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The use of high-resolution solid-state NMR spectroscopy and differential scanning calorimetry to study interactions of anaesthetic steroids with membrane

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Abstract

We have used a combination of high-resolution solid-state ^{13}C-NMR and DSC (differential scanning calorimetry) to study the distinctively different thermotropic and dynamic properties of the anaesthetic steroid alphaxalone and its inactive congener Δ^{16}-alphaxalone in dipalmitoylphosphatidylcholine (DPPC) model membranes. In the solid-state ^{13}C-NMR, the techniques included cross polarization (CP) and/or magic angle spinning (MAS). The observed data revealed the following important results. (a) DSC as a bulk method showed that the active steroid lowers the main phase transition temperature and broadens the pretransition more significantly than the inactive congener. The ^{13}C-CP/MAS experiments allowed us to detect the pretransition temperature in the alphaxalone-containing preparation, which was not discernible in DSC. (b) The chemical shift values varied with temperature, indicating different degrees of *trans*-gauche isomerization in the lipid acyl chains when the bilayer is in the liquid crystalline phase. (c) Only specific additional peaks appeared in the ^{13}C-CP/MAS spectra when each of the steroids was present in the preparation. Δ^{16}-alphaxalone gives rise to more additional peaks than alphaxalone, indicating a different mobility of the corresponding molecular moiety in the phospholipid bilayer environment. (d) The relative intensities of these peaks also confirmed that alphaxalone is fully incorporated in the bilayer, whereas Δ^{16}-alphaxalone is only partially so. These results suggest that the differential effects of these two analogues in the membrane may, at least in part, explain the reason for their different biological activities. © 1997 Elsevier Science B.V.

Keywords: Phosphatidylcholine; Bilayer; Anesthetic steroid; Nuclear magnetic resonance; Differential scanning calorimetry

1. Introduction

Our laboratory has been involved in an effort to understand the mode of action of anaesthetic steroids at the molecular level. The steroid compounds may have similar molecular features, but distinctively different biological efficacies. To account for the structural specificity, there have been two proposed mech-

Abbreviations: DSC, differential scanning calorimetry; MAS, magic angle spinning; CP/MAS, cross polarization magic angle spinning; NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine

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anisms of action at the membrane level. The first suggested that anaesthetic steroids act after binding to a distinct site on a target protein [1] or on the $GABA_A$ receptor complex [2–7]. The second mechanism hypothesized that the site of action of anaesthetic steroids is at the membrane lipids. Experimental results obtained from electron spin resonance (ESR) with spin-labelled bilayers containing cholesterol [8] and our study using several biophysical methods [9–12] showed that lipids are capable of high degree of structural discrimination. These experiments also showed that steroids with anaesthetic activity caused more perturbation in the lipid bilayer vis à vis the biological inactive steroids.

At the moment, there is no single technique that allows unambiguous determination of which of the two proposed mechanisms is true or more important. Since both mechanisms involve the membrane, it is interesting to study the effects of anaesthetic steroid on the membrane bilayer, starting with the information on the physical and chemical parameters that govern the interaction between the steroids and the model membranes. This information may be applied to investigations using more complicated systems, such as biological membranes.

In this paper, present a comparative study on alphaxalone and Δ^{16}-alphaxalone, an ideal pair for investigating structural specificity. Alphaxalone (5α-pregnane-3α-ol-11,20-dione) has potent properties and is used clinically as the main active component in the commercially available anaesthetic also known as Althesin. Δ^{16}-alphaxalone (5α-pregn-16-ene-3α-ol-11,20-dione), which differs from alphaxalone only by having a double bond in the C-16 position (Fig. 1), lacks the anaesthetic activity [13,14]. ^{13}C-CP/MAS and ^{13}C-MAS NMR spectroscopy have been applied to study the dynamic and thermotropic properties of these steroids in DPPC model membrane. When a steroid molecule is present in the phospholipid bilayer, it can cause the following changes to the ^{13}C-NMR spectrum. (a) Changes in peak intensity and line-width due to modified membrane fluidity. (b) Changes in chemical shift values of individual carbon nuclei of the membrane lipid due to modified phase transition profiles. (c) Appearance of a specific subset of peaks from the carbon nuclei of the incorporated steroid molecules. These spectral features will be analyzed both quantitatively and qualitatively, and the results will be combined with DSC experiments to gain better understanding of the membrane's thermotropic properties induced by each of the steroids. Solid-state ^{13}C-NMR and DSC are complementary methods, each providing one perspective, and when used in combination, are often fruitful in yielding consistent information.

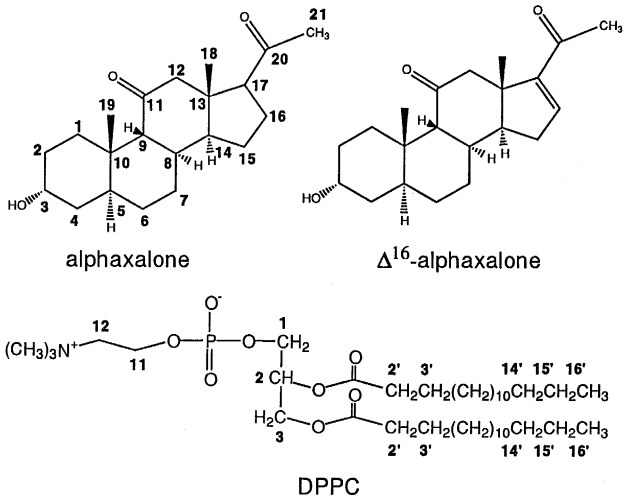

Fig. 1. Chemical structures of alphaxalone, Δ^{16}-alphaxalone, and DPPC.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids, Inc., Birmingham, Alabama, USA. Alphaxalone and Δ^{16}-alphaxalone were kindly donated by Professor Alexandros Makriyannis at the University of Connecticut. For all of the experiments in DSC, ^{13}C-CP/MAS, and ^{13}C-MAS NMR, identical procedures were used for preparing the following three samples: (1) DPPC bilayer; (2) DPPC bilayer containing alphaxalone; and (3) DPPC bilayer containing Δ^{16}-alphaxalone. In the latter two samples, the lipid-to-steroid molar ratio was 80:20, which will be designated as $x = 0.20$ in this paper. For each sample, appropriate amounts of DPPC and a steroid were dissolved in spectroscopic grade chloroform. After mixing, the solvent was evaporated by passing a stream of O_2-free nitrogen

over the solution at 50°C and the residue was placed under vacuum (0.1 mmHg) for 12 h. Distilled and deionized water was added to produce a 50% (w/w) lipid/water preparation. The final preparation was transferred to a ZrO_2 spinning rotor for the ^{13}C solid-state NMR experiments, and part of the same sample was subsequently transferred to a stainless-steel capsule and sealed hermetically for the DSC scans.

2.2. NMR spectroscopy

Solid-state ^{13}C-NMR spectra were obtained at 100.613 MHz on a Bruker MSL400 NMR spectrometer capable of high-power ^{1}H decoupling, $^{1}H-^{13}C$ cross polarization, and magic angle sample spinning. The spinning rate was 2.9 kHz, and was kept constant for all the temperatures (25–43°C) used in the experiments. Each spectrum was typically an accumulation of 10 000 scans for a temperature when the bilayer is in the gel phase, and of 2500 scans when the bilayer is in the liquid crystalline phase. The delay time was 4 s, the 90° pulse width was 5.0 μs, and the contact time for cross polarization was 1 ms.

Literature chemical shift values of DPPC bilayers published by Wu and Chi [15] were used as references in all of our ^{13}C-NMR spectra, which resembled theirs qualitatively and quantitatively.

2.3. Differential scanning calorimetry

DSC thermograms were obtained on a Perkin–Elmer DSC-7 instrument. The temperature scale of the calorimeter was calibrated by running standard samples (fully hydrated DPPC and pure indium) and using the known values of their transition temperatures. Each sample was scanned at least twice until identical thermograms were observed. An optimal scanning rate of 2.5°C/min was used to avoid distortions in the thermograms.

3. Results and discussion

3.1. DSC measurements

Hydrated DPPC lipids spontaneously form a bilayer, whose dynamic and thermotropic properties have been extensively studied by various biophysical methods, such as DSC and NMR [16–21]. The bilayer exists in the gel phase for temperatures lower than 35°C, and in the liquid crystalline phase for temperatures higher than 42°C. The transition is accompanied by several structural changes in the lipid molecules as well as systematic alterations in the bilayer geometry, but the most prominent feature is the *trans*-gauche isomerization taking place in the acyl chain conformation. The average number of gauche conformers indicates the effective fluidity, which depends not only on the temperature, but also on perturbations due to the presence of a drug molecule intercalating between the lipids.

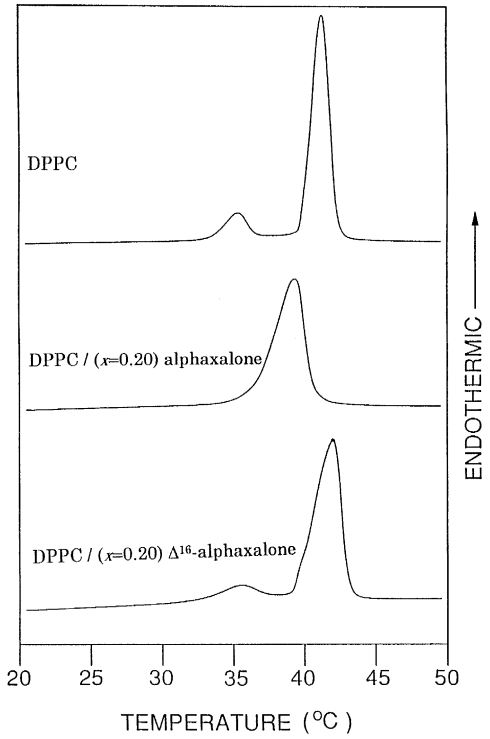

Fig. 2. Differential scanning calorimetry thermograms obtained from bilayer preparations of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alphaxalone.

Fig. 2 shows the thermograms of three preparations. The DPPC bilayer shows the characteristic pretransition with a low enthalpy-change and a sharp main transition, both at the expected transition temperatures of 35.3 and 41.2°C, respectively. However, the two steroid-containing bilayers have opposite effects on these two phase transitions. The active alphaxalone makes the pretransition non-discernible,

Table 1
Values of transition temperature (T_C), half-width ($T_{C1/2}$), and enthalpy-change (ΔH) of the three preparations studied

Sample	T_C (°C)	$T_{C1/2}$ (°C)	ΔH (cal/g)
DPPC	35.2, 41.2	1.8, 1.4	1.6, 12.5
DPPC + alphaxalone	39.3	1.4	9.0
DPPC + Δ^{16}-alphaxalone	35.3, 42.1	1.9, 1.2	0.9, 9.2

Those on the left of each column correspond to the pretransition, while on the right to the main phase transition.

and causes the main transition to appear as a broad peak at a lower temperature of 39.3°C. The inactive Δ^{16}-alphaxalone broadens the peaks, but, nevertheless, preserves the pretransition and, in fact, shifts the main transition peak to a higher temperature of 42.2°C. Quantitative data from these thermograms are listed in Table 1.

DSC results indicate that alphaxalone has a significant disordering effect on the bilayer, whereas Δ^{16}-alphaxalone seems to have a stabilizing effect. Because this method detects the absorbed heat by the entire bilayer, it gives only bulk information on the thermotropic properties, but not the detailed dynamic behaviour of different regions in the bilayer. To study the local changes induced by the steroids, solid-state ^{13}C-NMR spectroscopy is superior to DSC.

3.2. ^{13}C-CP / MAS spectra

Cross polarization and magic angle sample spinning techniques were applied to the three bilayer samples at various temperatures ranging from 25 to 43°C, which cover all the mesomorphic states of the DPPC bilayer. For the purpose of analysis, each spectrum will be divided into three regions, namely, carbon atoms in the hydrophobic region (10–40 ppm), those in the glycerol and backbone region (40–80 ppm), and the esterified carbonyls (near 170 ppm). Peak assignments were based on literature CP/MAS data of DPPC bilayer and high-resolution data of derivative molecules of alphaxalone and Δ^{16}-alphaxalone [22–24].

Fig. 3 shows the hydrophobic region of the ^{13}C-CP/MAS spectra of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alphaxalone. For each temperature, the chemical shift values of C-2′, C-3′, C-14′, C-15′, and C-16′ are individually measured and compared in Fig. 4. For DPPC, the chemical shifts of all the secondary carbons (C-2′ through C-15′) show a con-

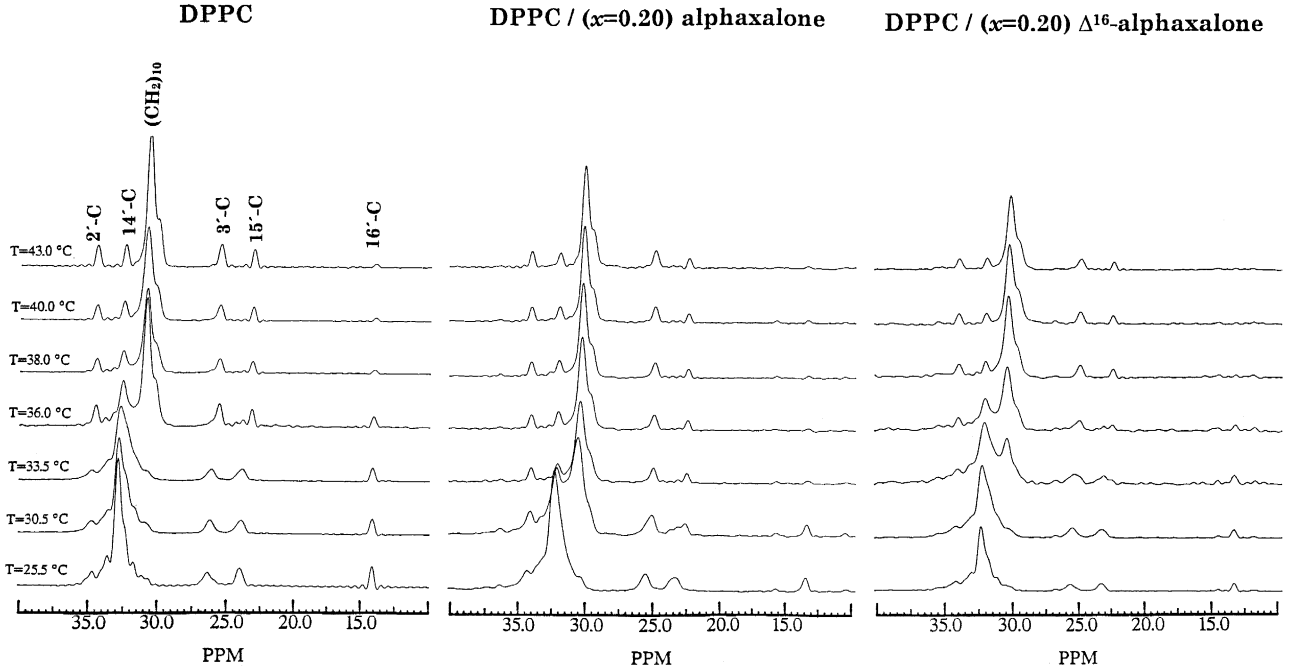

Fig. 3. The hydrophobic region of ^{13}C-CP/MAS spectra of bilayer preparations of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alphaxalone in the temperature range of 25–43°C.

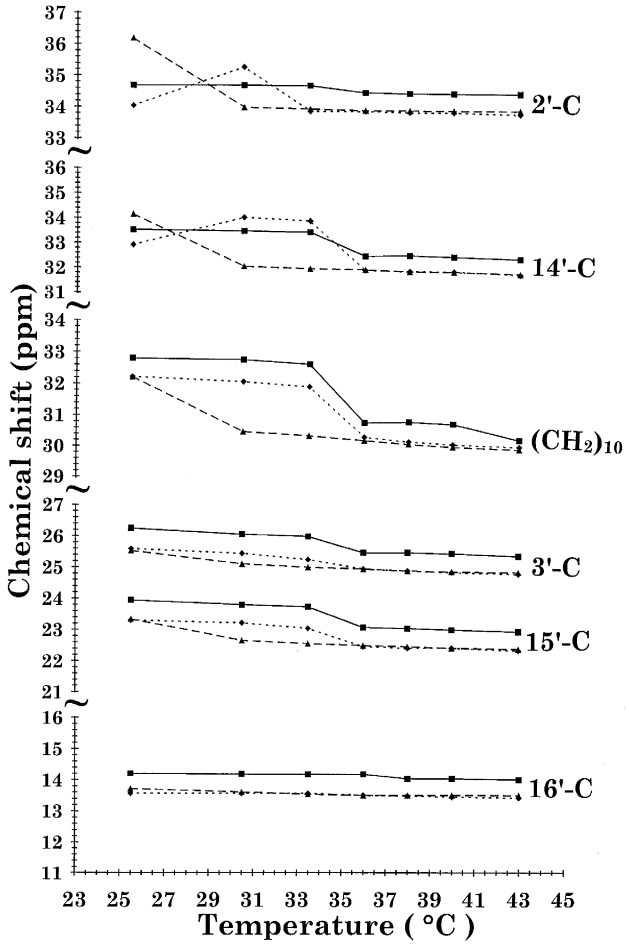

Fig. 4. Chemical shift changes vs. temperature of ^{13}C-NMR peaks in the hydrophobic region of the bilayer preparations of DPPC (■), DPPC + alphaxalone (▲), and DPPC + Δ^{16}-alphaxalone (♦) in the temperature range of 25–43°C.

sistent upfield shift taking place at 35°C, coinciding with the pretransition temperature. This represents the onset of the *trans*-gauche isomerization in the chain, resulting in an increased fluidity, a well-known effect [25]. For DPPC + Δ^{16}-alphaxalone, the upfield shift is observed at the same temperature of 35°C for the majority of sites (C-3′ through C-12′, and C-15′). This is consistent with the DSC results which show that Δ^{16}-alphaxalone preserves the pretransition at the same temperature, but causes it to broaden. The event of upfield shifting is somewhat irregular and occurs at lower temperatures for C-2′ and C-14′, which implies that they are probably more sensitive to the incorporation of Δ^{16}-alphaxalone. The most drastic effects on the chemical shift values were observed in the DPPC + alphaxalone preparation, where the upfield shifts for C-2′ through C-15′ are clearly visible near 29°C. This remarkable observation allowed us to detect the pretransition in the bilayer containing the active steroid, which did not offer any pretransition information in the DSC thermogram due to severe broadening. From the above chemical shift analysis, we have established a correlation between the chemical shift change and the bilayer pretransition, and observed the otherwise indiscernible pretransition of the bilayer perturbed by alphaxalone. Compared with Δ^{16}-alphaxalone, it is evident that alphaxalone induces more gauche conformers in the lipid acyl chains, not only because the isomerization starts at a much lower temperature (29 vs. 35°C), but also because alphaxalone causes consistent lower chemical shift values for C-2′ through C-15′ in the temperature range of 29–35°C. The primary carbon C-16′ has a constant chemical shift for all temperatures, due to its high mobility at the end of the chain. In the liquid crystalline phase, the two steroid-containing preparations have similar effects on the chemical shift of all the acyl chain carbons, shifting each peak towards upfield by about 0.7 ppm.

There are also other features in the spectra (such as intensity loss and multiple components) that indicate the onset of the pretransition. Intensity loss is observed as we compare the spectra from one sample for different temperatures. The spectral intensity reaches a minimum at 35.3, 30.5, and 33.5°C for DPPC, DPPC + alphaxalone and DPPC + Δ^{16}-alphaxalone, respectively. At these temperatures, each spectrum also shows at least two components for some of the acyl chain carbons (Fig. 5). These observations are due to the bilayer undergoing conversions between two mesomorphic states at a rate slower than the NMR time scale, and the two states may co-exist in the same membrane. The lower temperature at which this occurs in the DPPC + alphaxalone preparation confirms the conclusion that alphaxalone has more significant perturbing effect on the lipid chains than its inactive congener Δ^{16}-alphaxalone.

In the hydrophobic region of the spectra, we also observed additional peaks due to several carbons on the steroid molecules. For example, at 25.5°C, the C-18 and C-19 methyl groups appear at 10.8 and 15.9 ppm in the DPPC + alphaxalone spectrum, and at 11.9 and 14.7 ppm in the DPPC + Δ^{16}-alphaxalone

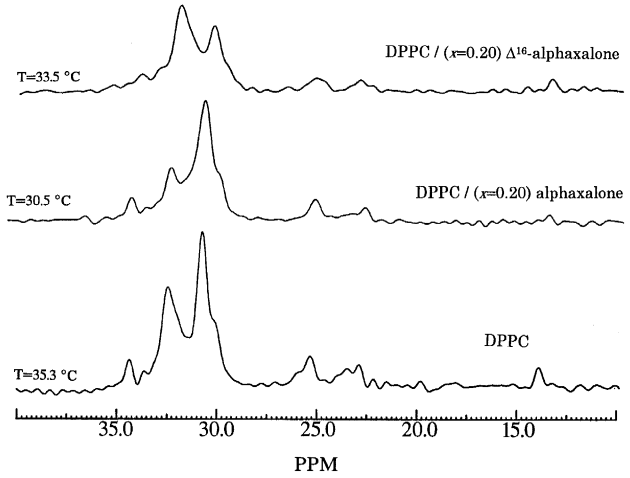

Fig. 5. The hydrophobic region of ^{13}C-CP/MAS spectra of bilayer preparations of DPPC at 35.3°C, DPPC + alphaxalone at 30.5°C, and DPPC + Δ^{16}-alphaxalone at 33.5°C.

spectrum. The C-21 methyl carbon is observed at 26.7 ppm only in the latter spectrum. This difference indicates that Δ^{16}-alphaxalone's C-21 has lower mobility than alphaxalone's counterpart. It may be a result of the particular phospholipid environment that restricts the motion of C-21 of Δ^{16}-alphaxalone.

The second spectral region (40–80 ppm) is due to the glycerol and backbone carbons of the lipids. Fig. 6 shows the spectra of the three preparations at various temperatures. None of the chemical shift values in this region changed significantly with temperature, indicating minimal change in local environment or motional rates during the phase transition. However, another set of additional peaks appeared in the spectra from the steroid-containing preparations. C-12 and C-14 of both steroids contributed to a broad peak around 55 ppm, and the C-13 gave rise to a sharp peak at 47.9 and 48.4 ppm from alphaxalone and Δ^{16}-alphaxalone, respectively. A peak at 67.0 ppm due to C-3 was clearly visible in the DPPC + Δ^{16}-alphaxalone spectrum, but not in DPPC + alphaxalone.

The third spectral region (downfield) contains the sp^2 carbons. We observed the carbonyl (C-1′) signal from the lipids near 172 ppm, and the C-16 and C-17 of Δ^{16}-alphaxalone at 148.3 and 151.4 ppm, respectively. These signals have relatively low intensities because the corresponding carbons have none or only one hydrogen atom attached and would not benefit from the ^{1}H–^{13}C cross polarization. In order to observe these carbons with the correct relative intensity compared to other carbon resonances, we also carried out the magic angle sample spinning experiments without cross polarization.

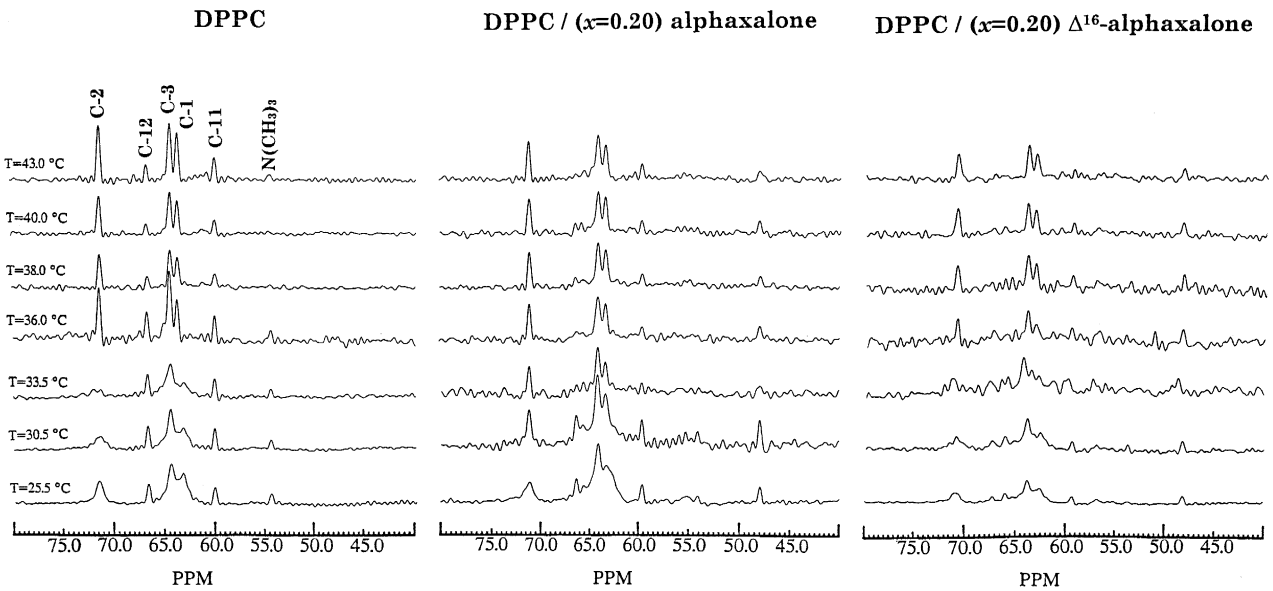

Fig. 6. The glycerol and backbone regions of ^{13}C-CP/MAS spectra of bilayer preparations of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alpaxalone in the temperature range of 25–43°C.

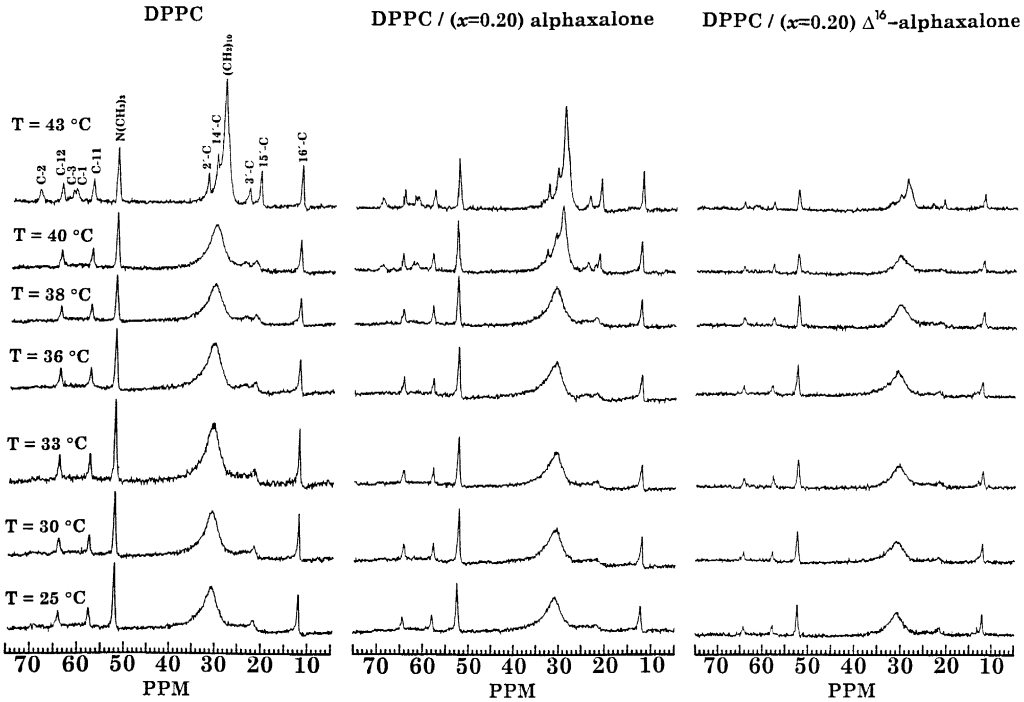

Fig. 7. The hydrophobic, glycerol and backbone regions of ^{13}C-MAS spectra of bilayer preparations of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alphaxalone in the temperature range of 25–43°C.

3.3. ^{13}C-MAS spectra

Without using cross polarization, the spectral intensity is proportional to the amount of carbon atoms, and does not strongly depend on molecular motions as it does in the CP/MAS experiments. Fig. 7 shows the upfield part of the ^{13}C-MAS spectra, where the $N(CH_3)_3$ and C-16′ carbons are restored to their righteous relative intensities. Graphs of peak intensities of several carbon sites vs. temperature are shown in Fig. 8, where we can extract the following two pieces of information. First, there is a trend that the intensity has a maximum in the DPPC preparation near its pretransition temperature. Second, the DPPC + Δ^{16}-alphaxalone preparation has a consistently lower intensity. This lower intensity can be explained by the notion that some of the Δ^{16}-alphaxalone molecules may complex with certain amount of lipid molecules in a form other than a bilayer, and this form may have unfavourable relaxation times, disallowing the observation of their NMR resonances. Similar effects of Δ^{16}-alphaxalone complexing with cholesterol and refusing full incorporation in the membrane were previously observed in our laboratories using DSC, X-ray diffraction and solid-state ^{2}H-NMR [25–27].

The downfield region is shown in Fig. 9, where

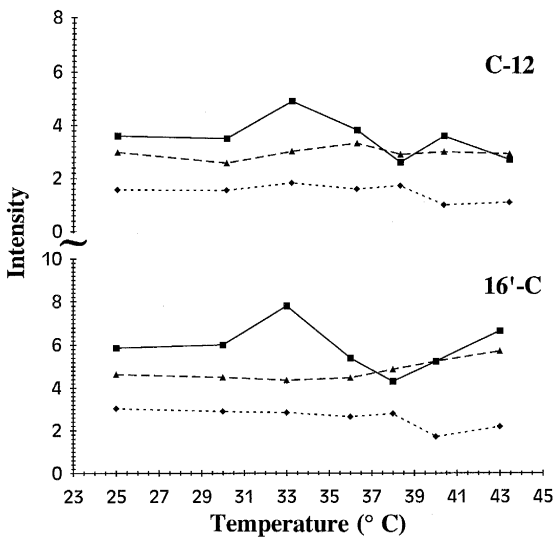

Fig. 8. Intensity changes of two representative carbon peaks over the temperature range of 25–43°C.

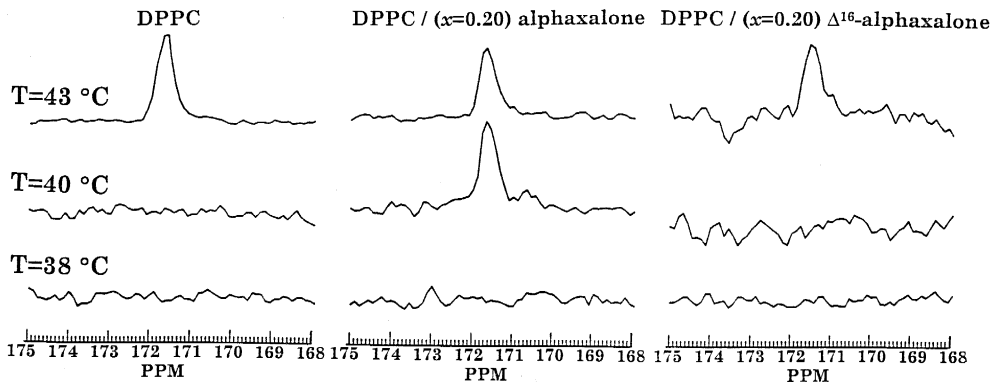

Fig. 9. The carbonyl region of ^{13}C-MAS spectra of bilayer preparations of DPPC, DPPC + alphaxalone, and DPPC + Δ^{16}-alphaxalone in the temperature range of 38–43°C.

the lone carbonyl peak emerges whenever the bilayer is in the liquid crystalline phase. Comparison among the spectra from the three preparations clearly shows that the main phase transition takes place at a lower temperature in DPPC + alphaxalone than in DPPC or DPPC + Δ^{16}-alphaxalone. This points to the conclusion that the active steroid alphaxalone interacts with the lipid backbone in a more pronounced manner than its inactive analogue Δ^{16}-alphaxalone.

4. Conclusions

In this study, CP/MAS and MAS techniques were applied in solid-state ^{13}C-NMR spectroscopy to investigate the differential effects of alphaxalone and Δ^{16}-alphaxalone on the thermotropic and dynamic properties of DPPC model membrane. The NMR methods provided local information, some of which was not available from DSC thermograms. Furthermore, ^{13}C-NMR uses a natural abundant isotope in the lipid and the steroid molecules, an important advantage over ^{2}H-NMR which usually requires elaborate ^{2}H-label synthesis, and over X-ray diffraction which also usually requires a heavy atom label, such as iodine. ^{13}C-CP/MAS and MAS spectra at various temperatures can provide data on the changes of chemical shift and peak intensities, which in turn allow us to understand the local environments and the amount of drug incorporation.

This study revealed that the active–inactive pair of anaesthetic steroids exert different effects on the phosphatidylcholine bilayers. A small structural difference in the steroid is recognized by the lipid such when having interactions in the bilayer. The phase transition of the DPPC model membrane is significantly affected by the active anaesthetic steroid alphaxalone, whereas the inactive Δ^{16}-alphaxalone has only marginal effects. The active steroid broadens the pretransition and lowers the onset temperature of the *trans*-gauche isomerization in the lipid acyl chains, as demonstrated by the results from both high-resolution ^{13}C-NMR and DSC. These corroborative results show that DSC mainly detects the thermotropic properties of the lipophilic region of the membrane. The NMR results also indicate that the membrane restricts the motion of these two steroids in different manners and the membrane accommodates them to different degrees. All these differences point to the conclusion that the membrane lipids are capable of discriminating the small structural difference in alphaxalone and Δ^{16}-alphaxalone, and the membrane lipids may constitute an important step in the mechanism of anaesthetic action.

Acknowledgements

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References

[1] C.D. Richards, K. Martin, S. Gregory, C.A. Keightlen, T.R. Hesketh, G.A. Smith, G.B. Warren, J.C. Metcalfe, Nature 276 (1978) 775–779.

[2] K.W. Gee, M.B. Bolger, R.E. Brinton, H. Coirini, B.S. McEwen, J. Pharmacol. Exp. Ther. 246 (1988) 803–812.

[3] D.M. Thurner, R.W. Ransom, J.S-J. Yang, R.W. Olsen, J. Pharmacol. Exp. Ther. 248 (1989) 960–966.

[4] J.J. Lambert, D. Belelli, C. Hill-Venning, J.A. Peters, Trends Pharmacol. Sci. 16 (1995) 295–303.

[5] J.J. Lambert, C. Hill-Venning, J.A. Peters, N.C. Sturgess, T.G. Hales, in: E.A. Bernard, E. Costa, (Eds.), Transmitter Amino Acid Receptors: Structure, Transduction and Models for Drug Development, Fidia Research Foundation Symposium Series, Vol. 6, Thieme, 1991, pp. 219–236.

[6] M. Bureau, R.W. Olsen, Mol. Pharmacol. 37 (1990) 407–502.

[7] G.L. Dunn, D.H. Morison, J. McChesney, W. Pine, C.R. Knmana, R.N. Gupta, J. Clin. Pharmacol. 22 (1982) 316–320.

[8] D.K. Lawrence, E.W. Gill, Mol. Pharmacol. 11 (1975) 280–286.

[9] A. Makriyannis, S.W. Fesik, J. Med. Chem. 26 (1983) 463–464.

[10] A. Makriyannis, C.M. DiMeglio, S.W. Fesik, J. Med. Chem. 34 (1991) 1700–1703.

[11] S.W. Fesik, A. Makriyannis, Mol. Pharmacol. 27 (1985) 624–629.

[12] A. Makriyannis, D.J. Siminovitch, S.K. Das Gupta, R.G. Griffin, Biochim. Biophys. Acta 859 (1986) 49–55.

[13] R.M. Atkinson, B. Davis, M.A. Pratt, H.M. Sharp, E.G. Tomich, J. Med. Chem. 8 (1965) 426–432.

[14] G.H. Phillipps, J. Steroid Biochem. 6 (1975) 607–613.

[15] W. Wu, L.-M. Chi, Biochim. Biophys. Acta 1026 (1990) 225–235.

[16] D.L. Melchior, J.M. Stein, Annu. Rev. Biophys. Bioeng. 5 (1976) 205–238.

[17] R.N. MeHlhaney, Chem. Phys. Lipids 30 (1982) 229–259.

[18] H.-J. Hinz, J.M. Sturtevant, J. Biol. Chem. 247 (1972) 6071–6075.

[19] M.J. Janiak, D.M. Small, G.G. Shipley, Biochemistry 15 (1976) 4575–4580.

[20] R.J. Witterbort, A. Blume, T.H. Huang, S.K. Das Gupta, R.G. Griffin, Biochemistry 21 (1982) 3487–3502.

[21] J.W. Blunt, J.B. Stothers, Organic Magnet. Reson. 9 (1977) 439–464.

[22] D. Dick, S. Pluskey, D.K. Sukumaran, D.S. Lawrence, J. Lipid Res. 33 (1992) 605–609.

[23] D.J. Ashworth, D.O. Adams, B.Y. Giang, M.T. Cheng, R.Y. Lee, Anal. Chem. 57 (1985) 710–715.

[24] J. Forbes, C. Husted, E. Oldfield, J. Am. Chem. Soc. 110 (1988) 1059–1065.

[25] A. Makriyannis, D.P. Yang, T. Mavromoustakos, in: Steroids and Neuronal Activity, Proceedings of Ciba Foundation Symposium No. 153, Wiley, Chichester, 1990, pp. 172–189.

[26] T. Mavromoustakos, D.P. Yang, A. Makriyannis, Biochim. Biophys. Acta 1194 (1994) 69–74.

[27] T. Mavromoustakos, D.P. Yang, A. Makriyannis, Biochim. Biophys. Acta 1239 (1995) 257–264.