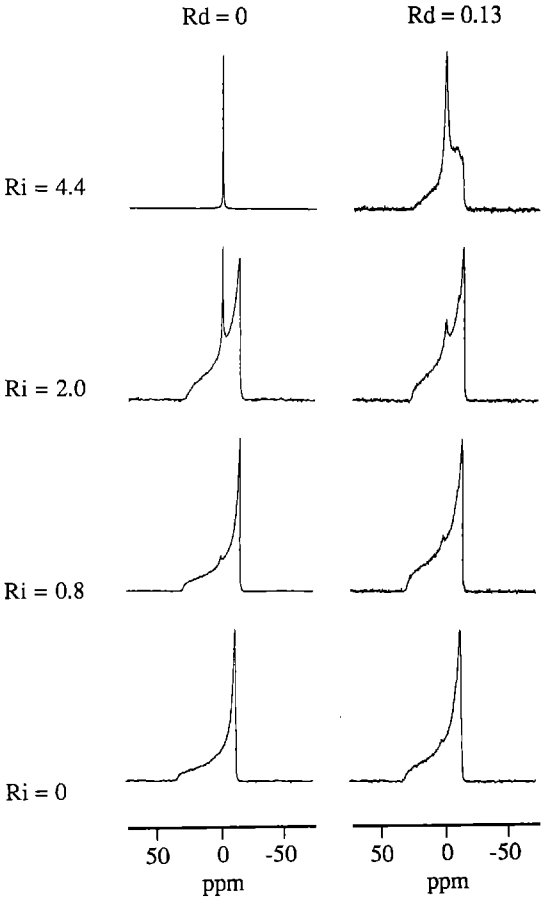

Fig. 4. Solid state ^{31}P-NMR spectra of various dantrolene/halothane/DPPC systems at 41 °C. R_i and R_d are indicated on spectra. Experimental parameters as in Fig. 1

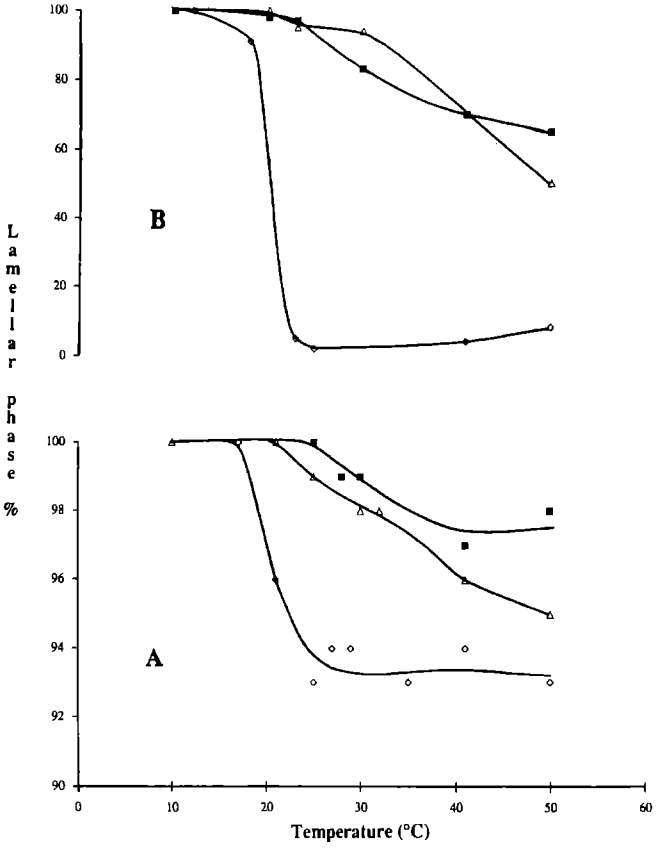

Fig. 5. Temperature dependence of the percentage of lamellar phase with various concentrations of dantrolene $R_d = 0$ (◇); 0.13 (△); 0.26 (■); for $R_i = 4.4$ (**A**) and 2 (**B**). The percentage is expressed relative to the total spectral area and obtained from simulation of ^{31}P-NMR spectra (see text). Experimental error in area determination is ca. 2%

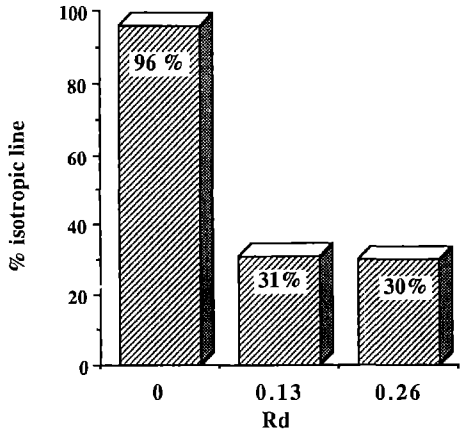

Fig. 6. R_d dependence of the percentage of isotropic line for the halothane/DPPC system $R_i=4.4$. The percentages are expressed relative to the total spectral area and obtained from simulation of ^{31}P-NMR spectra (see text). Experimental error in area determination is ca. 2%

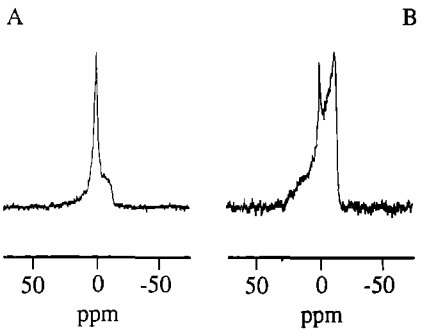

Fig. 7. Solid state ^{31}P-NMR spectra of various halothane/DPPC systems ($R_i=4.4$) without ($R_d=0$; **A**) or with dantrolene ($R_d=0.13$; **B**) at 41 °C. In the latter case, dantrolene was incorporated into the membrane prior to halothane addition. Experimental parameters as in Fig. 1

in this peak is very fast; then it reaches a plateau at about 30%. In spite of adding dantrolene, the isotropic line does not disappear completely. This plateau is reached for a halothane-to-dantrolene molar ratio (R_i/R_d) of about 34.

The second spectral moment (M_2) values have been calculated for DPPC dispersions in the presence of halothane and dantrolene. Representative values are plotted as a function of temperature at various R_d and R_i values (Fig. 2). Adding dantrolene to the $R_i=2$ systems leads (Fig. 2B) to an increase in the transition temperature T_c, as reflected by the variation of M_2. For comparison, we indicate by arrows T_c as estimated from DSC experiments. Moreover, the second spectral moment is always higher in the presence of dantrolene than in its absence. In the fluid phase this can be shown by the decrease in the isotropic line which has a very small second moment. In the gel phase, there is only one powder spectrum. Hence the increase in M_2 indicates a slowing down in the phospholipid head group dynamics. The same remarks can be made for the $R_i=4.4$ system, (Fig. 2C). The M_2 increase in the fluid phase is due to a very drastic decrease in the isotropic line content, whereas in the gel phase, the increase in M_2 indicates a slowing down in phosphate head group dynamics. However, the M_2 values never reach those of the pure lipid. This means that dantrolene only partially restores the membrane disturbed by halothane.

Experiments described above were performed using dantrolene incubated in the membrane before the addition of halothane. The effect of dantrolene was also studied on a membrane that had been previously treated with halothane. Only one experiment ($R_d=0.13$ and $R_i=4$) was carried out because of experimental difficulties. Figure 7 shows ^{31}P-NMR spectra, at 50 °C, for the halothane-DPPC system, before and after the addition of dantrolene. In the absence of dantrolene (A) treatment, the isotropic line represents 55% of the total spectrum area, whereas this percentage decreases to 3 after adding it (B). Therefore, the action of dantrolene on halothane-DPPC systems does not depend upon the order of application of the membrane effectors.

Discussion

These studies show the inhibiting effect of dantrolene against the macroscopic rearrangement induced by halothane on model membranes. This has been investigated both by differential scanning calorimetry, which reveals global changes of the entire system, and ^{31}P-NMR, which shows molecular and supramolecular membrane modifications.

DSC has revealed that addition of a small amount of halothane causes a decrease both in T_c and ΔH. The presence of dantrolene induces an increase of these parameters towards values of the pure model membrane system. With even more halothane, T_c decreases whereas ΔH increases towards a plateau value above that for pure DPPC (Gaillard et al. 1991). The presence of dantrolene in such a system leads to an increase in T_c and a decrease in ΔH. Again, the change is always towards values of the pure model membrane. This means that the perturbations induced by halothane, and reflected by these thermodynamic parameters, are drastically reduced by dantrolene.

Electron microscopy on freeze-fracture replicas have clearly demonstrated that halothane drastically modifies the membrane structure (Gaillard et al. 1991). New supramolecular entities (small unilamellar vesicles) made of halothane and lipids are formed. This is reflected in ^{31}P solid state NMR by the appearance of an isotropic sharp line. For $R_i=4.4$, this line dominates the spectrum and is characteristic of small vesicles. The presence of dantrolene reduces the amount of this line and a powder pattern typical of a lamellar phase appears. This indicates that the system has returned, in part, to large multilamellar dispersions. For a fixed value of halothane in the DPPC system, the more dantrolene the less isotropic line. However, the restoration of a powder pattern characteristic of multilamellar dispersions is never complete. The decrease in the proportion of small vesicles seems to end when R_d is greater than 0.13. This indicates that the best restoring

effect acts for 1 dantrolene molecule per ca. 34 molecules of halothane.

The amount of the isotropic line is less when halothane is added to the membrane previously treated with dantrolene and the isotropic line disappears when dantrolene is added as a water soluble mixture to the membrane pretreated with halothane. Although dantrolene is not added in exactly the same conditions in the second experimental procedure, (the presence of mannitol, a very water soluble molecule, is assumed not to affect the membrane) this means that the effect of the relaxing agent is both "curative" and "preventative" against halothane. The "curative" action could be due to a better solubility of dantrolene inside the lipophilic membrane (Harrison 1988), unlike halothane. Thus the halothane molecules would be expelled to the outside of the lipids, the small vesicles would melt away and the lipids would recover their thermodynamically stable state, i.e. multilamellar dispersions. As to the "preventative" action, the DSC and ^{31}P-NMR reveal that dantrolene slightly affects the structure and dynamics of DPPC. The only significant effect is a decrease in the enthalpy variation $\Delta H'$ of the pretransition and a slight decrease of the second spectral moment M_2, particularly in the gel phase. The structure of dantrolene is a three-ring system, and can be compared to cholesterol, a four-ring system. It is well known that cholesterol regulates membrane motions (Dufourc and Smith 1984; Dufourc 1988), i.e. it reduces membrane order in the gel phase and increases it in the fluid phase. This is reflected by a gradual decrease in the main transition enthalpy variation ΔH and a decrease in M_2 of phosphorus spectra both in the gel and the fluid phases (Leonard and Dufourc 1991). Although no ΔH change of the main transition is detected after dantrolene addition, the comparable M_2 decrease in the gel phase could mean that the relaxing agent possesses some of the rigidifying properties of cholesterol in the fluid phase. This description suits very well the "preventative" action of dantrolene against halothane, i.e., dantrolene would reinforce the membrane, and thus prevent halothane penetration. Since the structure of dantrolene is quite distinctive, further detailed information is required about its interactions with lipids in order to check this hypothesis. It will also be interesting to investigate the action of halothane on a cholesterol-containing membrane.

Although the mechanisms for molecular interactions in the halothane-dantrolene-membrane systems are not yet well understood, it is clear that dantrolene either prevents or repairs the de-structurating action of halothane on model membranes. The dantrolene-inhibiting effect on halothane-treated membranes is temperature dependent. In the presence of halothane only, the ^{31}P-NMR spectra exhibit a single isotropic sharp line characterizing the tumbling of the small vesicles. Above T_c, this sharp line is temperature independent. Indeed, its proportion with regard to the total spectrum area does not change with increasing temperature (Gaillard et al. 1991). In contrast, for given dantrolene and halothane contents in the DPPC system, the residual amount of the isotropic line increases with temperature. This indicates that the increase in temperature counterbalances the possible membrane reinforcing effect of dantrolene. It appears therefore that the higher the fluidity of the membrane, the stronger the halothane effect. This is also in agreement with our previous result according to which halothane interaction is greater with fluid phase than with gel phase model membranes (Gaillard et al. 1991). We have shown, through two different physico-chemical techniques i.e. DSC and NMR, that dantrolene "cures" or "prevents" the halothane-induced membrane re-structuring. This is linked to the competing abilities of both effectors to penetrate into the membrane, as well as to the possible reinforcer effect of dantrolene on membranes.

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